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Review

Protein O-mannosylation: one sugar, several pathways, many functions

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Recent research has unveiled numerous important functions of protein glycosylation in development, homeostasis, and diseases. A type of glycosylation taking the center stage is protein O-mannosylation, a posttranslational modification conserved in a wide range of organisms, from yeast to humans. In animals, protein O-mannosylation plays a crucial role in the nervous system, whereas protein O-mannosylation defects cause severe neurological abnormalities and congenital muscular dystrophies. However, the molecular and cellular mechanisms underlying protein O-mannosylation functions and biosynthesis remain not well understood. This review outlines recent studies on protein O-mannosylation while focusing on the functions in the nervous system, summarizes the current knowledge about protein O-mannosylation biosynthesis, and discusses the pathologies associated with protein O-mannosylation defects. The evolutionary perspective revealed by studies in the *Drosophila* model system are also highlighted. Finally, the review touches upon important knowledge gaps in the field and discusses critical questions for future research on the molecular and cellular mechanisms associated with protein O-mannosylation functions.

Key words: *Drosophila* model system; matriglycan; protein O-mannosylation; protein O-mannosyltransferases; receptor protein tyrosine phosphatase.

Introduction

Protein O-mannosylation (POM) is an evolutionarily conserved posttranslational modification present in a wide range of organisms, from yeast to mammals (reviewed in (Neubert and Strahl 2016; Sheikh et al. 2017; Endo 2019; Larsen et al. 2019)). Mammalian O-mannosyl glycans were first discovered on glycoproteins in rat brain lysate about 40 years ago, which suggested that POM may play an important role in the nervous system (Finne et al. 1979). This hypothesis was later confirmed by identifying genetic disorders associated with defects in the POM pathway that cause pronounced neurological abnormalities in humans (Yoshida et al. 2001; Beltran-Valero de Bernabe et al. 2002). One of the most common types of these disorders is classified as congenital muscular dystrophies (CMDs), a group of debilitating neuromuscular abnormalities that are present at birth or in infancy and rapidly progress with age. CMDs that involve POM defects are commonly associated with more severe neurological phenotypes. The involvement of POM in the regulation of the nervous system has been documented now by many studies. New enzymes that mediate POM were recently discovered and many proteins were found to be O-mannosylated (Vester-Christensen et al. 2013; Larsen et al. 2017a; Larsen et al. 2023; Monagas-Valentin et al. 2023). However, the mechanisms of POM functions are still not well understood and significant knowledge gaps are associated with the paucity of functionally characterized substrates and limited structure–function information on different O-mannose linked glycans. In this review, we will discuss the recent studies on POM while focusing on the known and proposed roles of this posttranslational modification in the nervous system. We will summarize the current knowledge about the biosynthesis of POM, review pathologies associated with POM abnormalities, and emphasize the evolutionary perspectives revealed by studies in the *Drosophila* model system. Our review will also highlight the gaps in understanding POM biosynthesis and posit important questions about molecular and cellular mechanisms associated with POM functions.

Biosynthesis of O-mannosyl glycan modifications of proteins

Enzymes initiating POM

Posttranslational modification of proteins with O-linked mannose was first described in yeast and later found to be widespread in other organisms, from fungi to mammals (Falcone and Nickerson 1956; Sentandreu and Northcote 1968; Finne et al. 1979; Chiba et al. 1997). Three families of glycosyltransferase enzymes that modify serine and threonine residues of proteins with O-mannose have been found in mammalian cells. They all localize to the ER and use dolichol-phosphate-mannose (Dol-P-Man) as an activated sugar donor to modify protein substrates, however their molecular targets are different (see below) (Fig. 1). The initially discovered family is comprised of two protein O-mannosyltransferases 1 and 2 (POMT1 and 2) that are highly conserved in metazoans and their origin can be traced in evolution to yeast PMT4 and PMT2 O-mannosyltransferases, respectively (reviewed in Nakamura et al. 2010a; Neubert and Strahl 2016). POMT1 and POMT2 work together as an obligatory enzymatic complex (Manya et al. 2004), in a presumed heterodimer configuration, analogous to the PMT1-PMT2 complex

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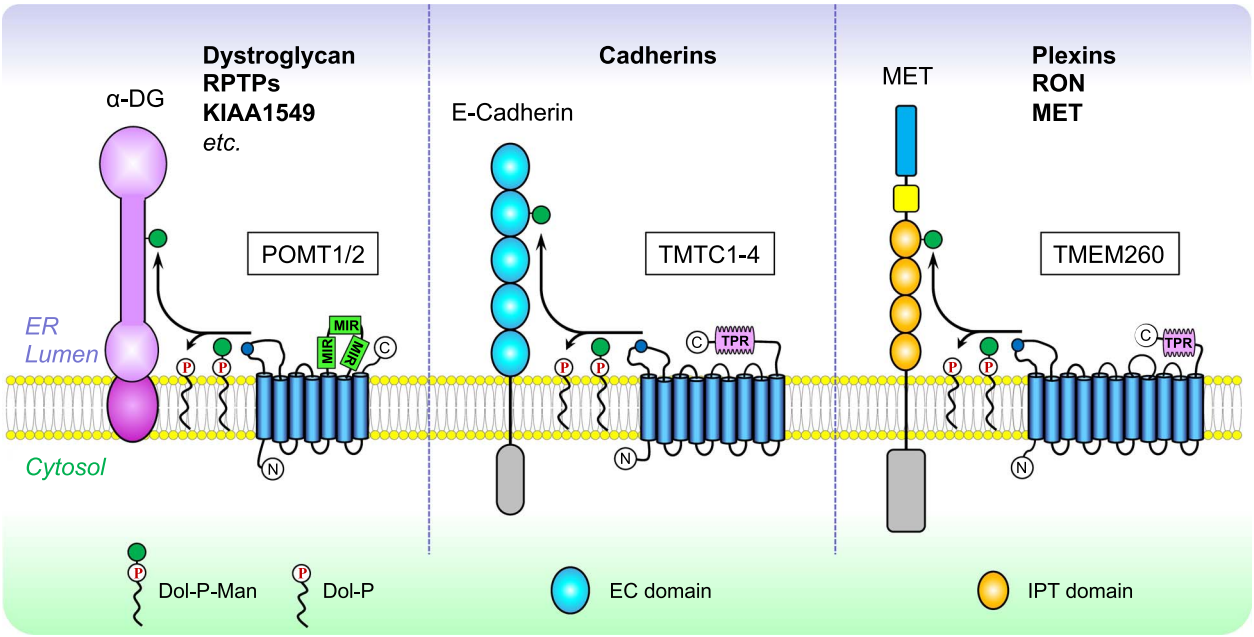

Fig. 1. Three families of enzymes mediating POM in animal cells. POMT1/2 O-mannosylate α-DG, RPTPs, KIAA1549, and some other proteins, whereas TMTC1–4 specialize in attaching O-mannose to the EC domains of cadherins. TMEM260, a recently discovered O-mannosyltransferase, is responsible for modifying the IPT domains of plexins and transmembrane receptor tyrosine kinases RON and MET. All these POM-mediating enzymes work in the ER and use Dol-P-Man as a donor substrate. They have a similar molecular architecture of integral membrane proteins with multiple membrane-spanning helixes, catalytically important aspartic acid residues in the first luminal loop (blue circles), and include different functional domains that are thought to be involved in substrate interactions (such as MIR and TPR). The substrate recognition of the enzymes remains not well understood. Modified from Larsen et al. (2019).

mediating POM in yeast (Bai et al. 2019). Several studies attempted to elucidate the substrate specificity of POMTs, however, no local consensus sequence recognized by these enzymes was determined as the substrate recognition appears to rely on some distant structural elements that remain poorly understood (as discussed elsewhere; Nakamura et al. 2010a; Neubert and Strahl 2016; Larsen et al. 2019). The number of known protein substrates of POMTs remain limited; they include dystroglycan (DG), receptor protein tyrosine phosphatases (RPTPs), KIAA1549, and some other proteins (Manya et al. 2004; Larsen et al. 2017b; Monagas-Valentin et al. 2023).

More recently, a second family of O-mannosyltransferases was discovered in human cells. It is represented by four structurally similar proteins originally known under the generic name transmembrane and tetratricopeptide repeat (TPR)-containing proteins 1–4 (TMTC1–4) (Larsen et al. 2017a). Mass-spectrometry (MS)-based glycoproteomic analyses combined with the SimpleCell technology (allowing efficient analyses of glycans in genetically modified mammalian cultured cells with simplified glycosylation) revealed that these enzymes add O-mannose to the extracellular cadherin (EC) domains of cadherins and related proteins (Fig. 1). Thus, to reflect their substrate specificity, TMTCs were renamed as transmembrane O-mannosyltransferases targeting cadherins. Remarkably, individual TMTCs can target distinct strands of the EC domains (Larsen et al. 2017a; Larsen et al. 2017b), suggesting that these enzymes have the substrate specificities finely tuned to recognize different structural features of the same EC fold. TMTCs are thought to interact with their protein substrates via TPR motifs located at TMTCs' C-termini, in a way analogous to the substrate recognition mediated by the TPR motifs of the O-GlcNAc transferase that carries out nucleocytoplasmic O-GlcNAcylation (Zachara et al. 2022).

