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Towards understanding the extensive diversity of protein *N*-glycan structures in eukaryotes

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ABSTRACT

N-glycosylation is an important post-translational modification of proteins that has been highly conserved during evolution and is found in Eukaryota, Bacteria and Archaea. In eukaryotes, *N*-glycan processing is sequential, involving multiple specific steps within the secretory pathway as proteins travel through the endoplasmic reticulum and the Golgi apparatus. In this review, we first summarize the different steps of the *N*-glycan processing and further describe recent findings regarding the diversity of *N*-glycan structures in eukaryotic clades. This comparison allows us to explore the different regulation mechanisms of *N*-glycan processing among eukaryotic clades. Recent findings regarding the regulation of protein *N*-glycosylation are highlighted, especially the regulation of the biosynthesis of complex-type *N*-glycans through manganese and calcium homeostasis and the specific role of transmembrane protein 165 (TMEM165) for which homologous sequences have been identified in several eukaryotic clades. Further research will be required to characterize the function of TMEM165 homologous sequences in different eukaryotic clades.

Key words: calcium homeostasis, endoplasmic reticulum, eukaryotes, glycosylation, glycosyltransferases, Golgi apparatus, manganese homeostasis, *N*-glycans, regulation, structural diversity

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I. INTRODUCTION

N-glycosylation is a co- and post-translational modification (PTM) of proteins resulting in the attachment of an oligosaccharide to the nascent protein through the formation of an *N*-glycosyl bond between the monosaccharide moiety and an asparagine residue belonging to the consensus sequence Asn-X-Ser/Thr/Cys (Gil, Velander & Van Cott, 2009; Zielinska *et al.*, 2010; Matsui *et al.*, 2011; Aebi, 2013). *N*-glycosylation represents the most abundant PTM of proteins (Khoury, Baliban & Floudas, 2011) and influences their physicochemical properties and biological functions (Lingg *et al.*, 2012; Varki, 2017a). Glycoproteomics studies have identified at least 2,000 glycoproteins in each of the following model organisms *Caenorhabditis elegans*, *Arabidopsis thaliana*, *Drosophila melanogaster* and *Danio rerio* (Zielinska *et al.*, 2012), with this number a likely underestimate. This process is universal and is found in Eukaryota, Bacteria, and Archaea (Nothaft & Szymanski, 2010). In bacteria, *N*-glycan processing starts in the cytoplasmic compartment and continues in the periplasm (Li *et al.*, 2017). By contrast, in eukaryotes, the *N*-glycosylation pathway is initiated in the endoplasmic reticulum (ER) and continues in the Golgi apparatus while glycoproteins travel along the secretory pathway (Colley, Varki & Kinoshita, 2015). In this review, we mainly focus on *N*-glycosylation pathways in eukaryotes, describing the main steps leading to the extensive diversity of *N*-glycan structures found in different organisms. We describe the more processed structures of *N*-glycans isolated from the main groups Amorphea, Archaeplastida, Excavates and TSAR (Telonemia, Stramenopila, Alveolata and Rhizaria). We also highlight recent findings regarding the regulation of this process. It was recently reported in mammals and plants that the biosynthesis of complex-type *N*-glycans is regulated through manganese (Mn^{2+}) and calcium (Ca^{2+}) homeostasis involving the newly identified transmembrane protein 165 (TMEM165). Future directions for research on protein *N*-glycosylation are also discussed.

II. *N*-GLYCAN BIOSYNTHESIS IN THE ER: A CONSERVED PROCESS DURING EVOLUTION

In eukaryotes, protein glycosylation involves hundreds of different molecular actors, including enzymes involved in the biosynthesis and transport of nucleotide sugars as well as glycosyltransferases (GTs) and glycosylhydrolases (GHs), called glycoenzymes. In humans for example, more than 500 glycoenzymes take part in *N*- and *O*-glycan processing including 114 enzymes and transporters involved in carbohydrate metabolism and transport, 76 glycosidases and more than 200 GTs (Neelamegham & Mahal, 2016). GTs involved in *N*-glycan biosynthesis or processing predominantly use activated nucleotide monosaccharides (also called nucleotide sugars) as donor substrates and catalyse their transfer to an acceptor substrate that can either be a lipid-linked oligosaccharide or an *N*-glycan (Rini & Esko, 2017). They represent between 1 and 2% of eukaryotic genomes (Lairson *et al.*, 2008). These actors are localised in different compartments, allowing efficient stepwise and well-controlled *N*-glycan processing. In eukaryotes, the synthesis of nucleotide sugars such as uridine diphosphate-galactose (UDP-Gal), guanidine diphosphate-fucose (GDP-Fuc), uridine diphosphate-*N*-acetylglucosamine (UDP-GlcNAc), uridine diphosphate-glucose (UDP-Glc) or guanidine diphosphate-mannose (GDP-Man) starts in the cytoplasm. By contrast, cytidine monophosphate-*N*-acetylneuraminic acid (CMP-Neu5Ac) synthesis occurs in the nucleus and uridine diphosphate-xylose (UDP-Xyl) in animals is formed in the Golgi apparatus from imported uridine diphosphate-glucuronic acid (UDP-GlcA). These nucleotide sugars are used as donor substrates in the different steps of *N*-glycan processing (Bar-Peled & O'Neill, 2011). *N*-glycan processing is initiated in the ER with the synthesis of an oligosaccharide intermediate precursor linked to a dolichol (Dol) lipid, commonly known as lipid-linked oligosaccharide (LLO), of diverse final structure ranging from $Glc_3Man_9GlcNAc_2$-P-P-Dol (Fig. 1) to a minimal form $GlcNAc_2$-P-P-Dol in protists (Samuelson & Robbins, 2015). LLO synthesis begins at the cytosolic face of the ER and requires the successive action of many different GTs called ALGs (asparagine-

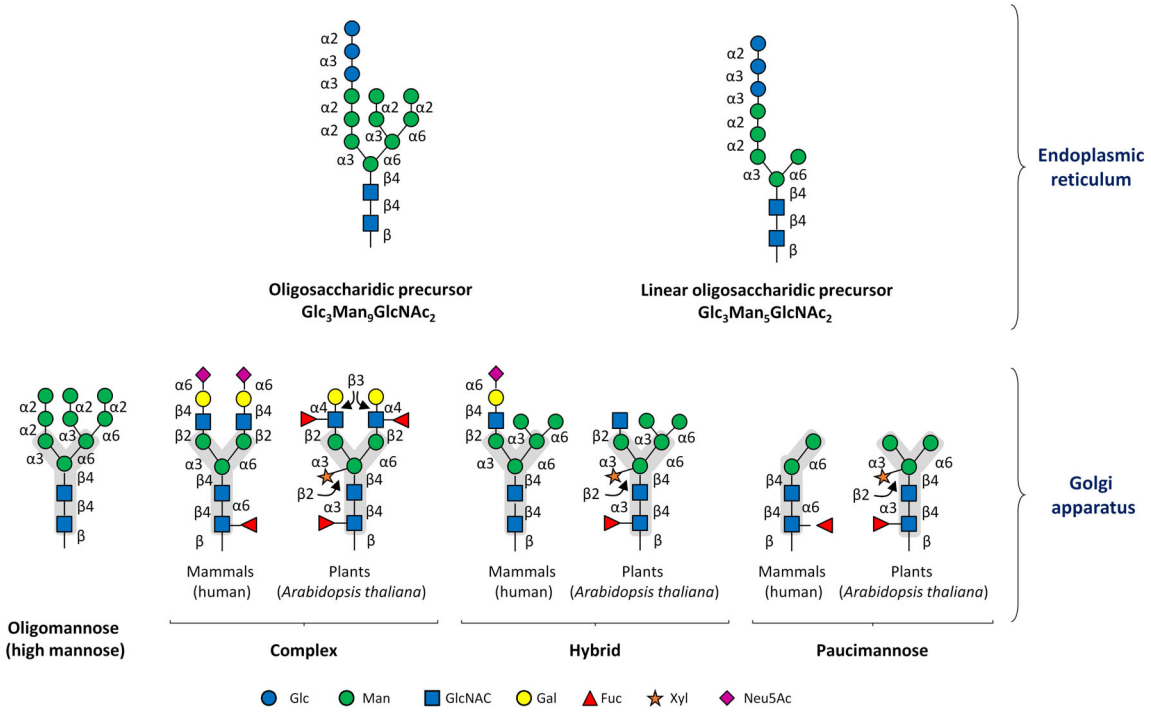


Fig. 1. Structures of oligosaccharidic precursors and examples of mature *N*-glycans. In the endoplasmic reticulum, the oligosaccharidic precursor is synthesized and then transferred *en bloc* to the nascent protein. This structure is found in almost all eukaryotes. However, in some protists and microalgae, the oligosaccharidic precursor can be linear and shortened. The oligosaccharide is then processed further in the Golgi apparatus to yield a completely different structure. As exemplified here, the four main types of human and plant *N*-glycans found in mature *N*-glycoproteins are oligomannose, complex, hybrid and paucimannose. The linkages between each monosaccharide are shown. *N*-glycan structures drawn according to the recently updated Symbol Nomenclature for Glycans (Varki, 2017b). Note that all eukaryotic *N*-glycans share the common core glycan structure $Man_{2\text{-}3}GlcNAc_2$ (shaded in grey). Fuc, fucose; Gal, galactose; Glc, glucose; GlcNAc, *N*-acetylglucosamine; Man, mannose; Neu5Ac, *N*-acetylneuraminic acid; Xyl, xylose.

linked glycosylation). These enzymes are embedded in the ER membrane and use activated nucleotide sugars and specific sugar acceptors to elongate the LLO (Aebi, 2013).

