

N-glycan antennal modifications are altered in *Caenorhabditis elegans* lacking the HEX-4 *N*-acetylgalactosamine-specific hexosaminidase

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Simple organisms are often considered to have simple glycomes, but plentiful paucimannosidic and oligomannosidic glycans overshadow the less abundant N-glycans with highly variable core and antennal modifications; *Caenorhabditis elegans* is no exception. By use of optimized fractionation and assessing wildtype in comparison to mutant strains lacking either the HEX-4 or HEX-5 β -*N*-acetylgalactosaminidases, we conclude that the model nematode has a total N-glycomic potential of 300 verified isomers. Three pools of glycans were analyzed for each strain: either PNGase F released and eluted from a reversed-phase C18 resin with either water or 15% methanol or PNGase Ar released. While the water-eluted fractions were dominated by typical paucimannosidic and oligomannosidic glycans and the PNGase Ar-released pools by glycans with various core modifications, the methanol-eluted fractions contained a huge range of phosphorylcholine-modified structures with up to three antennae, sometimes with four *N*-acetylhexosamine residues in series. There were no major differences between the *C. elegans* wildtype and *hex-5* mutant strains, but the *hex-4* mutant strains displayed altered sets of methanol-eluted and PNGase Ar-released pools. In keeping with the specificity of HEX-4, there were more glycans capped with *N*-acetylgalactosamine in the *hex-4* mutants, as compared with isomeric chito-oligomer motifs in the wildtype. Considering that fluorescence microscopy showed that a HEX-4::enhanced GFP fusion protein colocalizes with a Golgi tracker, we conclude that HEX-4 plays a significant role in late-stage Golgi processing of N-glycans in *C. elegans*. Furthermore, finding more “parasite-like” structures in the model worm may facilitate discovery of glycan-processing enzymes occurring in other nematodes.

As compared with most types of protein glycosylation that are generally based on step-by-step formation of an oligosaccharide chain, N-glycans are based on a build–remove–rebuild

principle. Thereby, after the initial transfer of a tetradecasaccharide to a subset of asparagine residues in most eukaryotes, selective removal of glucose and mannose is followed by addition of further monosaccharide units; in multicellular organisms, *N*-acetylglucosamine, fucose, and galactose are amongst the residues transferred in the Golgi apparatus to the antennal or core regions of the protein-bound N-glycan structure. Especially the addition of *N*-acetylglucosamine results in GO or NOGO signals for glycan modification events as respectively exemplified by the action of *N*-acetylglucosaminyltransferases I and III in vertebrates (1).

In many lower multicellular organisms (plants and invertebrates), *N*-acetylglucosaminyltransferase I (Enzyme Commission [EC] number: 2.4.1.101) is also a key enzyme, but often the transferred residue on the core α 1,3-mannose is absent from the mature glycoprotein, despite its necessity for core fucosylation. Thus, the concept of a processing hexosaminidase was introduced, which could account for fucosylated paucimannosidic glycans found in, for example, insects (2). Later, it was shown that the *Drosophila melanogaster fused lobes (fdl)* gene encodes a Golgi hexosaminidase, which specifically removes the *N*-acetylglucosamine transferred by *N*-acetylglucosaminyltransferase I (3).

Fucosylated paucimannosidic glycans also occur in nematode worms, such as *Caenorhabditis elegans*, whereby any of the core fucose residues can be galactosylated (4). Some core-modifying enzymes, especially the FUT-8 core α 1,6-fucosyltransferase (EC no.: 2.4.1.68), have a very definite bias toward substrates with an antennal *N*-acetylglucosamine residue on the core α 1,3-mannose even though it is then absent from the final glycan; however, the FUT-1 core α 1,3-fucosyltransferase (EC number: 2.4.1.214) will only act after removal of this *N*-acetylglucosamine (5). The relevant identified processing hexosaminidases (HEX-2 and HEX-3) are not paralogous to FDL but are members of a different subgroup (“clade B”) of the GH20 family (6). Indicative of their *in vivo* function, a highly simplified N-glycome results when knocking out both the *hex-2* and *hex-3* genes in *C. elegans*—core α 1,3-fucose is lacking from the reducing terminus in this mutant (7), whereas removal of the core α 1,6-mannose is apparently

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the signal for transfer of a third core fucose by FUT-6 (8). Thus, the processing for these core-modified fucose-rich glycans in *C. elegans* is actually rather complicated with multiple build–remove–rebuild steps.

