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Minireview

Paucimannose N-glycans in *Caenorhabditis elegans* and *Drosophila melanogaster*

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Dedicated to Professor Dr. Hans Kamerling on the occasion of his 65th birthday

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ABSTRACT

There is a rich diversity of paucimannose N-glycans in worms and flies, and these may play a role in the survival of these organisms. Although paucimannose N-glycans are not expressed in vertebrates, complex N-glycans may take over some of the functions of paucimannose N-glycans. Identification of the target proteins of β-1,2-*N*-acetylglucosaminyltransferase I (GnTI) in worms and flies and elucidation of their functions may thus lead to a better understanding of the role of GnTI-dependent glycoproteins in the survival/longevity of both invertebrates and vertebrates.

Introduction

Post-translational modifications such as glycosylation can greatly magnify the functions of a single gene.[1,2] The number of genes directly involved in glycan metabolism (glycogenes[3–6]) is relatively large; for example, there are ~250–500 human glycogenes representing ~1–2% of the total genome. Glycan biosynthesis takes place on a complex non-template assembly line in the rough endoplasmic reticulum and Golgi apparatus where many proteins work together to manufacture the final glycan product.[7,8]

N-Glycans are attached to protein by a GlcNAcβ1-*N*-Asn linkage. Inhibition of protein N-glycosylation is lethal for cells and for the intact organism.[9–15] All N-glycans share the same M3Gn2 pentasaccharide core structure (see Fig. 1 for abbreviated N-glycan names). The N-glycan synthetic pathway is comprised of three distinct stages. The first two stages up to the synthesis of the 'oligomannose' N-glycan M9Gn2 (Fig. 1) represent an ancient pathway expressed both in protozoa and metazoa.[16–19] The remainder of the second stage (conversion of M9Gn2 to M5Gn2, Fig. 1)[20–22] and initiation of the third stage (conversion of M5Gn2 to the 'hybrid' N-glycan GnM5Gn2, Fig. 1)[7,23–26] first appeared in evolution at about the same time as metazoa.

The synthesis of paucimannose N-glycans by worms and flies

UDP-GlcNAc:α3-D-mannoside β-1,2-*N*-acetylglucosaminyltransferase I (GnTI, encoded by *Mgat1*) plays a key role in N-glycan metabolism. GnTI adds GlcNAc in β-1,2-linkage to the non-reducing Manα1,3Manβ1,4 terminus of M5Gn2 to create the first hybrid N-glycan (GnM5Gn2, Fig. 1). The other hybrid N-glycans (GnM4Gn2, GnM3Gn2) are formed by the sequential action of α3,6-mannosidase II (Fig. 1). The vertebrate N-glycan synthesis pathway is directed toward 'complex' N-glycans (Gn2M3Gn2 and its derivatives, Fig. 1) via the action of UDP-GlcNAc:α6-D-mannoside β-1,2-*N*-acetylglucosaminyltransferase II (GnTII, encoded by *Mgat2*). GnTII adds GlcNAc in a β-1,2-linkage to the Manα1,6-Manβ-terminus of the hybrid N-glycan GnM3Gn2 (Fig. 1).[7,8,23,24] GnTI action is an essential prerequisite for GnTII action.

A specific β-*N*-acetylhexosaminidase expressed in *Caenorhabditis elegans*,[27] *Drosophila melanogaster*[28–30] and plants[31] hydrolyzes exclusively the GlcNAc residue attached to the Manα3Manβ terminus of GnM4Gn2 and GnM3Gn2 by GnTI (Fig. 1)[30] to form 'paucimannose' N-glycans (M4Gn2, M3Gn2, Figs. 1–3) and their fucosylated derivatives (M3Gn2F^3, M3Gn2F^6, etc, Figs. 2 and 3). Paucimannose N-glycan formation is therefore dependent on the prior actions of three important enzymes (GnTI, α3,6-mannosidase II, and β-*N*-acetylhexosaminidase) within the lumen of the Golgi apparatus. β-*N*-acetylhexosaminidase is not expressed in vertebrates, which is consistent with the fact that vertebrates