The first step during LLO biosynthesis is catalysed by dolichol-phosphate GlcNAc phosphotransferase 1 (DPAGT1), also known as ALG7, and results in the transfer of a phospho-GlcNAc group from UDP-GlcNAc on the dolichol phosphate (Dol-P). A GlcNAc and five Man residues then are sequentially added from UDP-GlcNAc and GDP-Man by the successive actions of multi-enzymatic complexes ALG13/14 and the three mannosyltransferases ALG1 (β(1,4)-mannosyltransferase), ALG2 (α(1,3)/(1,6)-mannosyltransferase) and ALG11 (α(1,2)-mannosyltransferase) (Gao, Nishikawa & Dean, 2004) to yield the dolichol pyrophosphate heptasaccharide $Man_5GlcNAc_2$-P-P-Dol. At this stage, the LLO undergoes a *trans* bilayer translocation across the ER membrane, requiring the activity of a flippase (Alaimo *et al.*, 2006; Rush, 2016; Verchère *et al.*, 2021). In humans, deficiency of the proposed flippase RFT1 leads to a severe decrease in *N*-glycosylation site occupancy on newly synthesized glycoproteins (Vleugels *et al.*, 2009). After flipping of $Man_5GlcNAc_2$-P-P-Dol, synthesis in most eukaryotes is completed on the luminal side of the ER (Breitling & Aebi, 2013) with four mannosylation steps respectively catalysed by ALG3, ALG9, ALG12 and ALG9. Then, three terminal Glc residues are consecutively added by ALG6, ALG8 and ALG10 to achieve the biosynthesis of the oligosaccharide precursor $Glc_3Man_9GlcNAc_2$-P-P-Dol that will be used as a donor for the *N*-glycosylation of specific Asn residues (Fig. 1). Its *en bloc* transfer from the lipid-linked precursor to newly synthesized proteins is catalysed by oligosaccharyltransferase (OST) (Silberstein & Gilmore, 1996; Lizak *et al.*, 2011; Breitling & Aebi, 2013). An *N*-glycosyl bond is then formed between the anomeric carbon of the proximal-reducing GlcNAc of the oligosaccharide and the nitrogen atom of the lateral chain of the asparagine belonging to the consensus *N*-glycosylation site of the protein (Kowarik *et al.*, 2002). In most organisms, OST is an enzymatic complex composed of several different subunits (Ruiz-Canada, Kelleher & Gilmore, 2009; Hamieh *et al.*, 2017; Wild *et al.*, 2018; Ramírez, Kowal & Locher, 2019). The oligosaccharide structure linked to the newly synthesized *N*-glycoprotein will then be further trimmed in the ER lumen by the action of specific glycosidases: α-glucosidase I, α-glucosidase II and eventually an ER-mannosidase leading to $Man_{8\text{-}9}GlcNAc_2$ oligomannosides. Interaction of glucosylated glycan intermediates with two ER-resident lectin-like chaperones, calnexin and calreticulin, ensures ER quality control of *N*-glycoproteins (Parodi, Cummings & Aebi, 2015).

Once correctly folded, glycoproteins leave the ER to reach the Golgi apparatus.

III. *N*-GLYCAN BIOSYNTHESIS IN THE GOLGI APPARATUS LEADS TO A RICH DIVERSITY OF *N*-GLYCAN STRUCTURES

Oligosaccharides ranging from $Man_{8\text{-}9}GlcNAc_2$ to the canonical $Man_5GlcNAc_2$ are present in most eukaryotes and are derived from the trimming of ER $Man_{8\text{-}9}GlcNAc_2$ by α-mannosidases. By contrast, a specific $Man_5GlcNAc_2$ structure has been recently identified in organisms synthesizing a truncated LLO $Glc_3Man_5GlcNAc_2$-P-P-Dol in the ER (Fig. 1), as reported for *Trichomonas vaginalis*, *Tetrahymena thermophila*, *Chlamydomonas reinhardtii* and *Trypanosoma brucei* (Jones *et al.*, 2005; Manthri *et al.*, 2008; Paschinger *et al.*, 2012; Levy-Ontman *et al.*, 2014; Lucas *et al.*, 2018). This non-canonical oligomannoside will be called 'linear' $Man_5GlcNAc_2$ herein since it exhibits a Manα(1,2)Manα(1,2)Man sequence α(1,3)-linked to the β-Man residue of the core.

After leaving the ER, oligomannosidic *N*-glycans (Fig. 1) are processed further in the Golgi apparatus. However, in contrast to the ER processing steps, Golgi *N*-glycan maturation involves a large diversity of glycosidases and GTs giving rise to a diversity of organism-specific complex, hybrid and paucimannosidic *N*-glycans (Fig. 1). This *N*-glycan diversity is discussed in the following subsections for Amorphea, Excavates, Archaeplastida and the TSAR supergroup.

(1) Amorphea

Amorphea are members of a taxonomic supergroup that includes fungi and animals and can be divided into two main subgroups: Amoebozoa and Obazoa. The latter includes Opisthokonta that can further be subdivided into two groups: Holomycota (fungi, yeasts) and Holozoa (animals and their closest single-cell relatives) (Fig. 2).

(*a*) *Amoebozoa*

To date, protein *N*-glycan profiles have been reported for only three amoebozoan species: two human parasites, *Acanthamoeba* strains (clade Discosea) and *Entamoeba histolytica* (clade Evosea), and the non-infectious slime mould *Dictyostelium discoideum* (clade Evosea). Amoebozoan species synthesize oligomannosides ranging from $Man_5GlcNAc_2$ to $Man_{9}GlcNAc_2$ with some additional substitutions (Fig. 2) (Magnelli *et al.*, 2008; Schiller *et al.*, 2009, 2012; Feasley *et al.*, 2010; Feasley, van der Wel & West, 2015; Obregón *et al.*, 2019). In *Acanthamoeba* strains, the major *N*-glycan structures are oligomannosides with 7–10 hexoses (Hex) partially substituted with methyl (Me) and pentose residues for which the position is still ambiguous (Fig. 2) (Schiller *et al.*, 2012). Further analyses have led to the biochemical characterization of a canonical $Man_5GlcNAc_2$ *N*-glycan substituted by an α(1,6)-Fuc residue capped with an α-linked Man (Schiller *et al.*, 2012). Similar substitutions of the core Fuc with a Hex have also been reported in nematodes (Gutternigg *et al.*, 2007b; Yan *et al.*, 2012; Paschinger & Wilson, 2015), cephalopods (Zhang *et al.*, 1997), gastropods (Wuhrer *et al.*, 2004) and platyhelminthes (Paschinger *et al.*, 2011). However, in these organisms, the Fuc residue is capped with a Gal residue instead of a Man residue.

The most abundant *N*-glycan found in *E. histolytica* is $Man_5GlcNAc_2$ (Magnelli *et al.*, 2008). *N*-glycome analysis of *E. histolytica* has also highlighted smaller oligomannosides from $Man_2GlcNAc_2$ to $Man_5GlcNAc_2$ carrying a Gal residue linked to the terminal mannoses. This Gal residue might eventually be substituted by an additional Glc (Magnelli *et al.*, 2008). *N*-glycans from *D. discoideum* are characterized by the presence of additional GlcNAc residues on oligomannoside *N*-glycans (Couso *et al.*, 1987; Schiller *et al.*, 2009; Feasley *et al.*, 2010; Nakagawa, Tojo & Fujii, 2011). Additional glycan modifications like fucosylation of the core proximal GlcNAc that bisects the GlcNAc have been reported, as well as substitutions on terminal monosaccharides with Fuc, sulfate or methylphosphate (Fig. 2) (Feasley *et al.*, 2010; Nakagawa *et al.*, 2011; Hykollari *et al.*, 2013, 2017). Concerning core fucosylation, both α(1-3)- and α(1-6)- linkages have been reported (Schiller *et al.*, 2009; Nakagawa *et al.*, 2011).