While the core modifications, including the bisecting galactose residue and the fucose on the distal *N*-acetylglucosamine, in *C. elegans* are quite well characterized (9), less attention has been given to the antennae, partly in the belief that complex glycans are not well represented in the worm's N-glycome. Only recently have *C. elegans* N-glycans been shown to possess LacdiNAc (GalNAc β 1,4GlcNAc) and chitobiose (GlcNAc β 1,4GlcNAc) antennal motifs (10), whereas the nematode BRE-4 enzyme related to mammalian β 1,4-galactosyltransferases is a proven β 1,4-*N*-acetylgalactosaminyltransferase (EC number: 2.4.1.244), which can modify N-glycans *in vitro* to form LacdiNAc motifs (11). On the other hand, in addition to HEX-2 and HEX-3, *C. elegans* has two other clade B GH20 enzymes designated HEX-4 and HEX-5, both with a distinct preference for *N*-acetylgalactosamine residues (6). While HEX-4 has optimal activity around pH 6 suggestive of a Golgi localization, HEX-5 is most active under acidic conditions (6). Considering also that nematodes with detectable LacdiNAc expression on glycoproteins, for example, *Trichinella spiralis* and *Trichuris suis* (12, 13), have only one clade B GH20 enzyme (unpublished data), we speculated that expression of HEX-4 may correlate with a lack of terminal LacdiNAc on N-glycans.

To explore our hypothesis further, we examined the N-glycomes of *hex-4* and *hex-5* mutant strains in comparison to the wildtype and reveal that *N*-acetylgalactosamine-capped N-glycans are more abundant in worms lacking HEX-4. Also, prefractionation of more “hydrophobic” N-glycans in the wildtype aids detection of multiantennary glycans with HexNAc_{2–4} motifs partly substituted with phosphorylcholine (PC) and/or fucose residues. Thus, we reveal that the N-glycome of *C. elegans* is even more complex than previously thought and that the Golgi-localized HEX-4 plays a role in determining the modification of N-glycan antennae in this model nematode.

Results

Transcription and deletion of different nematode hexosaminidases

Previously, we used promoter-driven *gfp* constructs to explore the expression pattern for five *C. elegans* GH20 hexosaminidase genes: whereas the *hex-1*, *hex-2*, *hex-3*, and *hex-5* reporters were expressed throughout the life cycle, the one based on the *hex-4* promoter was restricted to the embryonal and L1 stages (6). We have now mined extensive transcriptome data (14, 15), which suggest that *hex-2* versus *hex-3* and *hex-4* versus *hex-5* show contrasting expression patterns in up to 10 h embryos; all four are also expressed to varying extents in larvae and young adults but display different tissue expression (Fig. S1). *In vitro*, HEX-4 and HEX-5 remove terminal GalNAc residues from N-glycan and glycosaminoglycan-type chains (Fig. S2), but unlike HEX-2 and HEX-3 do not remove GlcNAc

residues (6, 16). While glycomic analyses showed that HEX-2 and HEX-3 are responsible for the removal of the nonreducing terminal β 1,2-linked GlcNAc from N-glycans (7), the *in vivo* molecular role of HEX-4 and HEX-5 remained unknown.

For the in-depth analysis of N-glycomic impact of deletions in the two β -*N*-acetylgalactosaminidase genes, we compared two *hex-4* strains (cop1517 and cop1518, generated using CRISPR–Cas9 technology) and one *hex-5* strain (tm3307, generated by random mutagenesis) with the parent wildtype N2 strain (Fig. S3). None of the hexosaminidase mutants had any obvious growth phenotype. As other glycomutant worm strains can have higher susceptibility or resistance to bacteria or their toxins, growth on *Pseudomonas* PA14 was tested; however, no major difference for *hex-4* and *hex-5* strains as compared with wildtype was observed (Fig. S3).

Thereafter, we focused on the biochemical role of HEX-4 and HEX-5 by comparing the molecular glycomic phenotypes of N2, *hex-4*, and *hex-5*. Following use of PNGase F and PNGase Ar for N-glycan release in combination with solid phase extraction on C18, fluorescent labeling and HPLC on an RP-amide column, we analyzed three pools of N-glycans (PNGase F-released water-eluted, PNGase F-released methanol eluted, and PNGase Ar-released post PNGase F) for each strain; each HPLC fraction was analyzed by MALDI-TOF mass spectrometry (MS), and the annotations are based on MS/MS before and after chemical or enzymatic treatments (Figs. 1–3, S4–S7, S9 and S10).