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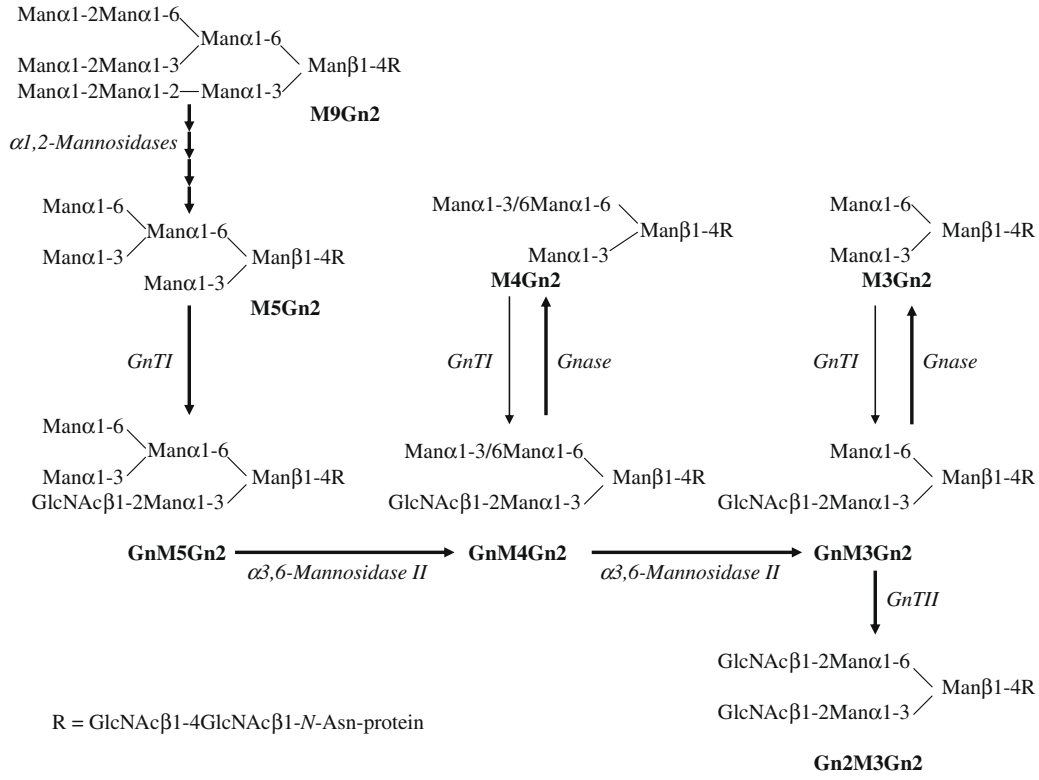

Figure 1. N-Glycan synthesis in wild-type *C. elegans* and *D. melanogaster*. The figure shows the conversion of oligomannose N-glycans to hybrid, paucimannose and bi-antennary complex N-glycans. The major N-glycan structures in worms[59] and flies[28] are shown. Abbreviated glycan names are in bold type (M = Man; Gn = GlcNAc): oligomannose (M9Gn2 to M5Gn2), hybrid (GnM5Gn2, GnM4Gn2, GnM3Gn2), paucimannose (M4Gn2, M3Gn2), and complex (Gn2M3Gn2). Fucosylated structures are shown in Figures 2 and 3. Enzyme names are in italics. *GnTI*[79] and *GnTII*[97,98] are defined in the text. *Gnase* is a β-*N*-acetylhexosaminidase that removes GlcNAc from GnM3Gn2 and GnM4Gn2.[27,29,30]

synthesize primarily complex and little, if any, paucimannose N-glycans, whereas invertebrates synthesize large amounts of paucimannose N-glycans at the expense of complex N-glycans.[27,28,32–40] *D. melanogaster* β-*N*-acetylhexosaminidase is encoded by the *fdl* (fused β-lobe) gene.[30] Mutant flies that do not express either β-*N*-acetylhexosaminidase[30] or GnTI[28] share the same fused β-lobe phenotype and paucimannose N-glycan deficiency. Mutant worms with a partial deletion of *hex-2*, a gene that encodes a *C. elegans* β-*N*-acetylhexosaminidase homolog, have been reported;[41] although these worms show a shift in the N-glycan profile, they still make appreciable amounts of paucimannose N-glycans possibly due, at least in part, to the presence of other active β-*N*-acetylhexosaminidase genes (*hex-3, -4, -5*).