(*b*) *Obazoa*

(i) Holomycota. Holomycota include yeasts and the large kingdom of Fungi. In this group, the main *N*-glycan structures found on glycoproteins are oligomannosides (Fig. 2). For the phylum Basidiomycota, the two species *Pseudozyma antarctica* and *Mallassezia furfur* synthesize oligomannosides composed of 3–9 Man residues (Flores *et al.*, 2019). Although oligomannosides are also observed in *Saccharomycotina* species (Gong *et al.*, 2009), substitutions by Gal residues have been reported (Ballou, Ballou & Ball, 1994; Ziegler *et al.*, 1999). Moreover, yeasts often extend their *N*-glycan structures to form large polymannosidic structures (de Pourcq, de Schutter & Callewaert, 2010). Concerning Leotiomyceta, especially *Penicillium* species, similar *N*-glycans have been identified with additional modifications such as the attachment of galactofuranose (Gal*f*) residues, phosphoethanolamine and phosphorylcholine (Fig. 2) (Hykollari *et al.*, 2016).

(ii) Holozoa (metazoans) (A) Porifera. Sponges were long considered members of the Archaeplastida kingdom but are now classified as a branch of Metazoa. *N*-glycosylation in Porifera seems not to be as complex as those of Bilateria described below (Fig. 2). The sponge *Haliclona caerulea* synthesizes protein *N*-linked glycans based on a $Man_7GlcNAc_2$ structure substituted with *N*-acetylhexosamine (HexNAc) and deoxyhexose (dHex) residues leading to a $HexNAc_{7\text{-}}Man_7dHex_2$ structure (Fig. 2). Larger *N*-glycans with up to 13 additional Hex residues have also been detected but these still require structural characterization (Carneiro *et al.*, 2013).

(B) Cnidaria. In Cnidaria, investigation of the protein *N*-glycan profiles of the freshwater *Hydra magnipapillata* revealed

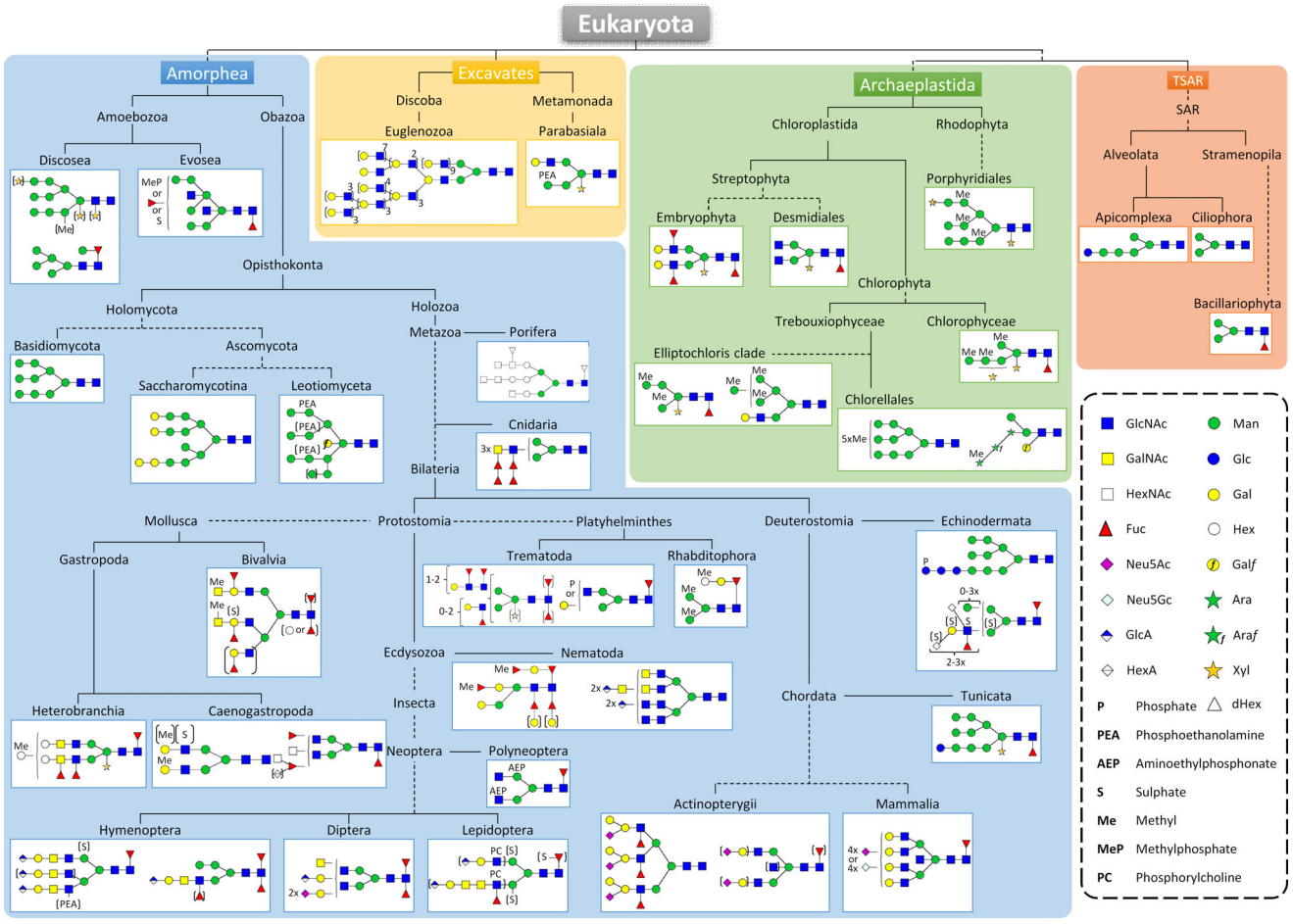


Fig. 2. The structural diversity of *N*-glycans synthesized in eukaryotes overlaid on the phylogenetic distribution of the different organisms. The most mature *N*-glycan structures are shown for each clade. *N*-glycan structures drawn according to the recently updated Symbol Nomenclature for Glycans (Varki, 2017b) using the Glycoworkbench tool v2.1. Phylogeny adapted from Burki *et al.* (2020) and http://lifemap.univ-lyon1.fr/explore.html. Ara, arabinose; Araf, arabinofuranose; dHex, deoxyhexose; Fuc, fucose; Gal, galactose; Galf, galactofuranose; GalNAc, *N*-acetylgalactosamine; Glc, glucose; GlcA, glucuronic acid; GlcNAc, *N*-acetylglucosamine; Hex, hexose; HexA, Hexuronic acid; HexNAc, *N*-acetylhexosamine; Man, mannose; Neu5Ac, *N*-acetylneuraminic acid; Neu5Gc, *N*-glycolylneuraminic acid; Xyl, xylose.

polyfucosylated LacdiNAc (GalNAcβ(1,4)GlcNAc) structures that play a role in its regenerative process (Sahadevan *et al.*, 2014).

(C) Bilateria. Bilateria is a very large group composed of Protostomia (arthropods, annelids, and molluscs) and Deuterostomia (echinoderms, chordates and hemichordates). The diversity of the *N*-glycan structures within this phylum is large, ranging from paucimannosidic to di-, tri- and tetra-antennary complex *N*-glycans (Fig. 2). In *Protostomia*, protein *N*-glycosylation is more sophisticated.

For molluscs, data have been reported mainly for two subgroups: Bivalvia and Gastropoda which includes Heterobranchia and Caenogastropoda. *N*-glycan analysis of these clades has revealed a large variety of structures, including oligomannosidic and complex *N*-glycans with an immense variety of modifications including core xylose, methylated Hex, Fuc and *N*-acetylgalactosamine (GalNAc) substitutions (Fig. 2) (Lommerse *et al.*, 1997; Dolashka-Angelova *et al.*, 2003; Gutternigg *et al.*, 2007a). The di- and tri-antennary structures can also be mono- or di-sulfated on the Hex residues or O-methylated on the terminal Gal, Man or GalNAc (Dolashka-Angelova *et al.*, 2003; Kurz *et al.*, 2013). A core difucosylation has been observed in the bivalve mollusc *Crassostrea virginica* in addition to a repertoire of sulfated and blood group-A epitopes (Kurz *et al.*, 2013). Alternatively, the core α(1,3)-Fuc residue can be replaced by a Hex residue on the proximal GlcNAc. Polyantennary *N*-glycans containing 4-*O*-methyl GlcA(1,4)GlcNAc(1,3)Fuc(1,4)GlcNAc have been described in the mollusc *Mytilus edulis* (Zhou *et al.*, 2013). Monofucosylation on the LacdiNAc motif associated with or without a core xylose has been reported in *Biomphalaria glabrata* (Lehr *et al.*, 2007). Within Mollusca, xylosylation of the core seems to be specific to the phylum Heterobranchia (Gutternigg *et al.*, 2007a). Other remarkable features are the presence in *Rapana venosa* of a HexNAc(HexA)Fuc motif linked to a terminal GlcNAc (Sandra *et al.*, 2007). In

Volvarina rubella, a carnivorous and scavenging marine gastropod, a complex *N*-glycome encompasses a range of oligomannosidic, paucimannosidic, core-modified and complex *N*-glycans. The latter include highly modified *N*-glycans bearing *N*-methyl-2-aminoethylphosphonate, phosphorylcholine, methyl, sulfate, or aminoethylphosphonate substitutions and also a core α(1,6)-Fuc capped with a Hex and an additional Fuc residue (Fucα(1,2)Galβ(1,4)Fucα(1,6)) (Eckmair *et al.*, 2016).