Typical oligomannosidic, paucimannosidic, and simple complex N-glycans

When performing MALDI-TOF MS of the N-glycan subpools eluted from C18 with water, the spectra are dominated by compositions of Hex_{3–9}HexNAc₂, Hex₃HexNAc₂Fuc₁, and Hex₃HexNAc_{3–4}Fuc_{0–1}. Indeed, upon offline LC–MS using an RP-amide column for separation prior to MS (Fig. 1), the major structure in each major peak was observed to have one of these compositions and their retention times in terms of glucose units as well as MS/MS fragmentation patterns were comparable with those from previous studies (17, 18). Furthermore, the “water-eluate” chromatograms for N2, tm3307, cop1517, and cop1518 strains are nearly superimposable; around 100 glycans were detected in each of these analyses, whereby 70 to 80% of the structures found were present in all samples. The chromatogram for the second *hex-4* mutant, cop1518, diverged in terms of the abundance of Man_{8–9}GlcNAc₂ (5–6 g.u.) as compared with the others, but the major differences between the water-eluted samples were rather in the presence or the absence of PC-modified glycans.

PC-modified N-glycans

Previously, Hanneman *et al.* (19), Haslam *et al.* (20), and we (21) reported zwitterionic N-glycans from the N2 strain with compositions of Hex₃HexNAc_{3–5}Fuc_{0–1}PC₁ (19–21), whereas we found Hex₅HexNAc₃Fuc_{0–1}PC₁ in the *aman-2* mutant, Hex₃HexNAc_{3–6}Fuc_{0–1}PC_{1–2} in the *hex-2;hex-3* mutant and