A third type of O-mannosylating enzymes was predicted to exist because O-mannose was also identified on the IPT (Ig-like, plexin, and transcription factor) domains of proteins, including plexins and receptor tyrosine kinases MET (mesenchymal-epithelial transition factor) and RON (receptor originated from Nantes), however, POMTs and TMTCs could not modify IPT domains (Larsen et al. 2017a). Combining bioinformatics, MS-based glycoproteomics, and CRISPR/Cas9 genetic engineering of cultured cells, an elegant study by Halim, Joshi and collaborators recently identified *TMEM260* as the gene responsible for IPT O-mannosylation (Larsen et al. 2023) (Fig. 1). Known targets of TMEM260 (transmembrane protein 260) include multiple plexins and plexin-related proteins, such as two homologous receptor tyrosine kinases, hepatocyte growth factor receptor MET (also known as c-MET) and RON (also known as macrophage stimulating one receptor (Larsen et al. 2023)).

The three families of animal protein O-mannosyltransferases are evolutionarily related and share the characteristic features of the GT-C superfamily of glycosyltransferases (Moremen and Haltiwanger 2019), the integral membrane enzymes that use isoprenoid-linked carbohydrate donor substrates. This superfamily also includes tryptophan C-mannosyltransferase, oligosaccharyltransferase, and ALG transferases working in the N-glycosylation pathway, and glycosyltransferases involved in the GPI (glycosylphosphatidylinositol) biosynthesis (Albuquerque et al. 2019; Bloch et al. 2020; Bai and Li 2021; Bloch et al. 2023). Although these glycosyltransferases do not show significant overall sequence homology, they have similar molecular architecture, including a conserved GT-C module with seven membrane-spanning helices and catalytic

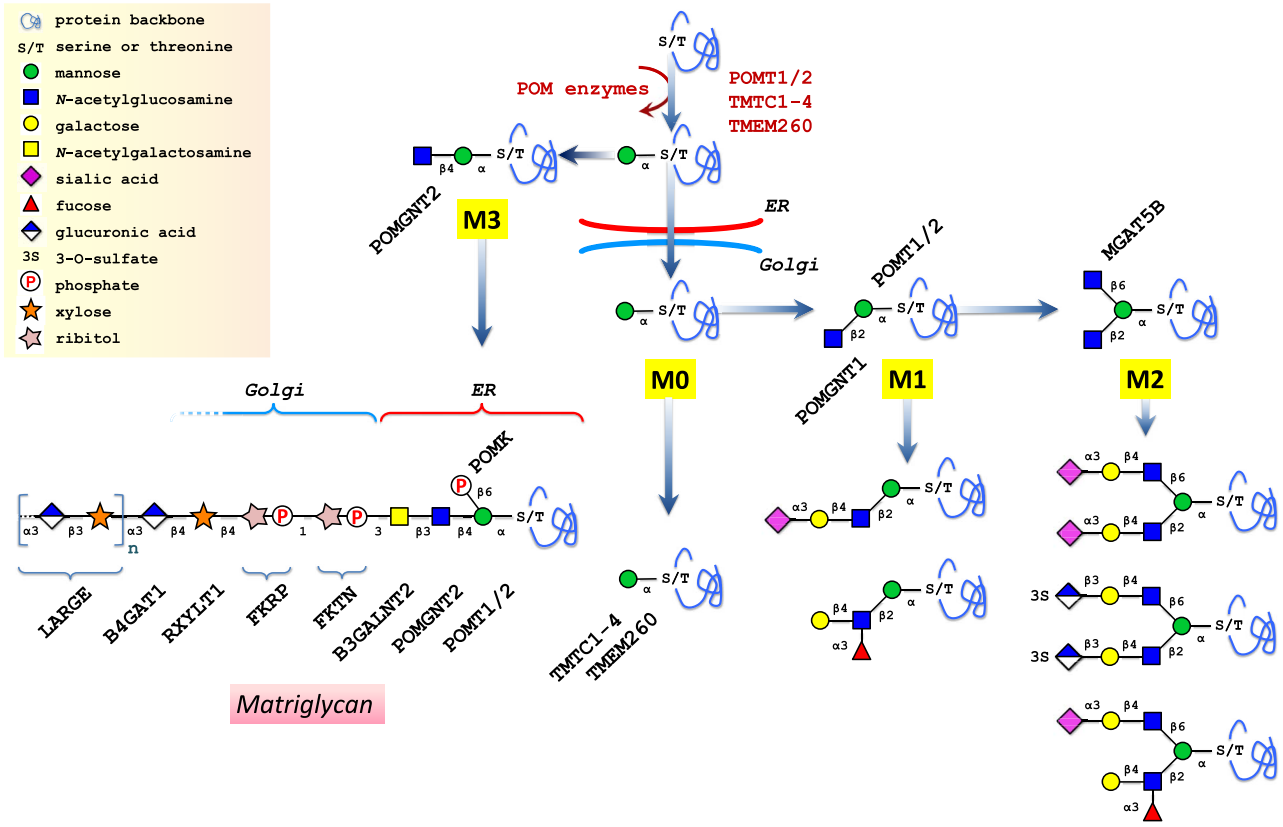

Fig. 2. Biosynthesis of POM. POM is initiated in the ER by three families of O-mannosyltransferases: POMT1/2, TMTC1–4 (transmembrane O-mannosyltransferases targeting cadherins), and TMEM260. Depending on a protein substrate, the O-mannose attached to a protein can remain non-elongated (M0 structure), or undergo further modification, such as elongation in the Golgi with β1,2-GlcNAc by POMGnT1 (protein O-mannose β1, 2-N-acetylglucosaminyltransferase 1), which creates core M1 structure, and additional modification with β1,6-GlcNAc by MGAT5B (α1,6-Mannosylglycoprotein 6-β-N-Acetylglucosaminyltransferase B), which creates core M2. M1 and M2 are further modified by enzymes that are not specific for POM, such as a galactosyltransferase, a sialyltransferase, etc., which results in structures with terminal sialic acid, HNK-1 (human natural killer 1 carbohydrate HSO_3-3GlcAβ1-3Galβ1-4GlcNAc-), or Lewis^X (Galβ1-4(Fucα1-3)GlcNAc-) epitopes. As an alternative to M1/M2 biosynthesis, the O-mannose can be modified in the ER with β1,2-GlcNAc by POMGnT2 (protein O-mannose β1,4-N-acetylglucosaminyltransferase 2), which creates core M3 and allows for further modification by the enzymes involved in the biosynthesis of matriglycan: B3GALNT2, POMK, FKTN (ribitol-5-phosphate transferase), FKRP (ribitol-5-phosphate transferase), RXYLT1, B4GAT1 (β1,4-glucuronyltransferase 1), and LARGE (β1,3-glucuronyltransferase and α1,3-xylosyltransferase, a bifunctional glycosyltransferase-polymerase that creates a long chain of -3GlcAβ1-3Xylα1- disaccharide repeats).

residues in its first luminal loop, as well as functional domains interacting with different substrates and regulating enzyme specificity and mechanism, such as MIR domains and TPR motifs (Bloch et al. 2020; Chiapparino et al. 2020; Bai and Li 2021). In contrast to POMTs that target unstructured mucin-like region of α-DG, TMTCs, and TMEM260 recognize specific folded domains (Endo 2019; Larsen et al. 2019; Larsen et al. 2023), suggesting distinct mechanisms of substrate recognition. How these different enzymes recognize and carry out O-mannosylation of their substrates remains an important focus of future studies.