The GlcNAc residue inserted by GnTI is a prerequisite for the fucosylation of hybrid N-glycans in *C. elegans* (Fig. 2) and *D. melanogaster* (Fig. 3),[33,42–44] and for the addition of phosphorylcholine to hybrid N-glycans in *C. elegans*.[45,46] These reactions must occur before β-*N*-acetylhexosaminidase removes the GnTI-dependent GlcNAc to convert hybrid to paucimannose N-glycans. *C. elegans*, surprisingly for an organism with a relatively simple anatomy, can synthesize about 150 different N-glycan structures,[47] a greatly more complicated pattern than is seen in *D. melanogaster*. This complexity is due in part to a variety of unusual fucosylation reactions and the addition of phosphorylcholine.[47] The phosphorylcholine-containing glycans have immune-modulating functions that may make nematodes immunologically invisible.[46,48]

Whereas mammals express five branching *N*-acetylglucosaminyltransferases (GnT I to V) that form a variety of complex N-glycans,[8,23] *C. elegans* expresses three branching GnTs (GnTI, II, V) and *D. melanogaster* expresses only two branching GnTs (GnTI, II). However, very small amounts of bi- and triantennary N-glycans are expressed in invertebrates due to the competing action of Golgi β-*N*-acetylhexosaminidase (Figs. 1–3). It is of interest that comparison of N-glycan structures between wild-type *D. melanogaster* and two fly mutants deficient in Golgi β-*N*-acetylhexosaminidase shows a 23 to 26-fold increase in GnM3Gn2F in the mutant flies (see Figs. 1 and 3 for structure nomenclature).[30] One mutant fly showed 2.0% and 5.6% occurrence of Gn2M3Gn2 and Gn2M3Gn2F, respectively. The other mutant showed a 3.8% occurrence of Gn2M3Gn2F but no data for Gn2M3Gn2. The per cent occurrence of Gn2M3Gn2 and Gn2M3Gn2F in wild-type flies could not be determined presumably because the amounts were too low.[30] The data are compatible with the concept that Golgi β-*N*-acetylhexosaminidase and GnTII compete for access to the hybrid N-glycans (Figs. 1–3).

C. elegans N-glycans have a high fucose content due to the expression of a large number of fucosyltransferases (FucTs), that is, one core α1,6-FucT homolog (FUT-8),[42,47] two established α-1,2-FucTs (CE2FT-1 and CE2FT-2, out of 25 putative genes),[49,50] and five α1,3/4-FucTs.[47,51,52]

Core α1,6-fucosylation is a conserved feature of N-linked oligosaccharides in both invertebrates and vertebrates.[42] An unsubstituted non-reducing terminal GnTI-dependent GlcNAc residue is an essential feature of the substrate for the core α1,6-FucT of both *C. elegans* and *D. melanogaster*. Both enzymes are unable to act on N-glycopeptides that carry a core α1,3-fucose residue. Therefore core α1,6-FucT requires the prior action of GnTI and must act before α1,3-FucT to make the difucosylated core structure (Figs. 2 and 3).