N-glycans of Platyhelminthes exhibit less-sophisticated structures compared to other bilaterians (Fig. 2). They perform core α(1,6)-fucosylation as well as galactosylation and phosphorylation of the terminal GlcNAc residues as reported for *Fasciola hepatica N*-glycans (Ravidà *et al.*, 2016). *N*-glycan structures identified on proteins of *Dugesia japonica* more commonly have a Galβ(1,4)Fuc disaccharide on the proximal GlcNAc residue and an additional methyl Hex linked to this motif (Paschinger *et al.*, 2011). More complex *N*-glycan structures (not shown in Fig. 2) have been observed in trematodes, notably the presence of polygalactosylation (up to five Gal residues, which may be methylated) on the core fucose. Methylation was also observed on the Man residues leading to polymethylated glycans in *Schmidtea mediterranea* (Subramanian *et al.*, 2018). In addition, LacdiNAc bearing three Fuc residues, Lewis x epitope (Fucα(1,3)-Galβ(1,4)), core xylose or even core difucosylation were identified in *Schistosoma* species (Khoo *et al.*, 1997; Wuhrer *et al.*, 2006a,b; Jang-Lee *et al.*, 2007; Mickum *et al.*, 2016).

Nematodes represent 80% of land animals (van den Hoogen *et al.*, 2019). The structural diversity of the *N*-glycans found in nematodes is very large, for example approximately 150 different *N*-glycan structures have been identified in *Dirofilaria immitis* (Martini *et al.*, 2019). A large complexity and diversity of *N*-glycans exist in the model organism C*aenorhabditis elegans*, which shares similar *N*-glycan features with other nematode species, such as Fuc residues or a Gal-Fuc disaccharide linked to the core, and attachment of phosphorylcholine which can also be found in *Ascaris suum*, *Oesophagostomum dentatum* and *Haemonchus contortus* (van Die *et al.*, 1999; Cipollo *et al.*, 2005; Pöltl *et al.*, 2007; Butschi *et al.*, 2010; Yan *et al.*, 2012, 2018; Paschinger & Wilson, 2015). However, *C. elegans* also has species-specific *N*-glycan modifications, such as the presence of a bisecting Gal motif that, to our knowledge, has never been reported in other organisms (Yan *et al.*, 2015). A detailed list of the diversity of *N*-glycan structures in *C. elegans* is provided by Paschinger, Yan & Wilson (2019) with the most processed structure being $MeFuc_5Gal_5Man_2GlcNAc_2$ (Fig. 2). Other structures such as poly-fucosylated GlcNAc motifs and tetra-antennary *N*-glycans with terminal GlcA have been identified in *D. immitis* (Martini *et al.*, 2019). Multi-antennary LacdiNAc structures have also been described in *D. immitis* and *Trichinella spiralis* (Fig. 2; Martini *et al.*, 2019; Morelle *et al.*, 2000).

Insects are divided into four main phyla: Polyneoptera, Hymenoptera, Diptera and Lepidoptera (Fig. 2). As reported for nematodes, insects have the ability to attach GlcA to their *N*-glycans. This residue has been found in all insect phyla investigated except Polyneoptera for which this substitution has not yet been described (Aoki & Tiemeyer, 2010; Stanton *et al.*, 2017; Hykollari *et al.*, 2018). The polyneopteran *Locusta migratoria* was the first example of an organism that can synthesise *N*-glycans exhibiting an aminoethylphosphonate motif (Hård *et al.*, 1993). This substitution was subsequently observed in glycoproteins from royal jelly and bee venom (Hykollari *et al.*, 2018). LacdiNAc motifs similar to those described above for Heterobranchia also have been reported. Surprisingly, Lepidoptera can synthesise protein *N*-glycans with phosphorylcholine substituents (Stanton *et al.*, 2017). Sulfation of Man and Fuc residues of the core *N*-glycan also have been observed in Lepidoptera. By contrast, in Hymenoptera, only Man sulfation has been reported (Stanton *et al.*, 2017). Such sulfation of *N*-glycans has also been reported in Crustacea, especially in the spiny lobster *Panulirus interruptus* (Van Kuik *et al.*, 1986, 1987). The presence of sialic acids substitutions in *Drosophila melanogaster* has also been reported (Aoki & Tiemeyer, 2010).

The super-phylum Deusterostomia is composed of echinoderms, chordates and hemichordates. Proteins from echinoderms carry oligomannosides up to $Man_9GlcNAc_2$ (Şahar & Deveci, 2017). Recently, mass spectrometry analyses demonstrated the presence of more complex *N*-glycans in *Holothuria atra*. Indeed, Vanbeselaere *et al.* (2020) identified oligomannoside structures (72%), the most sophisticated being an unusual *N*-glycan containing a phosphate (P) and three Glc residues ($P_1Glc_3Man_9GlcNAc_2$). Truncated forms containing only seven Man have also been identified. The remaining 28% of *N*-glycans are complex and hybrid structures exhibiting core α(1,6)-Fuc and a LacNAc (Galβ(1,4)GlcNAc) motif. This latter motif can also be fucosylated and substituted by two *N*-glycolylneuraminic acids (Neu5Gc), the first being α(2,3)-linked to the Gal residue and the second attached in α(2,6) to the GlcNAc of the antennae. The *N*-glycome of the brittle star *Ophiactis savignyi* was recently described. In this species, core fucosylation, and various sulfate and Neu5Gc substitutions that can be methylated have been reported. Structures with polyLacNAc motifs or a terminal GlcNAc bearing a phosphate residue are also synthesized in *O. savignyi* (Eckmair *et al.*, 2020).

Chordates include vertebrates (fish, amphibians, reptiles, birds, and mammals) and tunicates. The tunicate *Ciona intestinalis* synthesises mainly hybrid *N*-glycans containing nine Man residues, one Glc and with core β(1,2)-Xyl and core α(1,3)-Fuc residues attached (Yagi *et al.*, 2008). Species from the phylum Actinopterygii (ray-finned fishes) synthesize very complex poly-antennary *N*-glycans substituted with core α(1,6)-Fuc, bisecting GlcNAc, LacNAc motif with or without α(2,3)-linked sialic acid and/or β(1,4)-Gal and a Fuc on the outer GlcNAc residues (Taguchi *et al.*, 1994; Hanzawa, Suzuki & Natsuka, 2017). In mammals, complex *N*-glycans are di- to tetra-antennary structures composed of LacNAc sequences carrying a core α(1,6)-Fuc. These antennae are terminated by sialic acid residues, either Neu5Ac or Neu5Gc (Figs 1 and 2). Humans do not have Neu5Gc (Brinkman-Van der Linden *et al.*, 2000; Tangvoranuntakul *et al.*, 2003;

Bardor *et al.*, 2005). Some mammalian species, but not humans, are able to synthesize the 'alpha Gal' epitope that consists of α(1,3)-Gal linked to the LacNAc motif (Galili *et al.*, 1988; Galili, 2013).

(2) Excavates

Excavates are diverse single-cell flagellate organisms ranging from free-living species such as the freshwater alga *Euglena gracilis* to human parasites like *Trypanosoma brucei* (clade Euglenozoa) or *Trichomonas vaginalis* (clade Metamonada).

(*a*) *Euglenozoa*

The freshwater alga *E. gracilis* is able to synthesise oligomannoside *N*-glycans ranging from $Man_6GlcNAc_2$ to $Man_9GlcNAc_2$ (de la Canal & Parodi, 1985; O'Neill *et al.*, 2017), a small proportion of which are substituted by a putative aminoethylphosphonate moiety (O'Neill *et al.*, 2017). Oligomannosidic *N*-glycans have also been observed on the variant surface glycoproteins of the human pathogen *T. brucei* (Zamze *et al.*, 1990, 1991; Jones *et al.*, 2005; Manthri *et al.*, 2008; Damerow *et al.*, 2016). However, in contrast to *E. gracilis*, *T. brucei* synthesizes complex *N*-glycans with unusually large poly-LacNAc-containing structures (Fig. 2) (Zamze *et al.*, 1991; Mehlert *et al.*, 1998; Atrih *et al.*, 2005; Manthri *et al.*, 2008; Damerow *et al.*, 2016). Such poly-LacNAc-containing structures have only been observed on proteins of the bloodstream form but not in the procyclic form of *T. brucei* (Mehlert *et al.*, 1998; Nolan, Geuskens & Pays, 1999). Although rare, these poly-LacNAc structures have also been found in cancerous tissue in humans (Ichikawa *et al.*, 1999; Ishida *et al.*, 2005; Holst, Wuhrer & Rombouts, 2015).