Structure of O-mannosyl glycans

Depending on the context of a protein substrate, O-mannose can undergo further extension with additional sugar residues, resulting in linear oligo mannose structures in yeast, or more complex, heterogeneous structures in mammals (Neubert and Strahl 2016; Sheikh et al. 2017). Extended O-mannosyl glycans in mammalian cells can be built on POMTs-modified glycoproteins, whereas, so far, there is no evidence that O-mannose can be elongated on the EC and the IPT domains modified by TMTCs and TMEM260, respectively. The best-studied POMTs' substrate is DG, a highly glycosylated cell-surface glycoprotein modified with complex O-mannose glycans that are crucial for interactions with the extracellular matrix (ECM; Barresi and Campbell 2006). Extended O-mannose oligosaccharide structures were originally discovered on the extracellular subunit of DG (termed α-Dystroglycan) in bovine peripheral nerve, and since then, a motley of extended O-mannose-linked glycans have been found on α-DG and some other proteins, such as RPTPζ/phosphacan (Chiba et al. 1997; Morise et al. 2013; Dwyer et al. 2015), reviewed in Praissman and Wells (2014) and Endo (2019), revealing a remarkable complexity of possible elongation of O-mannose in mammalian cells (Fig. 2). Core O-mannosyl glycan structures are classified depending on their type of elongation as M0 (unextended O-mannose), M1 (O-mannose modified with β1,2-GlcNAc, non-branched structures), M2 (O-mannose modified with β1,2- and β1,6-linked GlcNAc, branched structures), and M3 (initiated on O-mannose by the addition of β1,4-GlcNAc). M1 is synthesized

on O-mannose by POMGNT1 (protein O-mannose β1,2-N-acetyl-glucosaminyltransferase 1) and can serve as a precursor for M2 that is built by the addition of β1,6-GlcNAc by the branching N-acetylglucosaminyltransferase MGAT5B (α1,6-mannosylglycoprotein 6-β-N-acetylglucosaminyltransferase B, also known as GNT-VB or GNT-IX). M1–2 cores are usually elongated with short sugar chains built by enzymes that are not specific for O-mannosyl glycans, thus creating terminal structures also present on other types of carbohydrate chains. The termini of M1 and M2 can be sialylated, carry Lewis^X, or modified with HNK-1 (Human Natural Killer-1) carbohydrate epitopes (Fig. 2) (Chiba et al. 1997; Smalheiser et al. 1998; McDearmon et al. 2006).

Much attention has been drawn to a particularly unique and functionally important structure called matriglycan, the phosphorylated M3 glycans carrying a long glycosaminoglycan-like polysaccharide chain composed of (-3GlcAβ1-3Xylα1-) disaccharide repeats (Inamori et al. 2012). Matriglycan is one of the most complex, elaborately built glycan structures found in mammalian cells. The enzymatic steps of its biosynthesis were elucidated by a combination of advanced MS, biochemical, and genetic approaches (Yoshida-Moriguchi et al. 2010; Kanagawa et al. 2016; Praissman et al. 2016), reviewed in (Yoshida-Moriguchi and Campbell 2015; Sheikh et al. 2017; Endo 2019). Unlike M1 and M2 that are synthesized on O-mannosylated glycoproteins after they are transferred from the ER to the Golgi, the M3 core is built in the ER by the addition of β1,4-linked GlcNAc mediated by POMGNT2 (protein O-mannose β1,4-N-acetylglucosaminyltransferase 2). This is the commitment step in M3 biosynthesis, thus POMGNT2 serves as a gatekeeper enzyme for building matriglycan (Yoshida-Moriguchi et al. 2013; Halmo et al. 2017). The β1,4GlcNAc of the M3 core is further elongated in the ER with β1,3-GalNAc by B3GALNT2 (β1,3-N-acetylgalactosaminyltransferase 2), and then the glycan undergoes phosphorylation of O-mannose at the C6 position, which is carried out by POMK (protein-O-mannose kinase). The following steps of matriglycan biosynthesis are mediated in the Golgi, first, by the orchestrated work of another three glycosyltransferases, Fukutin (FKTN), Fukutin-related protein (FKRP), and RXYLT1 (ribitol-5-phosphate β1,4-xylosyltransferase 1, also known as TMEM5). They build a substrate structure for LARGE (β1,3-glucuronyltransferase and α1,3-xylosyltransferase-polymerase, also known as "like-acetylglucosaminyltransferase"), a bifunctional glycosyltransferase-polymerase that synthesizes a long chain of (-3GlcAβ1-3Xylα1-) disaccharide repeats (Fig. 2). The mechanism of chain length regulation is not fully understood; however, recent studies suggested that several factors can play roles in this process. They include the modulation of LARGE activity by POMK-mediated phosphorylation of O-mannose, the regulation involving the N-terminal domain of α-DG, as well as the competition of LARGE for the GlcA termini of matriglycan with HNK-1 sulfotransferase that is known to target matriglycan termini in the nervous system (Sheikh et al. 2020; Walimbe et al. 2020; Okuma et al. 2023). An additional layer of regulation is possibly mediated by factors that affect activities of the enzymes involved in matriglycan biosynthesis, such as CDP-glycerol that inhibits Fukutin and FKRP (Imae et al. 2018), however, so far little is known about these mechanisms and how they can operate *in vivo*.

Function of O-mannosyl glycans and disorders associated with their defects

Matriglycan

So far, matriglycan has only been detected on α-DG, and it remains the best functionally studied O-mannosyl glycan. Defects in matriglycan biosynthesis are associated with severe muscular dystrophies, collectively termed dystroglycanopathies (Table 1), which emphasizes the importance of this carbohydrate structure for neuromuscular development and homeostasis.

Matriglycan is essential for binding between α-DG and LG (laminin-globular) domains of extracellular ligands that are usually embedded in the ECM, such as laminin, agrin, perlecan, neurexin, and pikachurin (Hohenester 2019). DG (encoded by the *Dag1* gene) is a central component of the dystrophin-associated glycoprotein complex (DGC) that provides an essential bridge between the ECM and the cytoskeleton via interactions with dystrophin and other DGC-associated proteins inside the cell (Fig. 3; Ibraghimov-Beskrovnaya et al. 1992; Ervasti and Campbell 1993), reviewed in Barresi and Campbell (2006). The size of LARGE-synthesized matriglycan correlates with the ability of α-DG to bind ECM ligands, which inversely correlates with the clinical severity of associated dystroglycanopathies (Goddeeris et al. 2013; Walimbe et al. 2020; Okuma et al. 2023).

Animal models of dystroglycanopathies significantly elucidated the relationship between pathomechanisms of these disorders and defects in matriglycan biosynthesis (Yoshida-Moriguchi and Campbell 2015; Nickolls and Bonnemann 2018; Endo 2019; Kanagawa 2021). Functional matriglycan structures can be detected on α-Dg using IIH6 antibody or ligand binding assays on western blots (e.g. a Laminin overlay assay; Ervasti and Campbell 1993; Michele et al. 2002). The size of a functional fully glycosylated α-DG is cell-specific: α-DG form corresponding to an $\sim$ 150–250 kDa band is present in skeletal muscles, a shorter $\sim$120 kDa form is more prevalent in the brain, whereas an intermediate size form ($\sim$180 kDa) is produced by some neurons in the cerebellum (such as Purkinje cells; Smalheiser and Schwartz 1987; McDearmon et al. 2006; Satz et al. 2010). These differences in the size probably reflect the function of matriglycan in cell-specific fine tuning of cell adhesion. Without proper interactions with N-terminal part of α-DG, LARGE synthesizes a short form of matriglycan ($\sim$100–120 kDa) that can still bind laminin and maintain the specific force of muscles; however, this causes a force deficit induced by lengthening contractions, which is also associated with dystrophic changes in muscles (Okuma et al. 2023). The size of matriglycan is important for the proper morphology of neuromuscular junctions (NMJ) and normal distribution of AChRs (acetylcholine receptors) at NMJ synapses, indicating that NMJ synaptic maturation requires a fully extended matriglycan (Nishimune et al. 2008; Okuma et al. 2023). Synaptic functions in the hippocampus are also affected by LARGE deficiency, as it is evident from reduced long-term potentiation of CA3-CA1 synapses in *LARGE^{myd}* mutant mice with a spontaneous mutation inactivating the gene (Lane et al. 1976; Satz et al. 2010). Severe dystroglycanopathies, such as Walker-Warburg Syndrome and Muscle-Eye-Brain (MEB) disease caused by defects in LARGE and other enzymes required for matriglycan biosynthesis, are usually associated with pronounced brain defects, including

Table 1. Congenital disorders and phenotypes associated with defects in POM.