The α1,2-FucTs are the largest family of glycosyltransferases in the *C. elegans* genome.[49] Recombinant CE2FT-1 α1,2-FucT[50] can utilize Galβ1-O-phenyl, Galβ1-4Xylβ-R, and Galβ1-6GlcNAc-R as

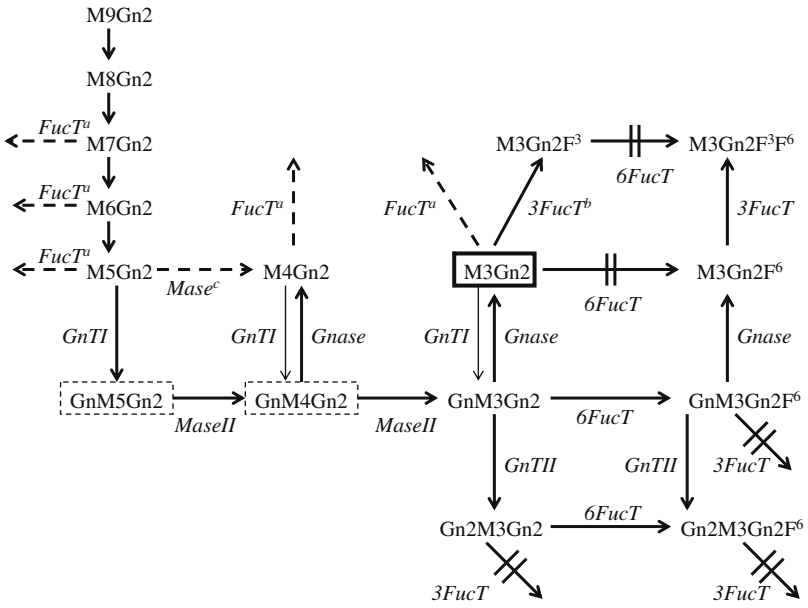

Figure 2. N-Glycan synthesis in wild-type *C. elegans*. This figure is based on the structural analysis of worm N-glycans.[41–43,47,68,70] N-Glycan names are defined in Figure 1. F^3 and F^6 refer to α1,3 and α1,6-linked core fucose, respectively. *Mase* and *FucT* are mannosidase and fucosyltransferase, respectively; other enzymes are defined in Figure 1. Reactions shown by continuous arrows have been established by in vitro or in vivo assays, whereas discontinuous arrows are hypothesized on other evidence. Arrows crossed with double lines indicate reactions that do not occur. The major structure in wild-type worms is M3Gn2 (boxed with a continuous line). Structures in boxes with discontinuous lines have not been detected using mass spectrometry, but are included in the figure on the basis of other evidence. The sites of action of the two core fucosyltransferases are indicated: 6FucT (α1,6-FucT, FUT-8) and 3FucT (α1,3-FucT, FUT-1). The fucosylation pathways are restricted by two important properties of the core FucTs: (i) α1,6-FucT must act before α1,3-FucT to make the difucosylated structure, and (ii) α1,6-FucT requires the prior action of GnTI, whereas α1,3-FucT acts only on structures with a non-substituted Manα1-3 residue on the N-glycan core. Structural analyses indicate that wild-type *C. elegans* can fucosylate M3-7Gn2 N-glycans with one or more Fuc residues. These Fuc residues are attached both to the N-glycan core and to peripheral residues by GnTI-independent FucTs (designated $FucT^a$ in the figure) that have not yet been characterized. The action of the core α1,3-FucT on M3Gn2 has been experimentally established and is therefore shown separately ($3FucT^b$) from the other FucTs acting on M3Gn2 ($FucT^a$). The fact that GnTI-deficient worms make M4Gn2 and M4Gn2F1 suggests that there must be a GnTI-independent mannosidase ($Mase^c$) that can act on M5Gn2 upstream of GnTI. Such mannosidases have been described in other species but not as yet in worms.[99,100] Many N-glycan structures that have been found in *C. elegans* are not shown in the figure, for example, the presence of methyl and phosphorylcholine groups and of galactose attached to core fucose residues.