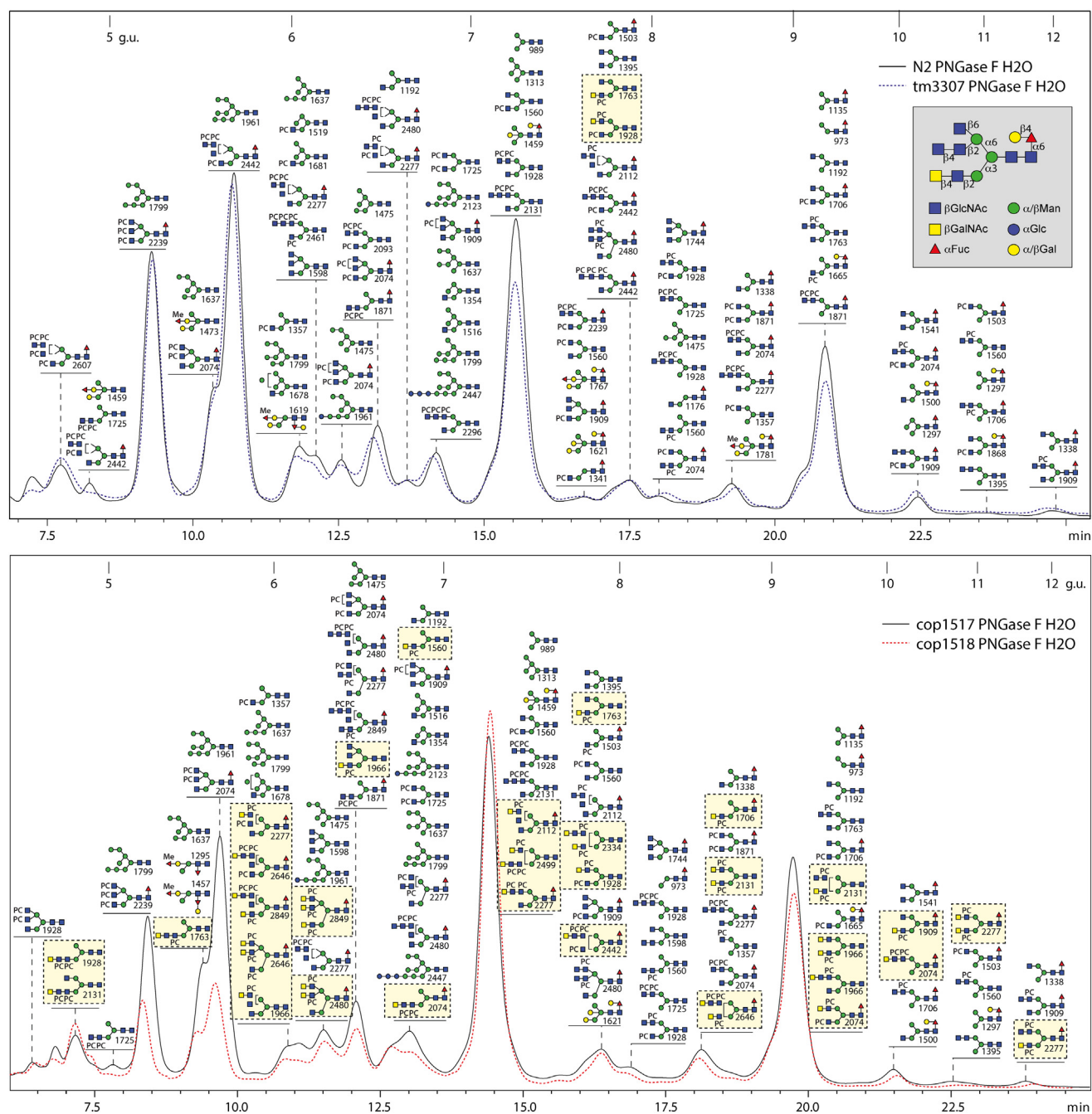


Figure 1. RP-amide chromatograms of PNGase F-released water-eluted N-glycans. Glycans from *Caenorhabditis elegans* strains (wildtype N2, *hex-5/tm3307*, *hex-4/cop1517*, or *hex-4/cop1518*) released by PNGase F and eluted from C18 solid phase extraction material with water were labeled with 2-aminopyridine. The RP-amide chromatograms, calibrated in terms of glucose units (g.u.), are annotated with glycan structures (in order of abundance in the relevant fraction) according to the Symbol Nomenclature for Glycans (mannose, galactose, or glucose is depicted by green/yellow/blue circles, GalNAc or GlcNAc by yellow/blue squares, fucose by red triangles, phosphorylcholine by PC and methyl by Me). The *m/z* for the protonated ions as detected by MALDI-TOF MS are also given with the depicted structures based on conclusions derived from elution time, chemical and/or enzymatic digestion, and MS/MS data of which selected examples are shown in Figures 4–7. As the *tm3307* and *cop1518* chromatograms are respectively similar to either the wildtype N2 or *hex-4 cop1517*, these are indicated in the panels by dashed lines. For the full annotations, refer to Figs. S4 and S5. The chromatograms of all four samples are very similar, despite differences in the detected N-glycans between the N2 and *hex-4* strains; the major glycans are identical, but the β 1,4-GalNAc-capped structures (proven by specific HEX-4 digestion; highlighted with light yellow boxes) are far more abundant in the *hex-4* strains. Based on previous studies, it is assumed that the phosphorylcholine is 6-linked to GlcNAc.

some “simple” Hex_{3–4}HexNAc_{3–4}Fuc_{0–1}PC_{1–2} structures in C18-HPLC fractions of glycomes from double fucosyl-transferase mutants (7, 18, 21). On the basis of typical PC-containing MS/MS fragments (*m/z* 369, 531, 572, etc.), we estimate that about one-half of the structures detected in the

water eluates (Fig. 1) and about two-thirds in the methanol eluates (Fig. 2) are modified with one or more zwitterionic PC residues; this bias toward elution from C18 with methanol is compatible with the late elution of PC-modified glycans on traditional reversed-phase material (10, 17). However,