Gene/protein	Associated congenital disorders[a]			Clinical findings and phenotypes	
				Neurological	Other
DAG1 Dystroglycan	1°	Dystroglycanopathies	Walker-Warburg Syndrome (WWS) *OMIM 236670*	Severe brain abnormalities: encephalocele, hydrocephalus, "cobblestone" cortex/agyria, absence of corpus callosum, fusion of cerebral hemispheres, atrophy of the cerebellar vermis. Eye abnormalities: unilateral or bilateral microphthalmia, buphthalmos, hypoplastic or absent optic nerves, retinal detachment, common anterior chamber malformations.	Severe recessive muscular dystrophy, elevated serum creatine kinase (CK) levels
POMT1–2 Protein O-mannosyl-transferases 1–2 ***POMGnT1*** Protein O-mannose β1,2-N-acetylglucosaminyltransferase ***POMGnT2*** Protein O-mannose β1,4-N-acetylglucosaminyltransferase ***B3GALNT2*** β1,3-N-acetylgalactosaminyl-transferase ***POMK*** Protein-O-mannose kinase ***RXYLT1*** Ribitol-5-phosphate β1,4-xylosyltransferase 1 ***B4GAT1*** β1,4-glucuronyltransferase ***FKTN*** Ribitol-5-phosphate transferase ***FKRP*** Ribitol-5-phosphate transferase ***LARGE*** β1,3-glucuronyltransferase and α1,3-xylosyltransferase	2°		Muscle-Eye-Brain Disease (MEB) *OMIM 253280*	Brain abnormalities: cerebellar hypoplasia, cortical dysplasia, "cobblestone" cortex, pachygyria/polymicrogyria/agyria, common epilepsy seizures, flattening of the pons and brainstem. Eye abnormalities: high myopia, retinal atrophy, retinal dysplasia, glaucoma, cataracts, retinal detachment. In rare cases: severe autistic features, tonic–clonic seizures (overall less severe than WWS)	Recessive muscular dystrophy, profound muscle hypotonia, impaired mobility, impared speach, muscle degeneration, fibrosis, elevated serum CK level.
			Fukuyama Congenital Muscular dystrophy (FCMD) *OMIM 253800, 608840*	Brain: cerebral and cerebellar micropolygyria, fibroglial proliferation of the leptomeninges, hydrocephalus, focal interhemispheric fusion, and hypoplasia of the corticospinal tracts. Eye abnormalities: microphthalmia, retinal detachment, buphthalmos malformations. Seizures, and mental retardation.	Recessive muscular dystrophy, generalized muscle weakness and hypotonia from early infancy, most patients are unable to walk without support, congenital cataracts, elevated serum CK levels.
CRPPA CDP-L-ribitolpyrophosphorylase A ***DPM1–3*** Dolichol-phosphate mannose synthases 1–3 ***MPI*** Mannose phosphate isomerase ***DOLK*** Dolichol kinase ***PMM2*** Phosphomannomutase 2 ***GMPPB*** GDP-mannose pyrophosphorylase B	3°		Muscular dystrophies with Brain and Eye Anomalies, and with Impaired Intellectual Development (MDDGA6, MDDGB6) *OMIM 613154, 608840*	Most severe cases represent LARGE-related WWS and MEB syndromes. Clinical findings include ventricular dilatation, absence of the inferior cerebellar vermis, hypoplastic cerebellum, optic atrophy, and retinal dysplasia. Some patients show hydrocephalus and Dandy-Walker malformation.	At birth, affected babies showed severe muscle dystrophy phenotypes, hypotonia, absent deep tendon reflexes, widened anterior fontanels, congenital cataracts, elevated serum CK levels.
			Limb-Girdle Muscular Dystrophy *OMIM 609308, 611588*	Mild mental retardation, microcephaly, essentially normal brain structure and morphology by MRI, with occasional mild hydrocephalus.	Muscle weakness, hypertrophy of the calf muscle, cardiomyopathy, elevated serum CK levels.
TMTC1 O-mannosyltransferase targeting cadherins 1	Schizophrenia[b] OMIM *617629*			Schizophrenia, schizoaffective disorder, cognitive impairment, moody disorder.	N/A
TMTC2 O-mannosyltransferase targeting cadherins 2	Sensorineural Hearing Loss (SNHL) Auditory Neuropathy Spectrum Disorder (ANSD)[c] *OMIM 615856*			SNHL, and ANSD as a subtype, associated with abnormal or absent auditory brainstem responses. Starts as high frequency hearing loss that progresses to severe-to-profound hearing loss over a period of several years.	N/A
TMTC3 O-mannosyltransferase targeting cadherins 3	Cobblestone Lissencephaly LIS8 *OMIM 617255*			Delayed psychomotor development, intellectual disability with poor or absent speech, early-onset refractory seizures, and hypotonia. Brain abnormalities include cobblestone lissencephaly, cortical gyral abnormalities and hypoplasia of the corpus callosum, brainstem, and cerebellum. Ocular atrophy.	Congenital cataracts, clubfeet
TMEM260 Transmembrane protein 260	Structural Heart Defects and Renal Anomalies Syndrome (SHDRA) *OMIM 617478*			Agenesis of the corpus callosum, microencephaly, undefined brain malformations.	Severe complex cardiac abnormalities, central cyanosis, heart murmur, general edema, renal and digit abnormalities, necrotizing enterocolitis

Mutations in a group of genes involved in the biosynthesis of matriglycan cause dystroglycanopathies, a type of congenital muscular dystrophies associated with hypoglycosylation of α-Dystroglycan. Depending on the role of a gene in the pathway, dystroglycanopathies are classified as primary (1°, *DAG1* mutations), secondary (2°, mutations in genes encoding enzymes that modify α-DG), or tertiary (3°, defects in genes indirectly required for matriglycan biosynthesis, such as genes involved in generating sugar donors for glycosyltransferases that build matriglycan). Note that this classification is not aligned with particular CMDs, such as WWS, MEB, and limb-girdle muscular dystrophies, as different mutations in a dystroglycanopathy gene can cause different CMDs with a range of severity, depending on the strength of alleles and other genetic factors. Mutations in *TMTC1–4* and *TMEM260* are not associated with defects in α-DG glycosylation but they cause defects in POM modifications of the EC domains of cadherins and IPT domains of plexins and some receptor tyrosine kinases, respectively. [a]Includes disorders that are caused by mutations in indicated genes (e.g. CMD disorders caused by dystroglycanopathy genes, LIS8 and SHDRA caused by mutations in *TMTC3* and *TMEM260*, respectively), as well as disorders with gene associations not yet analyzed by functional studies (e.g. schizophrenia and SNHL/ANSD associated with *TMTC1* and *TMTC2*, respectively). [b]Based on GWAS studies (Levinson et al. 2012; Schizophrenia Working Group of the Psychiatric Genomics Consortium 2014). [c]Included in OMIM as an association pending confirmation. The table is not intended to be comprehensive, and it highlights instead some characteristic examples of disorders and phenotypes. Based on selected publications discussing dystroglycanopathies (Imbach et al. 2000; Kim et al. 2000; Yoshida et al. 2001; Beltran-Valero de Bernabe et al. 2002; Kano et al. 2002; Moore et al. 2002; Taniguchi et al. 2003; Beltran et al. 2004; van Reeuwijk et al. 2005; van Reeuwijk et al. 2006; Godfrey et al. 2007; Martin 2007; van Reeuwijk et al. 2007; Clement et al. 2008; Messina et al. 2009; Vuillaumier-Barrot et al. 2009; Yoshida-Moriguchi et al. 2010; Clarke et al. 2011; Hara et al. 2011; Barone et al. 2012; Devisme et al. 2012; Manzini et al. 2012; Roscioli et al. 2012; Vuillaumier-Barrot et al. 2012; Willer et al. 2012; Buysse et al. 2013; Stevens et al. 2013; Riemersma et al. 2015; Radenkovic et al. 2021), disorders associated with mutations in *TMTCs* (Guillen-Ahlers et al. 2018; Jerber et al. 2016; Li et al. 2018; Mealer et al. 2020; Runge et al. 2016; Schizophrenia Working Group of the Psychiatric Genomics Consortium 2014), and TMEM260 (Ta-Shma et al. 2017; Pagnamenta et al. 2022).