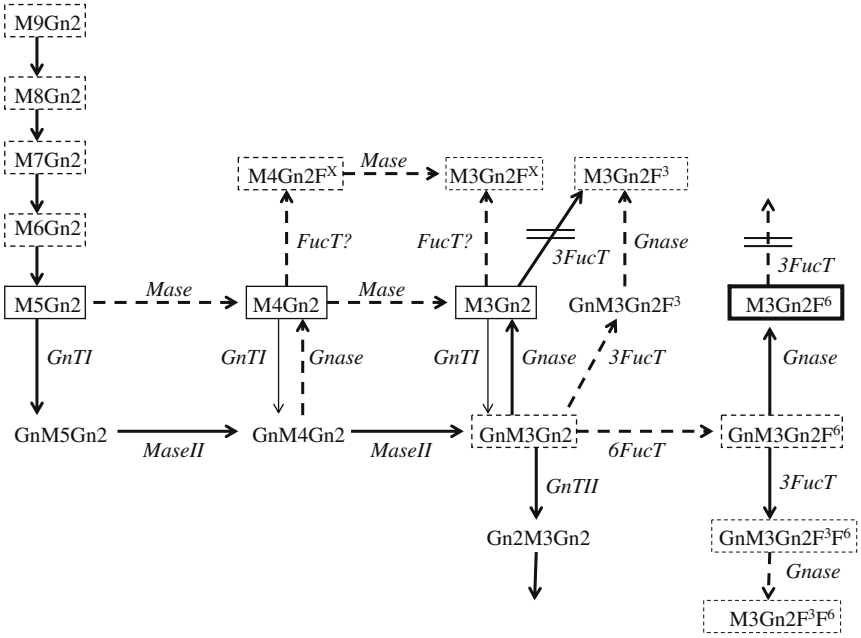

Figure 3. N-Glycan synthesis in wild-type *D. melanogaster*. This figure is based on the structural analysis of fly N-glycans.[28,33,41,42] N-Glycan and enzyme names are defined in Figures 1 and 2. The major structure in wild-type flies is $M3Gn2F^6$ (box with a thick continuous line). Other structures present in large amounts (32–68% of $M3Gn2F^6$) are boxed with thin continuous lines. The remaining boxed structures (discontinuous lines) are present in amounts less than 10% of $M3Gn2F^6$. Unboxed structures have not been detected by mass spectrometry but are included in the figure on the basis of other evidence.[42] The substrates, products, and reactions of core α1,6-FucT (6FucT)[42] and core α1,3-FucT (3FucT, FucTA)[44] are shown; both FucT enzymes are dependent on prior GnTI action. 6FucT cannot act on structures with a core α1-3-linked Fuc and must therefore act before 3FucT to make the small amounts of $M3Gn2F^3F^6$ in wild-type *Drosophila*.[33,42] GnTI-null flies make relatively large amounts of M3Gn2 and M4Gn2 suggesting that a GnTI-independent α-mannosidase (Mase) acts on M5Gn2 upstream of GnTI; such a mannosidase has been reported in *Spodoptera frugiperda*[100] but not in *Drosophila*.

an acceptor substrate but is unable to fucosylate either Galβ1-4Glcβ-R or free lactose. Recombinant CE2FT-2 α1,2-FucT[49] has a high activity toward Galβ1-3GalNAcα1-O-*p*-nitrophenyl acceptor but is inactive toward Galβ1-O-phenyl.

One of the five α1,3/4-FucTs (FUT-1, CEFT1)[43,51–53] adds Fuc in α1,3 linkage to the core GlcNAc residue, but only after the GnTI-dependent GlcNAc is removed and after prior action of the core α1,6-FucT (Fig. 2). FUT-1 is the only core FucT in plants and animals described to date that does not require the prior action of GnTI. Cross-reactivity with anti-horseradish peroxidase (HRP) antiserum is a feature of many glycoproteins from plants and invertebrates, and staining with this reagent has been used to track neurons in both worms and flies.[43,54] Worms with a deletion of *fut-1* (encoding core α1,3-FucT) show no reaction to anti-HRP,[43] proving that the cross-reaction with HRP is due to core α1,3-fucosylation.

Three α1,3-FucTs act on either Galβ1,4GlcNAc or GalNAcβ1,4GlcNAc moieties at the non-reducing ends of the N-glycan and one α1,3-FucT (FUT-4, CEFT5) was not active with any tested substrate.[51,55] Deletion mutants for each enzyme were analyzed.[51] While loss of CEFT-1 correlated with loss of core α1-3-fucosylated N-glycans in worms, loss of the other enzymes did not correlate with any phenotypic changes. These results suggest that each of the α1,3FTs in *C. elegans* has unique specificity and expression patterns.