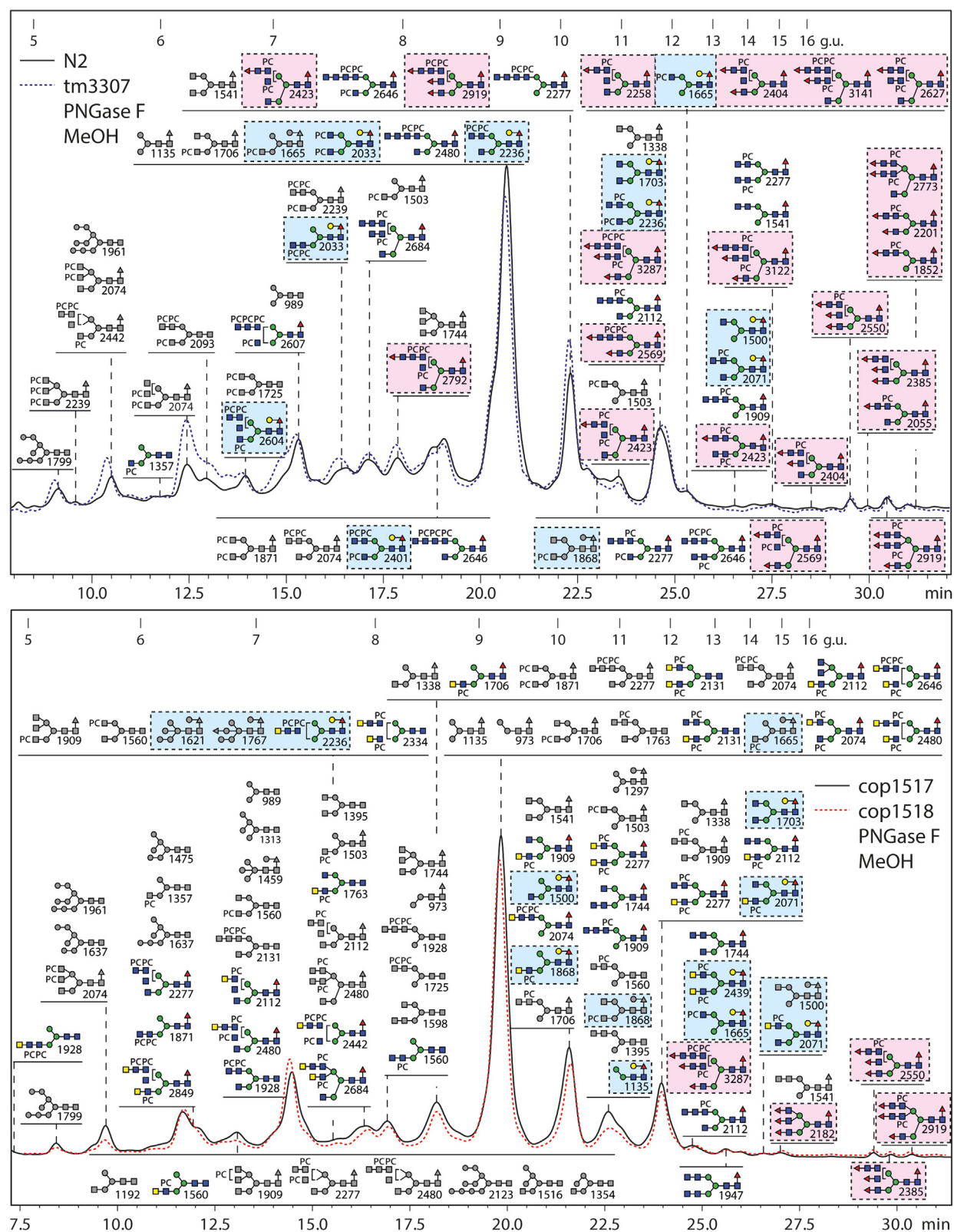


Figure 2. RP-amide chromatograms of PNGase F-released methanol-eluted N-glycans. Glycans from *Caenorhabditis elegans* strains (wildtype N2, *hex-5/tm3307*, *hex-4/cop1517*, or *hex-4/cop1518*) released by PNGase F and eluted from C18 solid phase extraction material with 15% methanol were labeled with 2-aminopyridine. The RP-amide chromatograms, calibrated in terms of glucose units (g.u.), are annotated with glycan structures (in order of abundance in the relevant fraction) according to the Symbol Nomenclature for Glycans; those in gray are also found in the water-eluted pools. MS/MS data of selected structures are shown in Figures 4–9. As the *tm3307* and *cop1518* chromatograms are respectively similar to the wildtype N2 and *cop1517*, these are indicated in the panels by dashed lines. For the full annotations, refer to Figs. S6 and S7. The chromatograms of all four samples are very similar, despite differences in the detected N-glycans between the N2 and *hex-4* strains. Glycans with multiple antennal phosphorylcholine and/or fucose residues,

subsequent separation on a fused-core column, such as the RP-amide used here, is more effective in enabling detection; generally, an increased number of PC residues leads to early elution on RP-amide, but this effect is counteracted by other structural modifications such as core α 1,6-fucosylation with or without galactose. As these glycans ionize especially well in positive mode due to the presence of the choline moiety absent from oligomannosidic and paucimannosidic structures, quantification as compared with nonzwitterionic glycans is not meaningful as the presence of such structures in every RP-amide fraction would lead to an overestimation of their abundance; thus, we only offer an estimate of the overall number of PC-containing glycans.

In the current study, other than the simple Hex₃HexNAc₃₋₅Fuc₀₋₁PC₁, hallmarked by m/z 369 and 531 B-ions (Hex₀₋₁HexNAc₁PC₁) upon MS/MS, we have found a variety of fragments indicative of longer antennae in the wildtype glycome with one or more PC modifications, for example, m/z 572, 737, 775, 940, 1105, 1143 and 1309 (HexNAc₂₋₄PC₁₋₃; Fig. 4, A–E); where two or more PC residues are present, then a loss of the trimethylamine from the choline moiety is observed ($\Delta m/z$ 59). The fragments of 899 (Hex₁HexNAc₂PC₂), 1102 (Hex₁HexNAc₃PC₂), 1267 (Hex₁HexNAc₃PC₃), and 1305 (Hex₁HexNAc₄PC₂) are compatible with two antennae attached to the same core α -mannose residue. Some of these fragments are reminiscent of those observed for N-glycans from *Oesophagostomum dentatum* or *Pristionchus pacificus* (17, 22), which like *C. elegans* are also predicted to have the antennal initiation enzymes MGAT-1, MGAT-2, and MGAT-5, but not MGAT-4; thus, the disubstituted mannose is predicted to be the “upper” α 1,6-linked one within the context of a triantennary structure (Fig. 4, F–J). Due to the diverging fragmentation of glycans found in different HPLC fractions, numerous isomeric structures could be identified as exemplified by m/z 2112, 2239, 2277, 2442, and 2480 (Figs. 4 and 5). The degree of isomeric diversity is high with, for example, 10 different isomers of Hex₃HexNAc₆Fuc₁PC₂ found in the wildtype and *hex-4* strains (m/z 2277; Fig. S8).