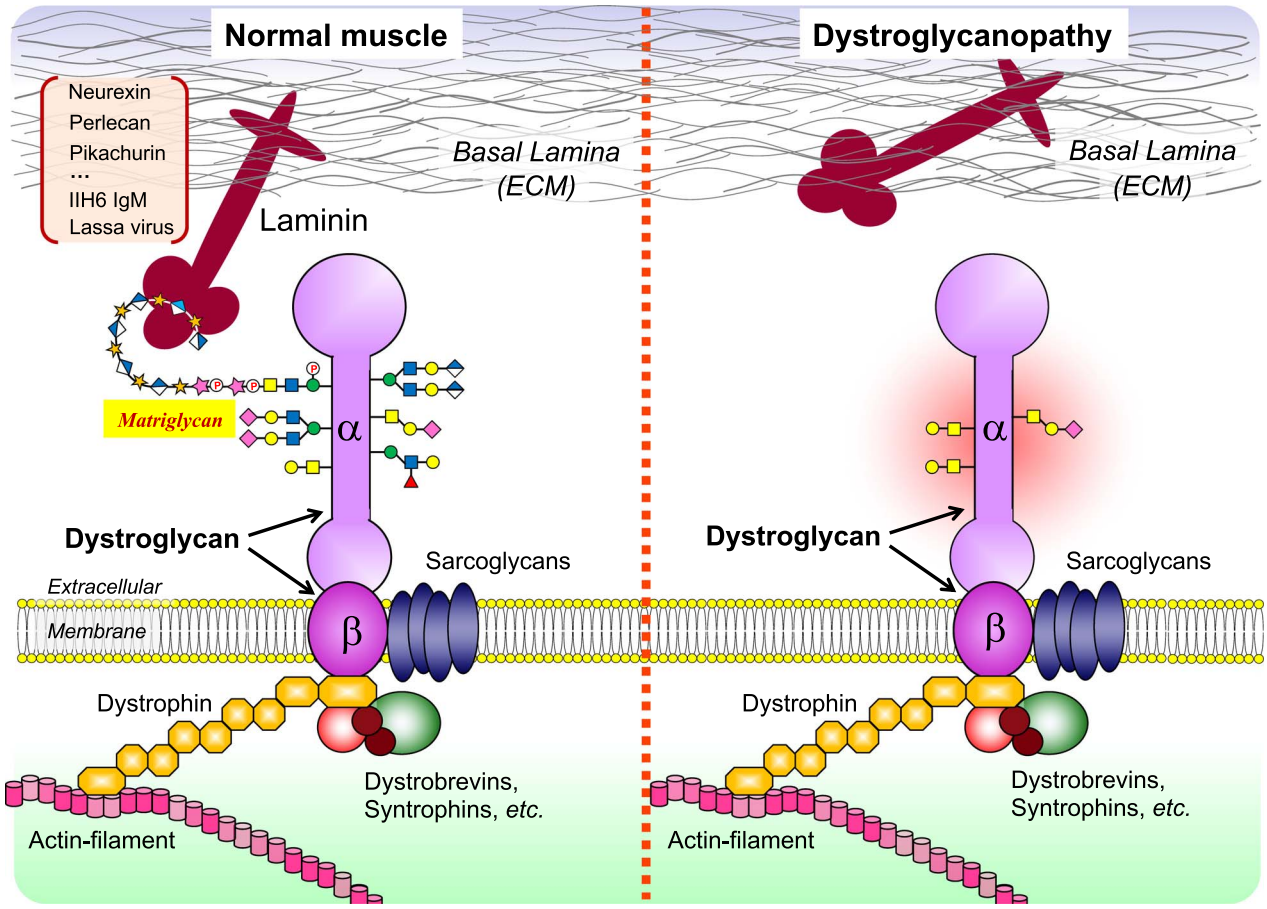

Fig. 3. Modification of α-DG with matriglycan is essential for interaction of the DGC complex with extracellular ligands. Left panel: α-DG ligands imbedded in the ECM, such as Laminin, Agrin, etc., bind to matriglycan-modified O-mannosyl glycans. Inside the cell, DGC interacts with actin filaments via dystrophin, which creates a DGC-mediated bridge between the basal lamina outside the cell and the cytoskeleton inside the cell. Note that matriglycan is also specifically recognized by IIH6 IgM antibody in vitro and by some viruses (e.g. Lassa virus) that use binding to matriglycan as a mechanism for cell infection. In *POMT* mutants (right panel), the O-mannosylation of α-DG is abolished, which disrupts α-DG interactions with the ECM ligands, leading to muscular dystrophy phenotypes (dystroglycanopathy).

cobblestone lissencephaly, hydrocephalus, cortical and cerebellar dysplasia, ocular defects, cognitive disability, and other neurological abnormalities (Table 1; Godfrey et al. 2007; Clement et al. 2008; Devisme et al. 2012).

The function of DG is particularly important for the integrity of the pial basement membrane, and O-mannosylation required for DG-laminin binding plays a central role in this process (Moore et al. 2002; Myshrall et al. 2012). Ruptures in the pial basement membrane, mislocalization of glial cells, and associated abnormal neuronal migration are common phenotypes of dystroglycanopathies, and they are recapitulated in mouse KO models with mutations in *LARGE*, *POMT1/2*, *POMGNT1*, *POMGNT2*, and *FKRP* (Michele et al. 2002; Hu et al. 2007; Li et al. 2008, 2011; Ackroyd et al. 2009; Chan et al. 2010; Hu et al. 2011; Nakagawa et al. 2015). Axon guidance defects in longitudinal axonal tracts in the hindbrain and the spinal cord are caused by mutations in genes encoding CRPPA (CDP-L-ribitol pyrophosphorylase A, also known as ISPD, an enzyme producing CDP-ribitol, the sugar donor for FKTN and FKRP (Fig. 2)) and B4GAT1, whereas *CRPPA* mutants also have disorganized optic chiasm axons, which largely phenocopies axon defects in *Dag1* conditional KO mutants (Wright et al. 2012; Clements and Wright 2018).

Unexpectedly, mutations in *POMGNT1* were also found to be associated with MEB syndrome and a defect in matriglycan, even though this gene encodes β-1,2-N-acetylglucosaminyltransferase that works in the biosynthesis of M1 structures and does not enzymatically participate in making matriglycan (Fig. 2; Yoshida et al. 2001). This paradox was explained by unveiling a non-enzymatic role of POMGNT1 in the recruitment of Fukutin to non-extended M3 structures via a direct protein complex formation and protein-carbohydrate interactions with M1 structures nearby, which potentiates further maturation of M3 (Kuwabara et al. 2016).

Taken together, these studies suggest that all known major functions of DG require its proper O-mannosylation. This notion was further supported by a recent elegant research that used enzymatic glycoengineering to synthesize matriglycan on an irrelevant carrier in the cells that lack DG, which restored Laminin binding, induced the IIH6 reactivity, and was able to support a Lassa-pseudovirus infection, (Sheikh et al. 2022), the key molecular/cell properties that normally require a functional DG (Fig. 3). This and other studies underscored the importance of thorough understanding of the biosynthesis and functions of O-mannosyl glycans for translational research. Recent progress in this area stimulated studies on

gene therapy and pharmacological approaches, which showed promising results. Mouse models of dystroglycanopathies, for example, demonstrated that even a partial restoration of DG glycosylation during fetal development can significantly ameliorate neurological phenotypes (Sudo et al. 2018), that *LARGE* gene transfer in older *LARGE*^{myd} mutant mice with severe muscular dystrophy restores skeletal muscle function, normalizes systemic metabolism, and greatly improves survival (Yonekawa et al. 2022), and that a CDP-ribitol prodrug can be an effective treatment for conditions with a defect in the biosynthesis of CDP-ribitol, the sugar donor required for FKTN and FKRP (Tokuoka et al. 2022). These proof-of-principle studies pave the way for future development of therapies for dystroglycanopathies.

RPTPs and M1/M2 glycans

RPTPζ (also known as phosphacan) represents the first target of POM discovered in metazoans (Krusius et al. 1986; Maurel et al. 1994; Dwyer et al. 2012). This RPTP belongs to the R5 subgroup of the big evolutionarily conserved family of transmembrane receptor-type protein phosphatases involved in the regulation of a wide spectrum of cell-adhesion and cell-signaling interactions (reviewed in Tonks 2006; Xu and Fisher 2012). RPTPζ is highly expressed in the mammalian brain and required for the development of perineuronal nets (Eill et al. 2020), the prominent aggregated ECM structures that surround the cell body and proximal neurites and are involved in brain plasticity and memory modulation (Fawcett et al. 2022). Earlier studies indicated that RPTPζ is modified with chondroitin sulfate chains and O-mannosyl glycans bearing keratan-sulfate (Maurel et al. 1994); however, these glycans were not well characterized and their detailed analysis awaits modern glycoproteomics approaches. More recent MS-based glycomics approaches revealed that RPTPζ is extensively modified with O-mannosyl glycans in the developing brain in a cell-specific manner. An array of different O-mannosyl glycans, including M0, M1, and M2 structures, was identified on RPTPζ (Pacharra et al. 2013; Trinidad et al. 2013; Dwyer et al. 2015; Bartels et al. 2016). M1 and M2 glycans were found with a variety of terminal modifications, including sialylated termini, Lewis^X and HNK-1 epitopes, and sulfo-LacNAc modifications; however, M3 structures were never detected on this glycoprotein (Dwyer et al. 2015).