Although the worm and fly N-glycan synthesis pathways have much in common (compare Figs. 2 and 3), flies do not attach phosphorylcholine to N-glycans and the fly's fucosylation pathways are less complex than those in the worm. *Drosophila* core α1,3-FucT requires the GnTI-dependent GlcNAc on its substrate (Fig. 3), whereas *C. elegans* core α1,3-FucT can act only after removal of the GnTI-dependent GlcNAc (Fig. 2). α3,6-mannosidase II, GnTII, and core α1,6-FucT (in vertebrates, worms and flies; Figs. 2 and 3) and addition of phosphorylcholine (in worms[45,46]) are all GnTI-dependent.

The phenotypes of *Mgat1*-null *C. elegans* mutants

C. elegans is a free-living nematode that undergoes a complex developmental process from embryogenesis through adulthood.[56–58] The mature adult is fertile for four days and lives for

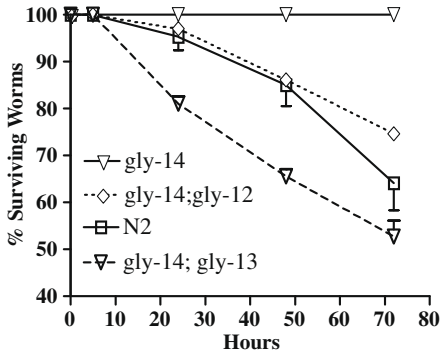

Figure 4. *C. elegans* survival. Three genes encoding enzymatically active *C. elegans* GnTI isoenzymes (*gly-12, gly-13, gly-14*) have been characterized.[71] Wild-type N2 worms and the three single-null mutants (*gly-12, gly-13, gly-14*), the three double-null mutants (*gly-12 gly-13, gly14;gly-12, gly-14;gly-13*), and the triple-null mutant (*gly-14;gly-12 gly-13*) were placed on *E. coli* OP50 growing on an enriched high-osmolarity peptone–glucose–sorbitol (PGS) medium.[59] Worms were loaded on the plates shortly after maturation to the adult form. Death was determined on failure to respond to prodding. All worms (only N2, *gly-14*, *gly-14;gly-12,* and *gly-14;gly-13* are shown in the figure) died rapidly with the exception of *gly-14* worms.[59] Lines are drawn through the averages of three experiments. Standard deviation bars are shown.

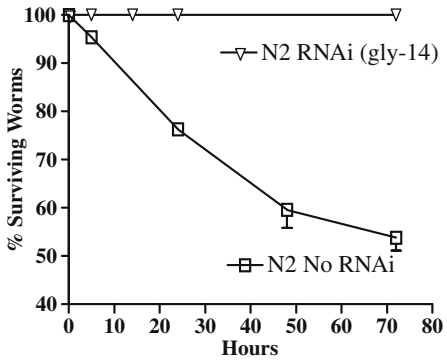

Figure 5. Production of the *gly-14* phenotype by RNA interference. N2 L4 larvae that had been subjected to RNA interference specific for the wild-type version of the *gly-14* gene and untreated N2 L4 larvae were placed on *E. coli* OP50 growing on PGS.[59] Downregulation of *gly-14* by RNAi had the same effect on survival (100% survival at three days) as was observed in the *gly-14* null worm (Fig. 4).

an additional 10–15 days when feeding on *Escherichia coli* OP50 grown on attenuated nematode growth medium (NGM). The complete genomic DNA sequence of *C. elegans* is known and contains sequences with significant similarities to a large number of mammalian glycogenes (at least 147) involved in the metabolism of protein-bound glycans.[59,60] The biosynthetic pathway for N-glycans in the worm is shown in Figure 2.