(*b*) *Parabasiala*

Protein *N*-glycosylation in the clade Parabasiala has received little attention to date and is restricted to the sexually transmitted parasite *T. vaginalis*. The main *N*-glycan of *T. vaginalis* is a linear $Man_5GlcNAc_2$ oligomannoside that results from the transfer in the ER of a truncated precursor $Glc_3Man_5GlcNAc_2$-P-P-Dol on the *N*-glycosylation site of the protein (Samuelson *et al.*, 2005; Paschinger *et al.*, 2012; Lombard, 2016). This linear $Man_5GlcNAc_2$ oligomannoside is then trimmed into $Man_4GlcNAc_2$ and $Man_3GlcNAc_2$ that are substituted by a pentose (likely Xyl), phosphoethanolamine or LacNAc motif, thus generating hybrid *N*-glycan structures (Fig. 2) (Paschinger *et al.*, 2012).

(3) Archaeplastida

The Archaeplastida are a major group of autotrophic eukaryotes comprising Rhodophyta and Chloroplastida. This latter subgroup is subdivided into organisms of the clade Streptophyta, which includes land plants and desmids, and the Chlorophyta clade containing the green algae.

(*a*) *Chloroplastida*

(i) Streptophyta. In addition to oligomannosides, Embryophytes (land plants and mosses) and Desmidiales are able to synthesise complex-type *N*-glycans (Lerouge *et al.*, 1998). To date, studies have mainly been carried out in land plants and mosses, but similar structures have been recently reported in the charophycean green alga *Penium margaritaceum* belonging to Desmidiales (Ruiz-May *et al.*, 2018). Complex *N*-glycans of streptophytes are characterized by the presence of a core $GlcNAc_2Man_3GlcNAc_2$ similar to that of mammals and insects that is substituted by a core α(1,3)-Fuc and a core β(1,2)-Xyl (Fig. 1). In land plants and mosses, additional modifications of *N*-glycans could result in the synthesis of the Lewis a (Le^a) epitope that results from the transfer of both an α(1,4)-Fuc and a β(1,3)-Gal to terminal GlcNAc residues (Figs 1 and 2). These complex *N*-glycan epitopes are highly conserved in land plants (Lerouge *et al.*, 1998) and also in mosses such as *Physcomitrella patens* (Fitchette *et al.*, 1999; Viëtor *et al.*, 2003; Mega, 2007). Proteins from land plants also carry truncated oligosaccharides consisting of mature *N*-glycans lacking terminal GlcNAc residues (Lerouge *et al.*, 1998). Such structures are called paucimannosidic *N*-glycans (Fig. 1). These glycans result from the action of hexosaminidases in the secretory system, the extracellular matrix or the storage organelles (e.g. vacuole) (Strasser *et al.*, 2007; Liebminger *et al.*, 2011; Shin *et al.*, 2017).

(ii) Chlorophyta. The clade Chlorophyta includes green algae such as *Chlorella* and *Chlamydomonas* species. Although phylogenetically close to Embryophytes, their protein *N*-glycan profiles largely differ from those of land plants. Oligomannosides represent the major *N*-glycans of proteins isolated from Chlorophytes. $Man_5GlcNAc_2$ oligomannoside found in *Chlamydomonas reinhardtii* exhibits a linear sequence (Vanier *et al.*, 2017). This non-canonical structure is derived from $Glc_3Man_5GlcNAc_2$-P-P-Dol occurring in the ER of this green microalga (Fig. 1) (Lucas *et al.*, 2018). In addition, oligomannosides from chlorophytes are partially mono- or di- *O*-methylated and substituted with deoxyhexoses and/or pentoses (Mathieu-Rivet *et al.*, 2013, 2014; Levy-Ontman *et al.*, 2014; Schulze *et al.*, 2017; Vanier *et al.*, 2017; Mócsai *et al.*, 2019; Lucas *et al.*, 2020; Oltmanns *et al.*, 2020). In *Chlamydomonas reinhardtii*, *N*-linked glycans are substituted by α(1,3)-Fuc residues on the proximal GlcNAc and Xyl residues linked to α-Man and β-Man residues of $Man_4GlcNAc_2$ and $Man_5GlcNAc_2$ oligomannosides (Lucas *et al.*, 2020). Two recent studies reporting the protein *N*-glycomes of *Chlorella* species (clade Chlorellales) revealed an unsuspected variety of new *N*-glycans composed of short oligomannosides substituted by various Gal, arabinose (Ara) and GlcNAc residues, some of which (Gal and Ara) exhibited either furanose or pyranose forms (Mócsai *et al.*, 2019, 2020a,b). In addition, *N*-glycans exhibiting GlcNAc at their non-reducing end have also been reported in *Botryococcus braunii* (clade Elliptochloris) (Schulze *et al.*, 2017). The presence of such GlcNAc-terminated glycans suggests the action of an *N*-acetylglucosaminyltransferase I in the *N*-glycan processing of these microalgae, a transferase that is absent in *C. reinhardtii* (Vanier *et al.*, 2017).

(*b*) *Rhodophyta*

In rhodophytes, protein *N*-glycosylation profiles are mainly from *Porphyridium* sp. Proteins from this red alga carry *O*-methylated oligomannosides substituted with one or two Xyl residues. Of particular interest, one of these two Xyl residues is described to be linked to the second GlcNAc of the core *N*-glycan structure (Levy-Ontman *et al.*, 2011).

(4) TSAR

The TSAR supergroup includes the clades Telonemia, Stramenopila, Alveolata and Rhizaria. To date, data regarding *N*-glycosylation have only been published for proteins isolated from alveolates and stramenopiles.

(*a*) *Alveolata*

The phylum Alveolata consists of unicellular organisms (protozoans) such as dinoflagellates, ciliates and apicomplexans (protozoan parasites including those responsible for the malaria and toxoplasmosis diseases). In contrast to dinoflagellates for which no information on protein *N*-glycosylation is available, several studies have focused on protein *N*-glycan structures in ciliates and apicomplexans. Short oligomannosides have been characterized in apicomplexans such as *Toxoplasma gondii* and *Cryptosporidium parvum*. For other apicomplexan species, such as the malaria parasite *Plasmodium falciparum*, only one or two GlcNAc residues are transferred to the asparagine of the *N*-glycosylation consensus site (Fauquenoy *et al.*, 2008, 2011; Garénaux *et al.*, 2008; Luk, Johnson & Beckers, 2008; Haserick *et al.*, 2017; Gas-Pascual *et al.*, 2019). Glucose-terminated linear $Man_5GlcNAc_2$ *N*-linked glycan has been identified in apicomplexan species (Taniguchi *et al.*, 1985; Fauquenoy *et al.*, 2008, 2011; Luk *et al.*, 2008; Calow *et al.*, 2016; Haserick *et al.*, 2017). More recently, unexpected structures like $Glc_2Man_6GlcNAc_2$ and $Man_6GlcNAc_2$ have been reported in *T. gondii* (Gas-Pascual *et al.*, 2019). These glycans derive from the trimming of a truncated $Glc_{2\text{-}3}Man_{5\text{-}6}GlcNAc_2$-P-P-Dol precursor synthesized in the ER (Taniguchi *et al.*, 1985; Garénaux *et al.*, 2008; Haserick *et al.*, 2017). In ciliates, shorter oligomannosides ($Man_3GlcNAc_2$) have been characterized, especially in *Tetrahymena thermophila* and *Tetrahymena pyriformis* (Taniguchi *et al.*, 1985; Becker & Rüsing, 2003; Weide *et al.*, 2006; Calow *et al.*, 2016).