Remarkably, RPTPζ is the major carrier of Lewis^X and HNK-1 epitopes in the developing brain (Stalnaker et al. 2011; Morise et al. 2014; Dwyer et al. 2015; Yaji et al. 2015). Considering that HNK-1 and Lewis^X structures are important for memory and learning, synaptic plasticity and brain development (Yamamoto et al. 2002; Yoshihara et al. 2009; Yaji et al. 2015), the modification of RPTPζ with O-mannosyl glycans are thought to play prominent parts in these processes; however, their underlying mechanisms remain not well understood. RPTPζ is notably hypoglycosylated in POMGNT1 mutants, indicating that it is modified with a significant number of M1 and M2 O-mannosyl glycans that require POMGNT1 for biosynthesis (Dwyer et al. 2012) (Fig. 2). This result also suggested that the phenotypes caused by defects in RPTPζ O-mannosylation (abnormal biosynthesis of M0-M2 glycans) may contribute to the neurological phenotypes of dystroglycanopathies associated with *POMT* and *POMGNT1* mutations.

The function of M2 structures is particularly interesting because they are thought to be strictly brain-specific as their biosynthesis depends on MGAT5B that shows a brain-limited expression (Inamori et al. 2003; Kaneko et al. 2003). Cell culture assays demonstrated that MGAT5B-mediated branching of O-mannosyl glycans promoted RPTPζ interactions with galectin-1 and the receptor dimerization, which inhibited its phosphatase activity and enhanced phosphorylation of β-catenin. These events are accompanied by decreased cell adhesion and increased cell migration, together suggesting a mechanism of O-mannosylation-mediated regulation of RPTPζ signaling and its effect on cell–cell and cell–ECM interactions (Abbott et al. 2008). However, MGAT5B knockout mice show no conspicuous neurological defects besides impaired astrocyte activation and abnormal axon remyelination in an induced demyelinating model (Lee et al. 2012; Kanekiyo et al. 2013), suggesting that M2 modifications function mainly in responses to neural insult and injury, whereas the involvement M2 structures in general regulation of neural cell adhesion is probably redundant *in vivo*.

RPTPζ remains an orphan substrate of O-mannosylation as so far there is no direct experimental evidence indicating what enzyme(s) is(are) responsible for its O-mannosylation. However, the presence of M1 and M2 structures suggests that RPTPζ is probably O-mannosylated by POMTs. This notion was recently reinforced by experiments using the *Drosophila* model that revealed that *Drosophila* RPTP 69D (PTP69D), a homolog of mammalian RPTPζ, is a substrate of POMT1–2 (Monagas-Valentin et al. 2023). Interestingly, the molecular architecture of PTP69D, including several Immunoglobulin-like (Ig) and Fibronectin type 3 (FN3) domains in its extracellular part, closely resembles that of LAR and other R2A-type RPTPs, such as PTPσ and PTPδ (Johnson and Van Vactor 2003; Coles et al. 2015), suggesting that these mammalian counterparts might also be substrates of POMTs. R2A-type RPTPs are involved in synaptogenesis, axon guidance and nerve regeneration, which indicates an intriguing possibility that these functions may be regulated by POMT-mediated O-mannosylation of the R2A receptors. Further biochemical and *in vivo* studies are required to understand the function of O-mannosyl glycan modification of RPTPs in greater detail.

Cadherins and TMTCs

The cadherin superfamily in mammals encompasses more than 100 cell surface receptor-type molecules that mediate cell adhesion and signaling and regulate a wide spectrum of processes, from separation of embryonic cell layers to synapse formation in the nervous system and to tissue homeostasis at the adult stage (Halbleib and Nelson 2006). Extracellular parts of cadherins include EC domains with a characteristic structure of a β-sandwich fold including ~110 amino acids, the functional units that mediate homophilic interactions and clustering of cadherins extending from apposed cells (Brasch et al. 2012; Troyanovsky 2023). Cadherins represent the largest group of known substrates of O-mannosylation in animals, with TMTCs being the enzymes that are dedicated to attaching O-mannose to their EC domains (Vester-Christensen et al. 2013; Larsen et al. 2017a; Larsen et al. 2017b). O-mannose on cadherins is not elongated (Lommel et al. 2013; Winterhalter et al. 2013; Larsen et al. 2017b), and different TMTCs are responsible for O-mannosylation of B- and G-strands located on opposite sides of the β-fold structure, suggesting that O-mannosylation of cadherins is a highly regulated process, and that O-mannose on distinct EC strands may have different functions ((Larsen et al. 2017a,

Larsen et al. 2017b), reviewed in Larsen et al. (2019). However, it remains unknown how O-mannose on cadherins can affect molecular interactions.

Mutations in *TMTC* genes are associated with severe neurological disorders, such as intellectual disability, epilepsy, brain malformations due to defects in neuronal migration, and microcephaly (*TMTC3*), sensorineural hearing loss and auditory neuropathy (*TMTC2*), and schizophrenia (*TMTC1*; Schizophrenia Working Group of the Psychiatric Genomics Consortium 2014; Runge et al. 2016; Farhan et al. 2017; Guillen-Ahlers et al. 2018; Hana et al. 2020) (Table 1). Knockout of *TMTC4* in mice results in acquired hearing loss, the condition analogous to noise-induced hearing loss in humans (Li et al. 2018), which is consistent with proposed involvement of *TMTC* defects in sensorineural hearing loss disorders (Guillen-Ahlers et al. 2018). Interestingly, defects in *TMTC3* were found to be associated with cobblestone lissencephaly and periventricular nodular heterotopia, the conditions known to result from abnormal neuronal migration (Jerber et al. 2016; Farhan et al. 2017; Liu et al. 2020). The defects in cell migration are consistent with the proposed role of TMTCs in regulation of cadherins that function in cell adhesion and migration, and these neurological phenotypes are hypothesized to result from defects in cadherin functions (Graham et al. 2020; Liu et al. 2020). Genetic manipulation of TMTCs and E-cadherin in human cultured cells indicated that TMTC3 can potentiate E-cadherin-mediated cell adhesion, which further supported the hypothesis that TMTC3 is required for proper cadherin-mediated cell interactions (Graham et al. 2020). However, the effect of TMTCs on cadherin functions has not been analyzed *in vivo* and the function of TMTC-mediated O-mannosylation remain not well understood. Furthermore, the phenotypes not directly linked to cadherins were also found to be associated with abnormalities in *TMTCs*, such as activation of the unfolded protein response in *TMTC4* mutants, abnormal ER calcium homeostasis associated with deregulation of *TMTC1* and *2*, and the involvement of possible non-cadherin substrates of TMTC1 in ovarian cancer malignancy (Sunryd et al. 2014; Li et al. 2018; Yeh et al. 2023), which further complicates the dissection of pathological mechanisms caused by defects in TMTCs. Studies in model organisms and tissue culture systems are expected to accelerate research in this area, shedding more light on the mechanism of O-mannose-mediated regulation of cadherin functions.

IPT-containing substrates

Mammalian plexins represent a family of nine cell surface signaling molecules that serve as major receptors for semaphorins and play essential roles in cell–cell interactions, affecting a broad spectrum of processes, such as axon guidance, neuronal migration, macrophage activation, the development of cardiovascular system and bone morphogenesis (reviewed in (Worzfeld and Offermanns 2014)). Plexins have been also implicated in different pathologies, including cancer (Gurrapu and Tamagnone 2019). IPT domains play an important part in plexin activation (Kong et al. 2016), suggesting an intriguing possibility that O-mannose may affect this process; however, the role of O-mannosylation in plexin regulations remains unknown. Mutations in *TMEM260*, the gene encoding the O-mannosyltransferase responsible for modification of IPT domains, were recently found to be associated with SHDRA (structural heart defects and renal anomalies) syndrome, a genetic disorder characterized by developmental heart defects, kidney abnormalities, neurological defects, and perinatal death (Ta-Shma et al. 2017; Pagnamenta et al. 2022). A recent study analyzed the effect of O-mannosylation on TMEM260 substrates in cultured cells, which revealed that TMEM260 is required for proteolytic maturation and ER exit of Plexin-B2 and RON (Larsen et al. 2023). However, no effect on MET was detected in similar experiments, suggesting that O-mannose may impact the function of IPT-containing substrates by different mechanisms. It is tempting to draw parallels between the promotion of maturation of RON and Plexin-B2 by O-mannose and the effect of O-fucose on the secretion of substrates with EGF (epidermal growth factor) and TSR (thrombospondin type 1) repeats, which is mediated via stabilization and acceleration of the substrate folding (Holdener and Haltiwanger 2019). However, whether the molecular mechanisms underlying functions of these different types of O-glycosylation are indeed similar remains to be elucidated.