To date, ~800 *C. elegans* proteins (~4% of the worm's proteins) have been identified with Asn-X-Ser/Thr sequons occupied by N-glycan structures.[61–66] Fine structural analyses, primarily by mass spectrometry, have shown that *C. elegans* makes about 150 individual N-glycan structures.[34,38–40,65–70] There is a predominance of oligomannose N-glycans (Fig. 2) identical to those found in vertebrates, but the complex and hybrid N-glycans that are highly abundant in vertebrates are present at very low levels in worms.[38–40,67,68] The *C. elegans* N-glycan profile is different for each developmental stage (L1–L4, Dauer, adult), suggesting a role for these glycans in worm development.[69]

Three genes encoding enzymatically active *C. elegans* GnTI isoenzymes (*gly-12*, *gly-13*, *gly-14*) have been characterized.[71] Worms with null mutations in all three GnTI genes develop into apparently normal adults.[70] The worms are viable and fertile, although worm extracts have no detectable GnTI activity and show a highly abnormal N-glycan pattern.[70] GnTI-dependent N-glycans are therefore not essential for the normal development of *C. elegans* grown under laboratory conditions.

C. elegans has a mean life span of 18–20 days at 20 °C[57] on *E. coli* OP50 growing on the attenuated low osmolarity nematode growth medium (NGM) normally used in the laboratory. However, the worm faces much harsher conditions in its natural environment. These conditions were simulated by growing worms on an enriched high-osmolarity peptone–glucose–sorbitol (PGS) medium.[59] Wild-type, triple-null, all three double-null, and two of the three single-null (*gly-12* and *gly-13*) worms die relatively quickly when feeding on *E. coli* OP50 on PGS (Fig. 4 and Ref. 59) presumably because the bacteria can synthesize virulence factors more effectively on this medium.[72,73] However, gene deletion (Fig. 4) or RNAi-mediated downregulation (Fig. 5) of the *gly-14* gene results in 100% survival over the 72-h period of the experiment. The *gly-14* gene is expressed only in gut cells from L1 to adults,[71] suggesting that survival may be due to an inability of the bacteria to adhere to the gut endothelium and/or to cross the endothelial barrier into the body of the worm. The rapid death of *gly-14;gly-12* and *gly-14;gly-13* double null mutants (Fig. 4) shows that GLY-12- and GLY-13-dependent N-glycans are both needed to prevent worm death. The data suggest that N-glycans modulate the worm's defense

system and/or survival capability. Deletion of one or more of the worm's three GnTI genes has different effects on *E. coli* OP50 pathogenicity[59] indicating that each GnTI isoenzyme has a distinct set of target proteins and a distinct role in the interaction of *C. elegans* with bacteria.

The phenotype of the *Mgat1*-null *D. melanogaster* mutant

D. melanogaster can be grown in large numbers relatively cheaply.[74] The fly has a short generation time (~2 weeks) and short life span (~60–80 days). Fly development has been characterized in detail, and excellent methods are available to generate transgenic flies. The small genome (~14,000 genes) is entirely sequenced. N-glycan structures[28,75–78] and the N-glycan biosynthetic pathway (Fig. 3) have been determined.

There is a single *Mgat1* gene in *D. melanogaster* encoding an enzymatically active protein with 52% amino acid sequence identity to human GnTI.[79] *Mgat1*-null flies have been generated by imprecise excision of a nearby transposable element.[28] Extracts from these mutant flies do not express GnTI enzyme activity and show dramatic changes on mass spectrometric analysis of N-glycan structures compatible with the absence of GnTI activity. *Mgat1* null mutants are viable but exhibit pronounced defects in locomotion. The mutants are also male sterile and have a severely reduced mean and maximum life span. RNAi knockdown of *Mgat1* in wild-type flies resulted in ~50% reduction in GnTI activity, locomotory defects, and reduced life span similar to the phenotype observed in *Mgat1* null mutants.[101]