(*b*) *Stramenopila*

Data on protein *N*-glycans in stramenopiles are restricted to *Phaeodactylum tricornutum*. In contrast to apicomplexan species, this diatom is able to synthesize oligomannoside *N*-glycans resulting from complete ER processing of the lipid-linked precursor up to $Glc_2Man_9GlcNAc_2$-P-P-Dol (Lucas *et al.*, 2018). After quality control events occurring in the ER, this glycan precursor is then trimmed into oligomannosides ranging from $Man_9GlcNAc_2$ to $Man_5GlcNAc_2$ *N*-glycans (Baïet *et al.*, 2011). These oligomannosides have recently been demonstrated to be identical to those of mammals and plants (Dumontier *et al.*, 2021). With regards to Golgi maturation, expression of an *N*-acetylglucosaminyltransferase gene [for α-1,3-mannosyl-glycoprotein 2-β-GlcNAc transferase (GnT I)] from *P. tricornutum* was demonstrated to be able to restore complex *N*-glycan maturation in the mammalian Chinese Hamster Ovary (CHO) cell line Lec1 mutant that lacks its endogenous GnT I (Baïet *et al.*, 2011). However, although this diatom expresses a functional GnT I and other molecular actors of *N*-glycan processing in the Golgi machinery (Zhang *et al.*, 2019), only small amounts of paucimannosidic *N*-glycans bearing a core α(1,3)-Fuc have been detected in *P. tricornutum* proteins.

IV. REGULATION OF THE *N*-GLYCOSYLATION PATHWAY

At the evolutionary level, glycosylation provides eukaryotes with a rich combinatorial system, generating an incredible diversity of *N*-glycan structures without prior genome modifications (Gagneux, Aebi & Varki, 2015; Varki & Gagneux, 2015). Fig. 2 shows the most common processed *N*-glycans for the different eukaryotic clades. However, within a given organism, *N*-glycoproteins exist as multiple glycosylation variants depending on their maturation level and number of *N*-glycosylation sites. Regulation of the glycoproteome therefore is essential for numerous biological and physiological functions and hence represents an unprecedented level of complexity. An important aspect of this regulation is that there is no gene encoding *N*-glycan structures *per se*, but 2% of the mammalian genome is estimated to encode genes involved in the synthesis of these *N*-glycan structures. As noted in Section II, a prodigious enzymatic arsenal secures the biosynthesis of *N*-glycan structures (Moremen, Tiemeyer & Nairn, 2012). Expression of these actors is a key parameter in the observed *N*-glycan structural diversity, depending on many cellular parameters such as regulation at the transcriptional (transcription factors, epigenetic regulation), post-transcriptional (microRNA, miRNA) (Nairn *et al.*, 2008; Antony *et al.*, 2014; Dewal *et al.*, 2015; Lau *et al.*, 2015), translational (speed of synthesis) and post-translational (conformation, modifications, interaction with other proteins) levels (Rabouille *et al.*, 1995; Tu & Banfield, 2010; Struwe & Reinhold, 2012; Gao *et al.*, 2014; Neelamegham & Mahal, 2016; Blackburn *et al.*, 2018; Liu, Doray & Kornfeld, 2018). Interestingly, the glycan structure itself, at least for the *N*-glycan processing, governs the abundance of certain glycoproteins. Indeed, for *N*-glycoproteins, quality control based on structures of the glycan chains takes place in the ER (Ruddock & Molinari, 2006; Xu & Ng, 2015). This quality control not only monitors *N*-glycoprotein folding but also discriminates correctly folded glycoproteins from poorly folded ones destined to be degraded. Folding state can be considered as a key element in governing the *N*-glycan structure generated

through the Golgi apparatus. The accessibility of the oligosaccharide side chains to GTs and glycosidases in the Golgi apparatus determines whether a specific *N*-glycan is processed into a complex-type *N*-glycan or not. This has been illustrated nicely for the well-known phytohemagglutinin-L (PHA-L), the lectin of the common bean *Phaseolus vulgaris*, which possesses a non-processed oligomannosidic *N*-glycan at the asparagine-12 and a complex-type *N*-glycan at asparagine-60 (Sturm & Chrispeels, 1986; Sturm, Bergwerff & Vliegenthart, 1992; Bardor *et al.*, 1999). Besides the notion of accessibility, the *N*-glycan structures generated through the Golgi apparatus are also dictated by the strong substrate specificities and sequential action of different Golgi GTs and GHs. This led to the concept of "Go/No Go" pathways proposed by H. Schachter, the best example being the activity of the GnT I considered as a 'Go' signal for the activity of the Golgi mannosidase II (Schachter, 1991; Ünligil *et al.*, 2000).

The spatiotemporal organization of the glycosylation actors in the secretory pathway is also crucial (Blackburn, D'Souza & Lupashin, 2019). The molecular mechanisms that govern this differential subcellular localization are far from being identified and understood. Nevertheless, about 10 years ago the importance of intra-vesicular Golgi trafficking in the regulation of protein glycosylation was highlighted in human patients (Wu *et al.*, 2004; Foulquier *et al.*, 2006, 2007; D'Souza, Taher & Lupashin, 2020). Many mutations affecting seven of the eight constituent subunits of the conserved oligomeric Golgi (COG) complex (COG3 being the exception) were subsequently identified in patients with congenital disorders of glycosylation (CDG), a rare metabolic disease affecting glycosylation (Reynders *et al.*, 2011; Péanne *et al.*, 2018). The COG complex has been studied in many different organisms (*Saccharomyces cerevisiae*, *A. thaliana*, *D. melanogaster*, *C. elegans*) and in most studied organisms, COG defects were shown to be associated with Golgi morphological disruption, protein glycosylation and vesicular trafficking defects (Blackburn *et al.*, 2019). The mechanisms by which glycosylation defects are generated have been the subject of numerous studies and have been partially elucidated. COG defects disturb the Golgi structure and the retrograde intra-Golgi trafficking of vesicles containing important proteins for the glycosylation process, such as GTs and/or nucleotide-sugar transporters (Reynders *et al.*, 2009, 2011). Instability of some GTs, resulting from inadequate lysosomal targeting, in particular GlcNAc transferases I and II) (MGAT), α-mannosidase class 2A member 1 (Man2A1), β-1,4-galactosyltransferase 1 (β4GalT1) and β-galactoside α-2,6-sialyltransferase 1 (ST6Gal1) has been reported in COG-depleted cells (Foulquier *et al.*, 2006; Reynders *et al.*, 2009; Pokrovskaya *et al.*, 2011). Altogether, these processes account for the observed microheterogeneity of the *N*-glycan structures on a specific glycoprotein. In addition, regulation of the Golgi *N*-glycan process through Mn^{2+} and Ca^{2+} homeostasis has been investigated very recently. A specific discussion of this recent and original regulation is provided below.

(1) Overview of the different GTs and their Mn^{2+} dependency within *N*-glycan processing

Human Golgi GTs can be classified into three main groups according to their structural folds: GT-A, GT-B and GT-C (Oriol *et al.*, 2002; Lairson *et al.*, 2008; Breton, Fournel-Gigleux & Palcic, 2012; Gloster, 2014; Albuquerque-Wendt *et al.*, 2019). GT-A and GT-B share a close topology and use nucleotide sugars as donor substrates. Structurally, GT-A possess two abutting secondary structures called Rossmann folds (alternative β-sheets and α-helices, β/α/β). By contrast, in GT-B, the two β/α/β Rossmann domains face each other and are flexibly linked. One of the main differences between GT-A and GT-B is the presence of a highly conserved DXD motif (aspartic acid–any amino acid–aspartic acid) in the active site of GT-A that is absent in GT-B. This motif is crucial as it allows the carboxylates to coordinate a divalent cation and/or a ribose required for the stabilization of the nucleotide sugar. Note that for a specific GT, GnT I, this motif is comprised of three specific amino acids: glutamate, aspartic acid and aspartic acid (EDD) (Breton *et al.*, 2012). GT-As are thus considered metal-ion-dependent enzymes while GT-Bs are generally metal-ion-independent. More recently, GT-Cs have been identified as a new structural family of GTs possessing several hydrophobic transmembrane domains and using lipid phosphate-linked sugar as the donor substrate (Oriol *et al.*, 2002; Gloster, 2014; Albuquerque-Wendt *et al.*, 2019). These specific GTs act in the ER during the so-called dolichol cycle. Like GT-As, GT-Cs possess a DXD or even a DD motif that is crucial for their enzymatic activity (Lommel *et al.*, 2011; Albuquerque-Wendt *et al.*, 2019). While magnesium (Mg^{2+}), zinc (Zn^{2+}) or cobalt (Co^{2+}) ions can be used as a cofactor by GT-A enzymes, Mn^{2+} is considered the most prominent. This has been investigated by following the kinetics of catalytic activity of β4GalT1 (EC 2.4.1.38), a key enzyme of *N*-glycan processing that catalyses, only in the presence of Mn^{2+}, the transfer of Gal from UDP-Gal to the non-reducing end GlcNAc of complex *N*-glycans (Ramakrishnan, Ramasamy & Qasba, 2006). β4GalT1 is a GT-A folded enzyme containing a $D_{248}XD_{250}$ motif located in the cleft of its catalytic domain, which serves as a Mn^{2+} binding site that then allows the binding of UDP-Gal (Arnold *et al.*, 2000; Ramakrishnan *et al.*, 2006). Fig. 3 summarizes the known and putative Mn^{2+}-dependent glycoenzymes that are involved in the *N*-glycan-processing pathway in mammals (human) and plants (*Arabidopsis thaliana*). The affinity of these enzymes for Mn^{2+} is not yet known and it could well be that according to their subcellular localization, a tight regulation of local Mn^{2+} homeostasis is required to sustain the corresponding glycosylation reactions. These Mn^{2+}-dependent enzymes are Golgi localized, highlighting the importance of Golgi Mn^{2+} homeostatic regulation in *N*-glycan processing. Although surprising, the molecular mechanisms of Golgi Mn^{2+} homeostasis were unknown until recently.