Evolutionary perspective from the *Drosophila* model

The functions of DG and POMTs are conserved in *Drosophila*

Drosophila genome encodes all essential protein components of the DGC complex, but they are represented by fewer homologs and DGC is predicted to show reduced complexity (Nakamura et al. 2010a). Unlike mammals that have a sole DG, *Drosophila* produces three different DG isoforms that are generated by the same *Dg* gene via alternative splicing (Deng et al. 2003). One of these isoforms includes a mucin-like region, the structural feature important for O-mannosylation of the mammalian counterparts (Deng et al. 2003; Nakamura et al. 2010b). Although POMT1 and POMT2 are conserved in flies (see below), *Drosophila* does not have close homologs of mammalian enzymes mediating the biosynthesis of extended O-mannosyl glycans, which is consistent with the fact that only non-elongated O-mannose has been identified in *Drosophila* (Aoki et al. 2008; Nakamura et al. 2010b; Sheikh et al. 2017; Monagas-Valentin et al. 2023). *Dg* was shown to regulate several developmental processes in flies, such as planar polarity of the basal actin stress fibers and oriented basement membrane fibrils in the ovarian follicle, as well as wing vein development (Deng et al. 2003; Christoforou et al. 2008; Mirouse et al. 2009; Cerqueira et al. 2020). *Dg* mutants have muscle defects during larval stages, and decreased mobility and age-dependent muscle degeneration as adult flies, thus showing the phenotypes reminiscent of dystroglycanopathies, which highlights the evolutionary conservation of DG function between *Drosophila* and mammals (Haines et al. 2007; Shcherbata et al. 2007). In the nervous system, *Dg* is required for photoreceptor axon pathfinding and normal synaptic transmission at larval NMJs where *Dg* affects glutamate receptor subunit composition and is required to maintain the normal level of Laminin and Dystrophin (Dys) (Shcherbata et al. 2007; Bogdanik et al. 2008; Wairkar et al. 2008). *Dg* and *Dys* show strong genetic interactions, affect similar functions, and usually have similar mutant phenotypes, further supporting the notion that the DGC function is conserved in flies (Shcherbata et al. 2007; van der Plas et al. 2007; Bogdanik et al. 2008; Christoforou et al. 2008; Wairkar et al. 2008; Marrone et al. 2011).

Drosophila orthologues of mammalian POMT1 and POMT2 are encoded by the *rotated abdomen (rt)* and *twisted (tw)* genes that were discovered due to the same conspicuous mutant phenotype of misalignment of abdominal segments ("abdomen rotation") in adult flies (Bridges and Morgan 1923; Martin-Blanco and Garcia-Bellido 1996; Ichimiya et al. 2004; Lyalin et al. 2006). RT and TW are structurally and functionally similar to mammalian counterparts (Fig. 4A); they have non-redundant functions and work together in the ER as an enzymatic heterocomplex that modifies *Drosophila* DG with O-mannose (Ichimiya et al. 2004; Lyalin et al. 2006; Nakamura et al. 2010b). *Drosophila POMT* mutants have abnormal synaptic transmission at larval NMJs and defects of muscle morphology, the phenotypes that they share with *Dg* mutants (Martin-Blanco and Garcia-Bellido 1996; Haines et al. 2007; Wairkar et al. 2008). In vivo expression experiments unveiled several interesting features of RT-TW activity: (i) they produce O-mannose that is apparently not extended but nevertheless can modulate DG function; (ii) they add numerous O-mannose residues to the mucin-like domain of DG in a processive manner; (iii) they generate O-mannose modifications that can compete with O-GalNAc addition to the same serine or threonine residues; and (iv) RT-TW complex can modify sites outside of the unstructured mucin-like region, at the locations with a defined predicted structure (Ichimiya et al. 2004; Nakamura et al. 2010a; Nakamura et al. 2010b). These results have important implications for understanding POMT1/2 function, suggesting, for example, that *POMT* mutations may cause phenotypes due to abnormal modification of O-mannosylation substrates with O-GalNAc (O-mannose potentially competes with O-GalNAc addition to serine/threonine residues), which may result in an aberrant conformation and ectopic molecular interactions (Nakamura et al. 2010a, 2010b; Tran et al. 2012; Borgert et al. 2021). The processive activity of RT-TW toward DG is consistent with structural studies of yeast homologs that proposed a carbohydrate-binding role of MIR domains that may underlie the processivity of the POMT1–2 complex (Chiapparino et al. 2020).

PTP69D as a new type of POMT1–2 substrates

Although *Drosophila POMTs* and *Dg* have several similar mutant phenotypes, *POMT* mutants also show phenotypes that cannot be explained by abnormalities in *Dg* function. *POMT* mutants have prominent defects in sensory axon wiring, the neurological phenotype leading to abnormal muscle contractions and body torsion during embryonic and larval stages (Baker et al. 2018); however, these phenotypes are not observed in *Dg* mutants. Furthermore, *Dg* is not epistatic to *rt* and *tw* in producing the rotation phenotype, and *Dg* functions at NMJs postsynaptically, whereas *rt* is required on both sides of the synapses (Wairkar et al. 2008; Nakamura et al. 2010a; Baker et al. 2018). Taken together, these data indicate that, besides DG, POMTs have other important targets in the nervous system (Nakamura et al. 2010a). Indeed, we recently found that PTP69D, one of *Drosophila* receptor-type protein phosphatases, is a functional substrate of POMTs (Monagas–Valentin et al. 2023). *POMT1/2* and *Ptp69D* mutants have similar wiring defects of sensory axons in the larval ventral ganglion, whereas mutant alleles of *POMTs* and *Ptp69D* show prominent genetic interactions in producing the wiring phenotype, as well as abdomen rotation (Monagas-Valentin et al. 2023). *Ptp69D* is known to regulate axon guidance and connectivity of different types of neurons, including motoneurons, photoreceptors, and neurons of the giant fiber (Desai et al. 1996; Garrity et al. 1999; Lee and Godenschwege 2015), suggesting that O-mannosylation may also affect PTP69D function in these contexts; however, the role of *POMTs* in these processes has not been analyzed. *Ptp69D* is structurally and functionally related to the R2A subfamily of mammalian RPTPs (Tonks 2006; Hatzihristidis et al. 2015; Fukai and Yoshida 2021). Structural and functional similarities between PTP69D and R2A-type RPTPs (characterized by a large extracellular part including N-terminal Ig domains followed by FN3 domains) suggest an intriguing possibility that other members of this subfamily may be modified and regulated by O-mannosylation. Numerous different O-mannosyl glycans were found on RPTPζ, a more distant mammalian homolog of PTP69D, however, their attachment sites were not well characterized and their *in vivo* function remain to be elucidated (Dwyer et al. 2015). *Drosophila* experiments reinforced the hypothesis that these glycans play important role in RPTPζ regulation. Remarkably, O-mannose was found in the membrane-proximal region (MPR), as well as on Ig and FN3 domains of PTP69D, the structural folds that are very different from the mucin-like region modified in DG, which highlights that POMTs have a complex, not well-understood mechanism of protein substrate recognition (Monagas-Valentin et al. 2023). Thus, the *Drosophila* studies shed new light on POMTs' functions and raised a number of important research questions, which warrants further investigation in flies and mammals and is expected to unveil novel conserved functions of POM in the nervous system.

POMT1/2 - independent O-mannosylation

TMTCs and their cadherin substrates are well-conserved in *Drosophila*. Functional and bioinformatic analyses identified 17 cadherin genes in *Drosophila* ((Hynes and Zhao 2000; Hill et al. 2001; Li et al. 2022), and FlyBase information (Gramates et al. 2022)). A number of them are known to play essential conserved roles in cell interactions, including important functions in the nervous system, such as control of targeting choices of photoreceptor axon (N-cadherin and atypical cadherin Flamingo; Schwabe et al. 2013), protection of photoreceptors from neurodegeneration (atypical cadherin Fat; Napoletano et al. 2011), neuroblast niche positioning (DE-cadherin; Doyle et al. 2017). So far, however, O-mannosylation of cadherins has not been analyzed in *Drosophila*.