In order to gain more insight into the underlying structures, chemical and enzymatic treatments were performed. To verify the position of PC within the antennae, three types of hexosaminidase were used to ascertain whether nonreducing terminal nonsubstituted HexNAc residues were present: (i) the β 1,3/4-specific *Streptomyces* chitinase, which can remove either N-acetylgalactosamine or N-acetylglucosamine residues (23), (ii) the nonspecific jack bean hexosaminidase, and (iii) the GalNAc-specific *C. elegans* HEX-4 enzyme (16, 24), which can remove GalNAc from LacdiNAc motifs modified with PC on the subterminal GlcNAc residue (23). In case of wildtype HexNAc₂PC₁ motifs, the chitinase can remove one HexNAc residue resulting in a shift in the fragments from m/z 572 to m/z 369 (Fig. 5, A, B, L and M), indicative that the innermost GlcNAc of the motif is carrying the zwitterionic modification; in contrast, HEX-4 was generally not effective on wildtype

glycans, suggestive that the outermost HexNAc was not GalNAc as on some parasitic glycans (24), but GlcNAc. On the other hand, jack bean hexosaminidase removed unsubstituted antennal GlcNAc residues (Fig. 5, C, D, N and O). As an independent indication for the presence of phosphodiester modifications, we employed treatment with hydrofluoric acid; this resulted in loss of all predicted PC modifications as well as shifts in the MS/MS fragmentation patterns (Fig. 5, E–K and P–V).

N-glycans with terminal N-acetylgalactosamine residues

In contrast to the wildtype or *hex-5* glycomes where the vast majority of the PC-modified HexNAc₂₋₄ motifs had N-acetylglucosamine as the terminal residue, the *hex-4* mutant strains had a large number of N-acetylgalactosamine-capped glycans. HEX-4 could remove one HexNAc residue from HexNAc₂PC₁, HexNAc₃PC₁, and HexNAc₃PC₂ motifs present on a variety of hybrid, biantennary, and triantennary N-glycans in these strains (Fig. 6, A–H); MS/MS showed shifts in m/z 572, 734, 775, 940, and 1305 B-ion fragments to m/z 369, 531, 572, 737, and 899 (Fig. 6, I–R). These data indicated that the terminal residue was GalNAc, but that the subterminal HexNAc in the case of HexNAc₃PC₁ was GlcNAc. Further variants of biantennary and triantennary glycans with terminal GalNAc included HexNAc₄PC₁₋₂ motifs or structures with α 1,6-mannose residues substituted with one “short” and one “long” PC-containing antenna (Fig. 7). Forms of these antennae lacking the zwitterionic modification were extremely rare, but N-glycans with one chitobiosyl and one LacdiNAc (both PC substituted) were also found, whereby these eluted slightly later than the isomers with two PC-substituted LacdiNAc motifs (e.g., m/z 2131 or 2277; Fig. 1). In total, only two GalNAc-capped N-glycans were detected in the N2 and *hex-5* strains as compared with over 50 in either *hex-4* mutant (Fig. S8).

N-glycans with antennal fucose residues

Another surprise in the methanol-eluted subpools was the presence of complex glycans of between 2200 and 3300 Da with multiple fucose residues. Previously, *C. elegans* glycans with more than one fucose residue were generally shown to be of the paucimannosidic type, some carrying up to five fucose and five galactose residues associated with the mannosylchitobiosyl core (4). In the present study, particularly the wildtype N2 and *hex-5* strains contained glycans with compositions such as Hex₃HexNAc₆Fuc₄ (m/z 2385, >16 g.u.). Here, m/z 349 and 552 B-ion HexNAc₁₋₂Fuc₁ fragments were found (Fig. 8A), indicative that the outermost HexNAc carried a fucose residue. Variants of these multiply fucosylated structures also carried PC residues as indicated by the B-ions such as m/z 718 or 1087 (HexNAc₂₋₃PC₁₋₂Fuc₁; Fig. 8, B–F). Hydrofluoric acid treatment, known to effectively cleave both PC and α 1,3/4-fucose residues, altered the MS and MS/MS

especially detected in the wildtype, were previously not described for *C. elegans*. Highlighted are glycans with antennal fucose (pink) and reducing-terminal Gal β Fuc (blue) epitopes.