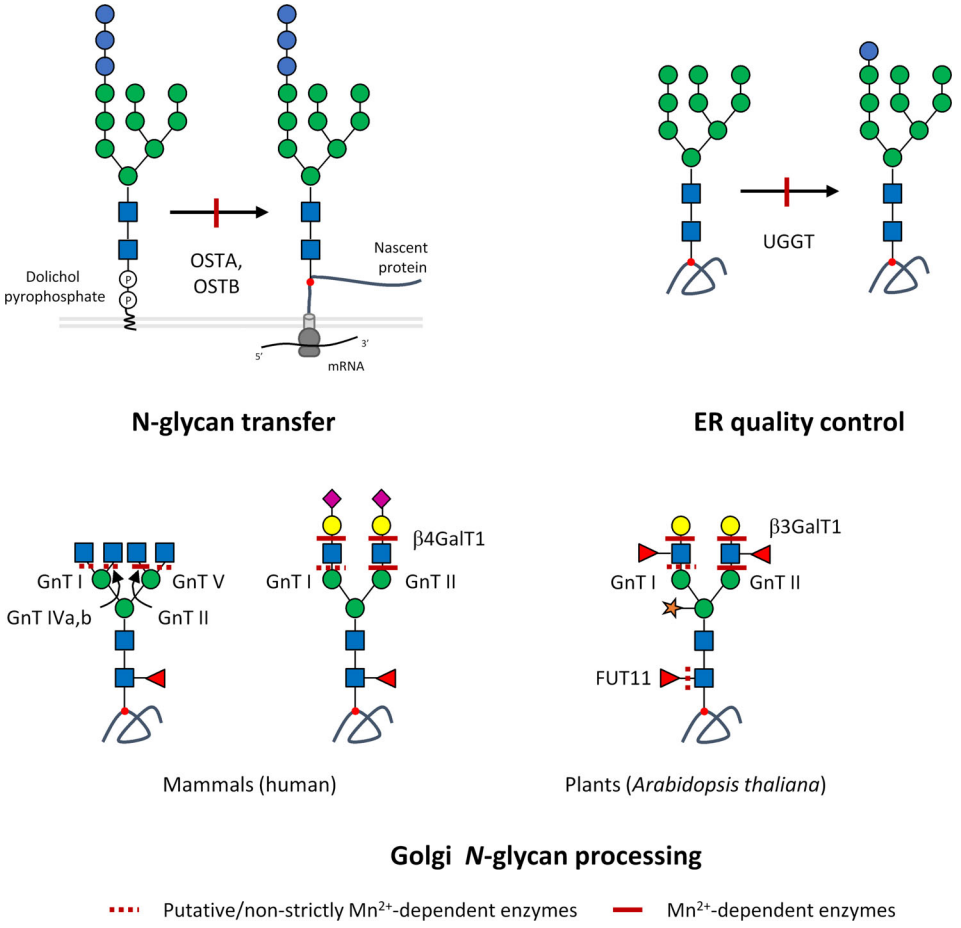


Fig. 3. Mn^{2+}-dependent glycoenzymes of *N*-glycosylation processing. During the biosynthesis of *N*-linked glycans, the oligosaccharyltransferase (OST) complex ensures the transfer of the oligosaccharide precursor to the nascent protein. The catalytic activity of this complex is driven by subunits STT3A for co-translational glycosylation and STT3B for co- and post-translational glycosylation. Both subunits act as glycosyltransferases (GTs) and are Mn^{2+} dependent. Afterwards, during endoplasmic reticulum (ER) quality control of *N*-glycoproteins, UGGT activity also requires Mn^{2+} ions as well as GnT I, GnT II, β4GalT1, GnTIVa,b and GnTVb involved in Golgi *N*-glycan processing in mammals and GnT I, Gn T II, FUT11 and β3GalT1 in *Arabidopsis thaliana*. Symbol nomenclature of monosaccharides follows Varki (2017b), see Fig. 1 for key. The red dot indicates the asparagine residue from the Asn-X-Ser/Thr/Cys consensus sequence. β3GalT1, β(1,3)-galactosyltransferase 1; β4GalT1, β(1,4)-galactosyltransferase 1; FUT11, α(1,3)-fucosyltransferase; GnT I, mannosyl α(1,3)-glycoprotein β(1,2)-*N*-acetylglucosamine transferase; GnT II, mannosyl β-(1,6)-glycoprotein β(1,2)-*N*-acetylglucosamine transferase; GnT IVa,b, mannosyl α(1,3)-glycoprotein β(1,4)-*N*-acetylglucosamine transferase 4a and 4b; GnT V, mannosyl α(1,6)-glycoprotein β(1,6)-*N*-acetylglucosamine transferase 5; mRNA, messenger RNA; UGGT, UDP-glucose glycoprotein glucosyltransferase 1.

(2) TMEM165, an essential protein in Golgi *N*-glycan processing and Mn^{2+} homeostasis

The discovery of *TMEM165* as a gene related to human CDG (Houdou & Foulquier, 2020) began a new era in the understanding of the regulation of Golgi Mn^{2+} homeostasis (Foulquier *et al.*, 2012). An understanding of the crucial role of TMEM165 in Golgi glycosylation maintenance arose unambiguously from the identification of CDG patients with mutations in *TMEM165*. All TMEM165-CDG patients were diagnosed as type II CDG based on serum-transferrin isoelectric focusing. In addition, mass spectrometry analysis of total *N*-glycans from patients' sera highlighted strong Golgi glycosylation defects with an increased level of abnormal *N*-glycan structures lacking both Gal and sialic acid residues (Foulquier *et al.*, 2012). This was confirmed in *TMEM165* knock-out cell lines (HEK cells) (Morelle *et al.*, 2017) where synthesised *N*-glycans provided evidence for a strong galactosylation defect, a mild GlcNAcylation defect and a slight sialylation defect. It is now clear that *TMEM165* deficiency not only affects *N*-glycan processing but also all other Golgi Mn^{2+}-dependent glycosylation pathways such as glycosaminoglycans and glycolipid synthesis (Bammens *et al.*, 2015; Morelle *et al.*, 2017; Haouari *et al.*, 2020). TMEM165 was also recently found to be involved in lactose biosynthesis during milk production (Snyder *et al.*, 2019).

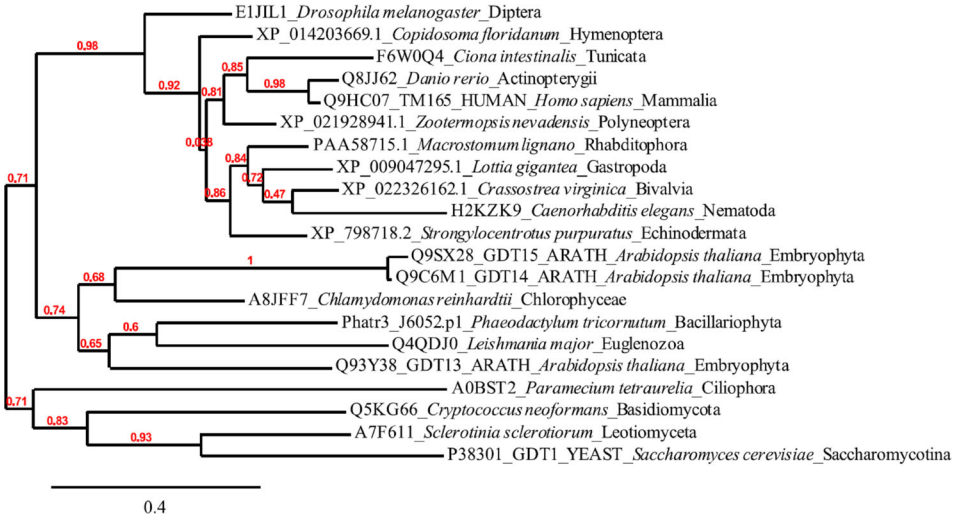


Fig. 4. Phylogenetic tree showing a selection of transmembrane protein 165 (TMEM165) homologs in Eukaryotes. Putative TMEM165 protein sequences from organisms belonging to the clades depicted in Fig. 2 were retrieved in the subfamily PTHR12608:SF1 (TRANSMEMBRANE PROTEIN 165) of the PANTHER database version 16 (Mi *et al.*, 2021; http://www.pantherdb.org/). A basic local alignment tool for proteins (BlastP) search with the human TMEM165 protein sequence (accession number Q9HC07) was also carried using the non-redundant protein sequences (https://blast.ncbi.nlm.nih.gov/) or in the Ensembl-Protists database (*Phaeodactylum tricornutum*), to identify additional similar sequences. Phylogeny created using the Phylogeny.fr platform (Dereeper *et al.*, 2008; http://www.phylogeny.fr/index.cgi) with the selected 21 sequences. The 'One Click' mode was chosen to perform the analysis with the following steps: the protein sequences were aligned using MUSCLE 3.8.31 (Edgar, 2004), a curation step by Gblocks 0.91b (Castresana, 2000) was included and the phylogenetic tree was subsequently built using the PhyML 3.1/3.0 aLRT program (Guindon & Gascuel, 2003; Anisimova & Gascuel, 2006). Graphical representation and editing of the phylogenetic tree were done with TreeDyn 198.3 (Chevenet *et al.*, 2006). The red numbers show the percentage of replicate trees in which the associated taxa clustered together, thus reflecting evolutionary distances based on sequence homologies.