Like mammals, flies have four *TMTC* genes, *TMTC1–4*. Interestingly, *Drosophila* apparently does not have a true orthologue of mammalian *TMTC1* that was probably lost in evolution. *Drosophila TMTC1* and *TMTC2* appear to be paralogues that arose from a gene duplication of an ancestral gene related to mammalian TMTC2 (Fig. 4B). It will be important to elucidate the functional relationship between *Drosophila* and mammalian TMTCs, which will shed light on the evolution of the TMTC family of transferases in animals. So far, only *TMTC3* has been studied in flies, which indicated that its function is conserved in *Drosophila* (Farhan et al. 2017). The neuronal knockdown of *Drosophila TMTC3* resulted in susceptibility to induced seizures, the phenotype analogous to epilepsy caused by *TMTC3* mutations in humans, whereas the transgenic expression of the human counterpart in flies

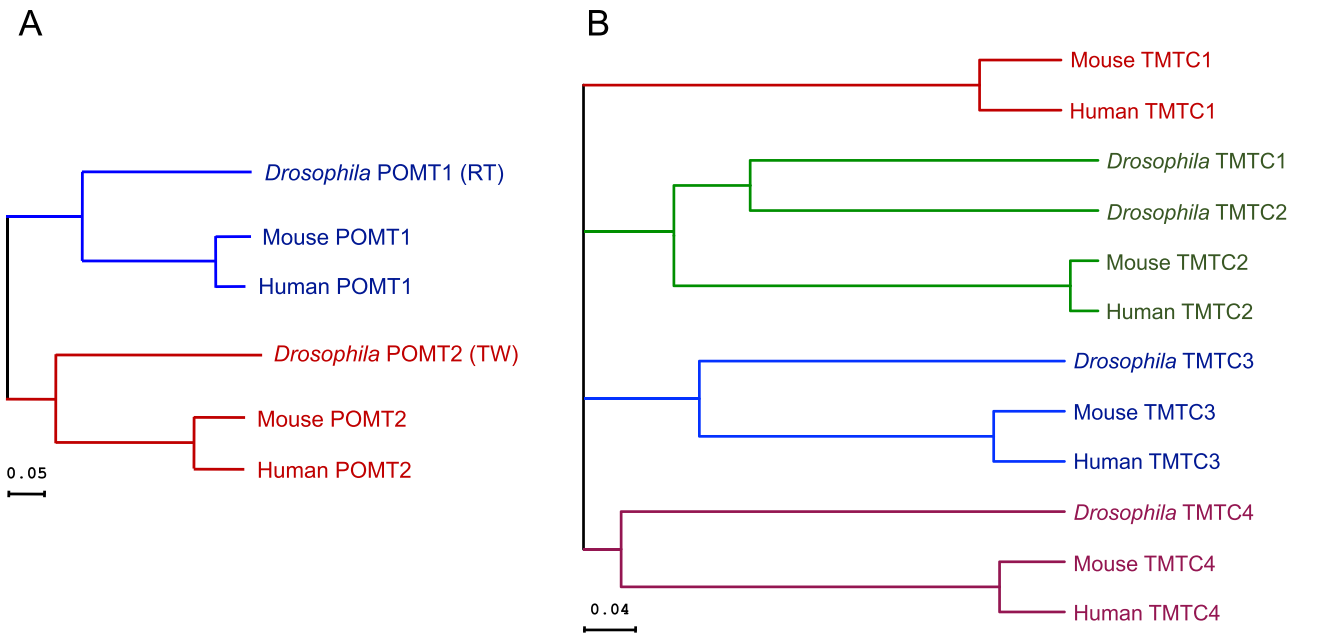

Fig. 4. Phylogenetic trees of animal POMT1–2 and TMTC1–4 enzymes. A, Phylogenetic tree of *Drosophila*, mouse, and human POMTs. RT (Rotated Abdomen), *Drosophila* POMT1; TW (Twisted), *Drosophila* POMT2 (modified from Nakamura et al. 2010a). (B) Phylogenetic tree of *Drosophila*, mouse, and human TMTCs. The trees were built based on multiple sequence alignments performed using the EMBL-EBI Clustal Omega server, followed by distance-based construction of phylogenic trees using a neighbor joining algorithm ((https://www.ebi.ac.uk/Tools/msa/clustalo/). The trees were visualized using the FigTree software (http://tree.bio.ed.ac.uk/software/figtree/). Scale bars, phylogenetic distance expressed as substitutions per site.

could rescue the seizure phenotype (Farhan et al. 2017). Considering these intriguing results, *Drosophila* is expected to be a useful model system to unveil the function and mechanisms of TMTC-mediated O-mannosylation and shed light on analogous mechanisms in mammals. However, how cadherin functions are affected by TMTCs in *Drosophila* remains an important open question.

Drosophila possesses two homologs of mammalian plexins, Plexin A and B, which play conserved roles in axon guidance as receptors for semaphorins (Zlatic et al. 2009). They participate in creating positional cues for axons in the developing ventral nerve cord along the dorsoventral axis and mediate trans-synaptic signaling to control presynaptic homeostatic plasticity (Zlatic et al. 2009; Orr et al. 2022). However, whether fly plexins are substrates for O-mannosylation is not known, and functional homologs of TMEM260 have not been identified in protostomes, including *Drosophila*.

Concluding remarks

Recent research highlighted the importance of POM for crucial biological functions and elucidated several key steps in the biosynthesis of O-mannosyl glycans in animals. The studies revealed numerous new substrates and discovered novel enzymes involved in the pathway. This progress has been driven in no small part by advances in MS-based glycomics and glycoproteomics, as well as genetic engineering approaches using cultured cells, such as the SimpleCell technology. However, the mechanisms of substrate recognition of different POM enzymes are not well understood, whereas non-enzymatic functions of these proteins were also suggested. Furthermore, the functions of different O-mannosyl glycans besides matriglycan remain largely unstudied, particularly *in vivo*. Various core M1 and M2 structures are present on α-DG, and they were also identified on several other glycoproteins with important functions in the nervous system, including CD24, neurofascin, and lecticans (Bleckmann et al. 2009; Stalnaker et al. 2011; Pacharra et al. 2012; Pacharra et al. 2013); however, the role of these modifications remains to be investigated. Yet, other important open questions are about the crosstalk between POM and other glycosylation pathways, and the cooperation of different enzymes involved in POM biosynthesis. Considering significant advantages of model systems due to experimental amenability, simplified glycosylation, and powerful genetic approaches, studies in *Drosophila* and other models are expected to help fill these knowledge gaps. The evolutionary conservation of POM and its substrates suggests that O-mannose modifications play similar roles in a wide range of animal organisms, from *Drosophila* to humans. Remarkably, all types of POM substrates are known to play prominent roles in the nervous system, which is consistent with the facts that O-mannosyl glycans are abundant in the brain, and that defects in the POM pathway are associated with severe neurological abnormalities. Thus, POM appears to be especially important for the function of the nervous system, and more neurological disorders associated with POM defects are expected to be identified. Modeling these human pathologies in simplified, genetically tractable systems, such as *Drosophila*, can help overcome obstacles encountered by research dealing with exceedingly complex nervous system and intricate glycosylation pathways in mammalian organisms. When applied together, different models can efficiently unveil pathomechanisms, test therapeutic strategies, and provide guidance for translational and clinical research in

developing treatments for debilitating POM disorders that currently have no available cure.

Abbreviations

AChRs, acetylcholine receptors; B3GALNT2, β1,3-N-acetylgalactosaminyltransferase 2; CMDs, congenital muscular dystrophies; CRPPA, CDP-L-ribitol pyrophosphorylase A; *DAG1*, *dystroglycan* (gene); DG, dystroglycan; DGC, dystrophin-associated glycoprotein complex; Dol-P-Man, dolichol-phosphate-mannose; Dys, dystrophin; EC, extracellular cadherin (domain); ECM, extracellular matrix; EGF, epidermal growth factor (repeat); ER, endoplasmic reticulum; FKRP, Fukutin-related protein; FKTN, Fukutin; FN3, fibronectin type 3 (domain); GPI, glycosylphosphatidylinositol; HNK-1, Human natural killer-1; Ig, Immunoglobulin-like (domain); IPT, Ig-like, plexin, and transcription factor (domain); KO, knockout; LARGE, β1,3-glucuronyltransferase and α1,3-xylosyltransferase-polymerase; LG, laminin-globular (domain); MEB, Muscle-Eye-Brain (disease); MET, hepatocyte growth factor receptor; MGAT5B, α1,6-mannosylglycoprotein 6-β-N-acetylglucosaminyltransferase B; MIR, protein mannosyltransferase, inositol 1,4,5-trisphosphate (IP_3) receptor, and ryanodine receptor (domain); MPR, membrane-proximal region; MS, mass-spectrometry; NMJ, neuromuscular junction; POM, protein O-mannosylation; POMGNT1, protein O-mannose β1,2-N-acetylglucosaminyltransferase 1; POMGNT2, protein O-mannose β1,4-N-acetylglucosaminyltransferase 2; POMK, protein-O-mannose kinase; POMT1 and 2, protein O-mannosyltransferases 1 and 2; PTP69D, receptor protein tyrosine phosphatase 69D; RON, receptor originated from Nantes (*or* macrophage stimulating 1 receptor); RPTP, receptor protein tyrosine phosphatase; *rt*, *rotated abdomen*; RXYLT1, ribitol-5-phosphate β1,4-xylosyltransferase; SHDRA, structural heart defects and renal anomalies (syndrome); TMEM260, transmembrane protein 260; TMTC1–4, transmembrane O-mannosyltransferases targeting cadherins (*or* transmembrane and TPR-containing proteins) 1–4; TPR, tetratricopeptide repeats (domain); TSR, thrombospondin type 1 repeat; *tw*, *twisted*.

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