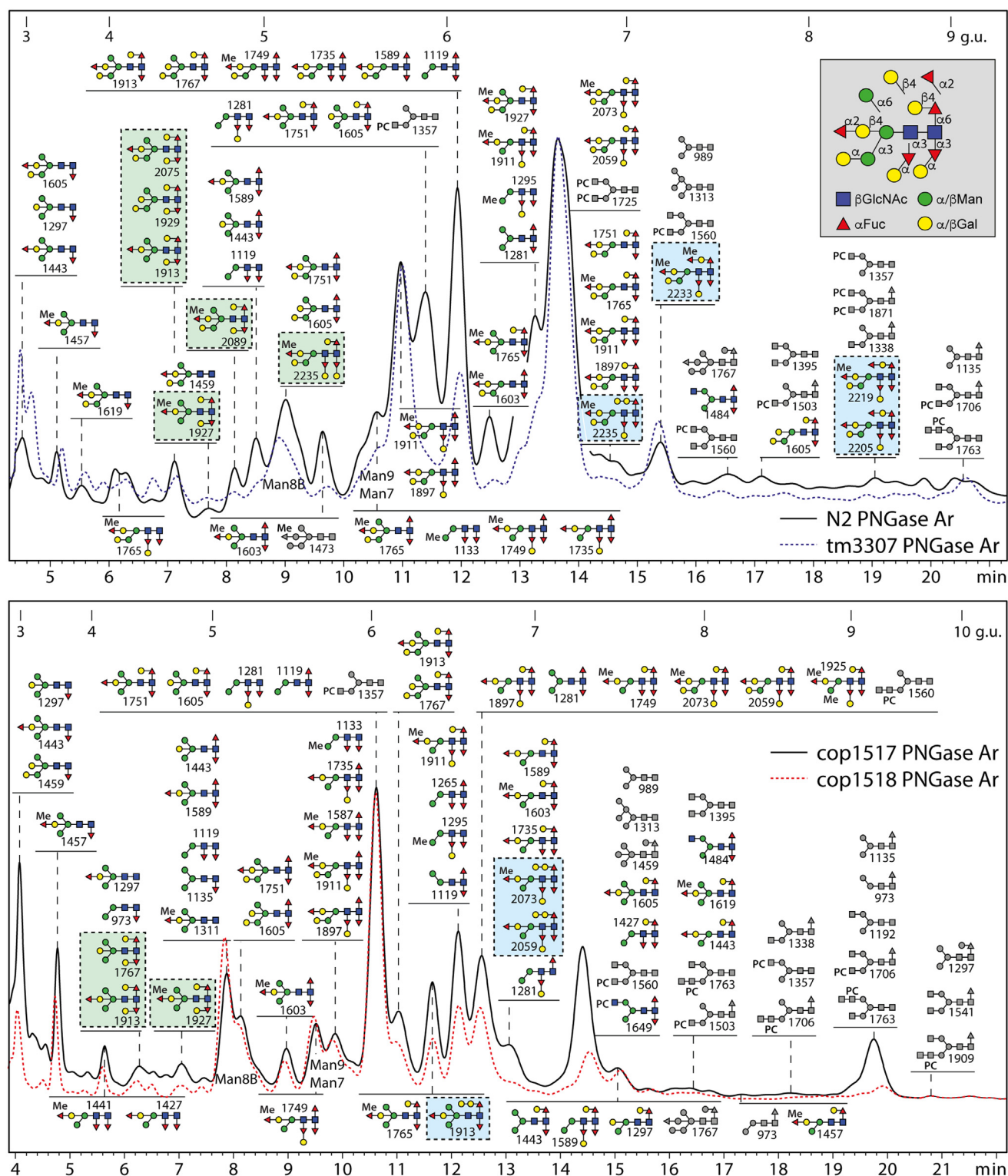


Figure 3. RP-amide chromatograms of PNGase Ar-released water-eluted N-glycans. Glycans from *Caenorhabditis elegans* strains (wildtype N2, *hex-5/tm3307*, *hex-4/cop1517*, or *hex-4/cop1518*) released by PNGase Ar were labeled with 2-aminopyridine. The RP-amide chromatograms, calibrated in terms of glucose units (g.u.), are annotated with glycan structures (in order of abundance in the relevant fraction) according to the Symbol Nomenclature for Glycans; those in gray are also found in the PNGase F-released pools. The *m/z* for the protonated ions as detected by MALDI-TOF MS are also given with the depicted structures based on conclusions derived from elution time, chemical and/or enzymatic digestion, and MS/MS data of which selected examples are shown in Figures 10 and 11. As the *tm3307* and *cop1518* chromatograms are respectively similar to the wildtype N2 and *cop1517*, these are indicated in the panels by dashed lines. For full annotations, refer to Figs. S9 and S10. The chromatograms surprisingly differ, especially between the N2 and *hex-4* strains in terms of the detected N-glycans, with a shift to fewer α -galactosylated and a higher abundance of certain trimannosylated structures (e.g., *m/z* 1751 at 6 g.u.) in the latter. Highlighted are glycans with reducing-terminal Gal α Fuc3 (green) and Gal- or Fuc-extended Gal β Fuc6 epitopes (blue).