Microscopic examination of *Mgat1*-null[28] and *fdl*[30] adult *Drosophila* brains showed the presence of fused β-lobes within the mushroom bodies. The mushroom bodies are prominent structures in the *Drosophila* brain that have been shown to play a vital role in memory and learning.[80–83] Failure of β-lobes to stop at the brain midline has been associated with mutations in the *Drosophila dfmr1* gene, the ortholog of the human *Fmr1* gene responsible for Fragile X mental retardation 1.[84] The highly conserved ubiquitin ligase Neuralized (*Neur*) gene is expressed in the adult *Drosophila* mushroom body α/β-lobe peripheral neurons and is a limiting factor for the formation of long-term memory.[85] Heterotrimeric G(o), one of the most abundant proteins in the fly brain, has been shown to be part of a signaling pathway in mushroom body neurons that is required for the formation of associative memory.[86]

C. elegans and *D. melanogaster* life span and learning ability

C. elegans mutants such as *age-1* (phosphatidylinositol-3-OH kinase[87]), *daf-2* (insulin-like/IGF-1 receptor[88]) and *daf-16*[89,90] display an extended life span, an increased resistance to stress, and a greatly reduced metabolic rate. The *age-1* and *daf-2* mutants also show a marked delay in the age-related decline of isothermal tracking, a thermotaxis learning behavior associated with temperature and food.[91,92] The *age-1* mutant shows a dramatic 3-fold extension of life span and improved thermotaxis learning behavior that suggest strong neuroprotective actions during aging. The *age-1* and *daf-2* mutants show resistance to multiple forms of stress and an upregulation of the genes involved in reactive oxygen species scavenging, heat shock, and P450 drug-detoxification. *C. elegans* therefore provides a promising tool to study the prevention of age-related deficits in the nervous system and in learning behaviors. Mutations of components of the insulin/insulin-like growth factor-1 signaling pathway (IIS) show life extension and increased stress resistance not only in *C. elegans* but also in *Drosophila* and mice.[93–96] The above studies and many others suggest that the biological mechanisms underlying aging are conserved across species and that model organisms such as worms and flies can provide approaches to the characterization of the mechanisms that regulate human aging and possibly even memory and learning.

The GnTI-dependent survival of the *C. elegans gly-14* mutant (Figs. 4 and 5),[59] the fused β-lobes within the mushroom bodies of *Mgat1*-null[28] and *fdl*[30] adult *Drosophila* brains, and our recent construction of a mutant fly with a GnTI-dependent two-fold increase in life span[101] suggest that GnTI-dependent N-glycans may play a role in the ability of invertebrate organisms to survive and learn. Although the major GnTI-dependent N-glycans are different in invertebrates (paucimannose) and vertebrates (complex), GnTI-dependent N-glycans may play a role in longevity, and possibly also in memory and learning, in both invertebrates and vertebrates.

The role of GnTI in invertebrate longevity

Two approaches can be used to identify GnTI-dependent candidate glycoproteins that may be responsible for the phenotypes observed in *gly-14 C. elegans*,[59] *Mgat1*-null flies[28], and long-lived GnTI-dependent *Drosophila* mutants.[101] The first approach begins with identification of the target proteins of GnTI in worms and flies by a modification[102] of previously reported mass spectrometry-based glycoproteomics approaches.[65,66] We have validated this method by showing that analysis of wild-type worms and flies identifies a large number of glycopeptides (Asn converted to Asp), whereas similar analyses of *gly-14;gly-12 gly-13* triple-null worms, and *Mgat1*-null flies show few, if any, glycopeptides. The second approach is based on genes (such as IIS pathway genes) previously shown to be involved in longevity and survival. The *C. elegans* and *Drosophila* GnTI high-survival mutants described above will be probed with candidate genes from the above approaches to identify genes that alter the respective phenotypes.

Conclusions

This review has described the rich diversity of paucimannose N-glycans in worms and flies and their possible role in the survival of these organisms. Although paucimannose N-glycans are not expressed in vertebrates, complex N-glycans may have taken over some of the functions of paucimannose N-glycans during the process of evolution. Identification of the target proteins of GnTI in worms and flies and elucidation of their functions may lead to a better understanding of the role of GnTI-dependent glycoproteins in the survival/longevity of both invertebrates and vertebrates.

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