The glycosylation defect is not restricted to TMEM165 but can be extended to all investigated uncharacterized protein family 0016 (UPF0016) members, a conserved family of transporter to which TMEM165 belongs (Demaegd *et al.*, 2014; Thines, Stribny & Morsomme, 2020). One of the best examples is Gdt1p, the yeast ortholog of TMEM165. The phenotype of the yeast null mutant *gdt1Δ* is a strong growth defect when cultured in the presence of high external calcium chloride concentration that can be correlated to strong *N*- and *O*-glycosylation abnormalities (Demaegd *et al.*, 2013). In addition to yeast Gdt1p, Yang *et al.* (2021) recently characterized the function of PML3 (photosynthesis-affected mutant 71 like 3; At5g36290), one of the five UPF0016 family members found in *Arabidopsis thaliana*, and demonstrated that PML3 played a critical role in Golgi glycosylation and cell wall biosynthesis under Mn^{2+}-deficient conditions. They also demonstrated interestingly a lower abundance of core α(1,3)-Fuc and β(1,2)-Xyl on the *N*-glycan structures isolated from protein extracts of *pml3* mutants. This defect is completely suppressed when the plant mutants are grown under Mn^{2+}-supplemented hydroponic nutrient solution (Yang *et al.*, 2021).

(3) TMEM165 structure and functional domains

TMEM165 belongs to the UPF0016 family, a highly conserved family of membrane proteins. A phylogenetic analysis of TMEM165 homologous sequences among different eukaryotic clades shows strong sequence homologies (Fig. 4). The sequence alignments reveal the presence of two copies of the hydrophobic domain E-Ø-G-D-(KR)-(ST) (where Ø indicates any hydrophobic residue) oriented in an antiparallel manner and highly conserved throughout species independently of the synthesis of complex-type *N*-glycans. This specific orientation could result from an ancient gene-duplication event as proposed by Demaegd *et al.* (2014) based on bacterial UPF0016 members. Many UPF0016 homologous sequences can be found in databases but only a few have been characterized, including the eukaryotic TMEM165 (human, mouse, zebrafish), Gdt1p (*Saccharomyces cerevisiae*) (Foulquier *et al.*, 2012; Demaegd *et al.*, 2013, 2014; Reinhardt, Lippolis & Sacco, 2014; Bammens *et al.*, 2015; Snyder *et al.*, 2019) and PAM71 (photosynthesis-affected mutant 71), CMT1 (chloroplast manganese transporter 1) and PML3–5 (plant *A. thaliana*) (Schneider *et al.*, 2016; Hoecker, Leister & Schneider, 2017; Eisenhut *et al.*, 2018; Hoecker *et al.*, 2020). During the last decade, many studies have attempted to unravel the exact functions of these different members (Demaegd *et al.*, 2013; Reinhardt *et al.*, 2014; Bammens *et al.*, 2015; Colinet *et al.*, 2016; Potelle *et al.*, 2017; Snyder *et al.*, 2017, 2019; Thines *et al.*, 2018; Stribny *et al.*, 2020; Wang *et al.*, 2020). So far, all these studies agree upon a function as a cation transporter required to maintain Ca^{2+}, Mn^{2+} and H^{+} homeostasis in the Golgi apparatus. Site-directed mutagenesis within the two consensus motifs

E-Ø-G-D-(KR)-(ST) demonstrated the crucial involvement of amino acid residues in these consensus motifs for Ca^{2+} and Mn^{2+} transport activities. The resulting altered transport function could be due to impaired cation affinity or pocket conformation changes, emphasizing that the amino acids of the two conserved motifs constitute the cation binding sites of the UPF0016 members.

(4) TMEM165 homologous sequences in eukaryotic clades and future research

For TMEM165 homologous sequences, the two consensus domains E-Ø-G-D-(KR)-(ST) are highly conserved across the different eukaryotic clades (Fig. 4) although an extensive diversity of *N*-glycan structures can be found. Some species clearly show evidence for Golgi glycosylation maturation while some only possess oligomannosidic-type *N*-glycans resulting from ER glycosylation. As shown for *A. thaliana*, TMEM165 orthologs are required for proper maturation of *N*-glycan structures (Yang *et al.*, 2021). The function of TMEM165 in the eukaryotic clades synthesizing only oligomannosidic-type *N*-glycans can be questioned. We noted from our phylogenetic analysis that the highest variability and heterogeneity of TMEM165 proteins is found in the variable length of the *N*-terminal region which does not show any obvious conservation. According to the species, the *N*-terminal extension of the eukaryotic UPF0016 members may thus have a role in targeting to reach their proper final destination. The observed diversity and variability in the *N*-terminal part of the eukaryotic UPF0016 members could then explain their different subcellular localizations. Specific targeting systems have certainly evolved to ensure the proper localization of proteins and the inherent complexity of the *N*-terminal region found in TMEM165 homologous sequences may coincide with the evolution of protein translocation systems used for membrane insertion and targeting. In terms of function, differential subcellular localization could allow the transport of Mn^{2+}/Ca^{2+} from the cytoplasm to specific luminal compartments such as the ER/Golgi in order to enable specific enzymatic reactions such as glycosylation and/or to protect the cytoplasm from Mn^{2+}excess and toxicity. Further studies are required to understand better the contribution of TMEM165 homologous sequences throughout the different eukaryotic clades and its possible involvement in regulation of *N*-glycosylation.

V. CONCLUSIONS

(1) The extensive diversity observed for most mature *N*-glycan structures, summarized in Fig. 2, indicates that *N*-glycan processing steps in the Golgi apparatus involve a large repertoire of organism-specific glycosidases and GTs, as well as other enzymes required for specific sugar substitutions (e.g. methyl, sulfate, methylphosphate, phosphoethanolamine, phosphorylcholine, etc.).

(2) The initial investigations of *N*-glycosylation have mainly focused on mammalian proteins, attempting to unravel this essential protein PTM and to understand the functions of mature *N*-glycans in human physiology and pathology. More recently, *N*-glycosylation of proteins from other eukaryotes has been investigated. The emergence of sensitive and efficient analytical technologies for the structural identification of mixture of complex oligosaccharides have allowed in-depth study of *N*-glycan profiles from numerous organisms, even for low amounts of available proteins, highlighting the rich structural diversity of mature *N*-glycans among eukaryotes.

(3) The functions of such *N*-glycan diversity of secreted proteins in eukaryotes are diverse. To date, numerous studies carried out on the functions of protein *N*-glycosylation in mammals have demonstrated their importance in cell–cell interactions, cell–protein recognition and interaction, and in human pathology (Varki, 2017a). The diversity of mature *N*-glycans in other eukaryotes suggests that *N*-glycosylation of proteins in all of these organisms does not solely function to ensure their proper folding in the ER. Indeed, considering the energy cost for each step of the *N*-glycan pathway, we can assume that the *N*-glycans resulting from Golgi maturation play important biological functions in organisms belonging to the different clades.

(4) Beyond the elucidation of the biological relevance of such *N*-glycan diversity, the regulation of the Golgi processing steps is a key issue. Indeed, such regulation is multifactorial. Moreover, recent studies have demonstrated that in some organisms, complex *N*-glycan processing steps are regulated through Golgi Mn^{2+} homeostasis, including molecular actors like TMEM165. More research is needed to understand the regulation of the *N*-glycan processing steps in other eukaryotes and to establish whether regulation of *N*-glycosylation through Mn^{2+} homeostasis is universal.

(5) In this context, future studies should attempt to decipher the roles of TMEM165 in the regulation of *N*-glycan processing in organisms that synthesise oligomannosides or mature *N*-glycans.

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