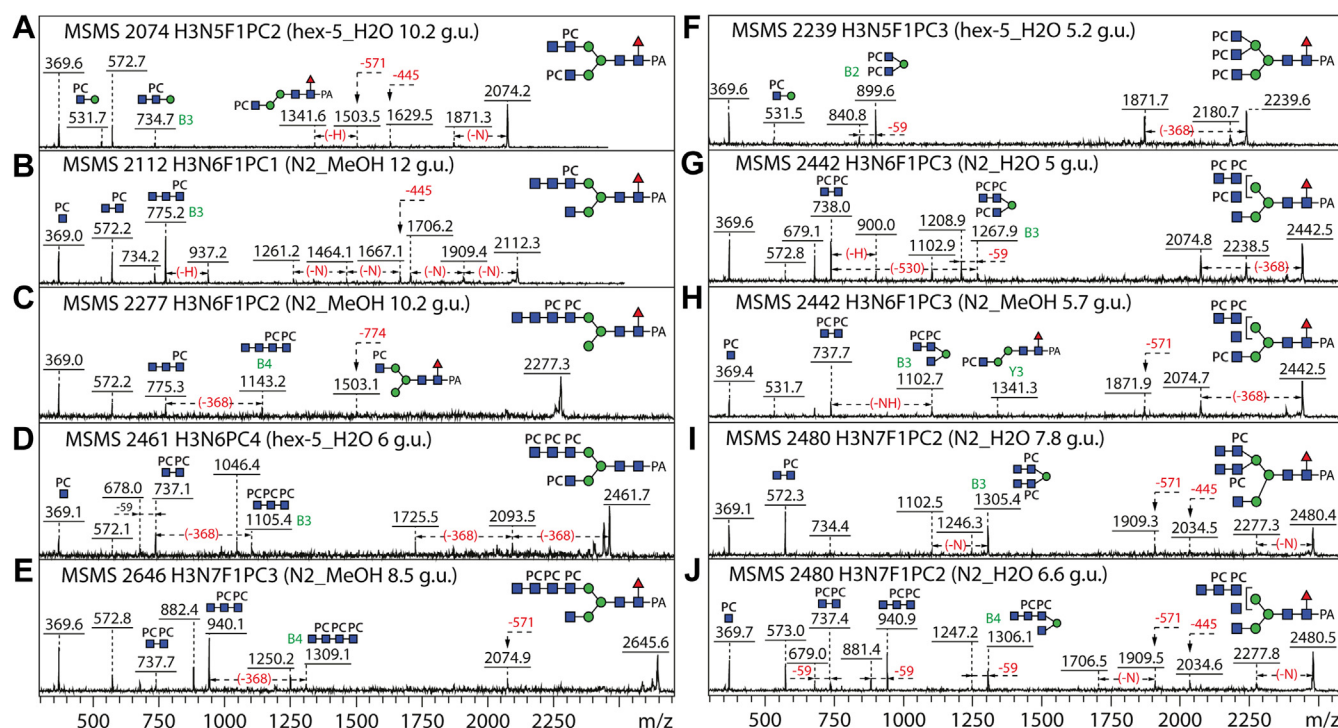


Figure 4. MS/MS of phosphorylcholine-modified N-glycans from *Caenorhabditis elegans* N2 and *hex-5* strains. A–J, positive mode MS/MS of selected RP-amide fractions of water- or methanol-eluted glycans (Figs. 1 and 2). Neutral losses of residues or differences in spectra are indicated with dashed lines (F, fucose; H, hexose; N, N-acetylhexosamine; PC, phosphorylcholine); key B-ion fragments are also annotated. The difference of 59 Da is due to loss of choline; brackets indicate antennal ambiguity. The occurrence of multiple PC-modified chito-oligomer antennae on biantennary/triantennary glycans is demonstrated by the B fragment ions such as m/z 775, 940, and 1105 (HexNAc₃PC₁₋₃), 899, 1102, or 1305 (Hex₁HexNAc₂₋₄PC₂), and 1267 (Hex₁HexNAc₃PC₃) or Y ions resulting from the loss thereof. Because of the excellent ionization of the PC-containing fragments, the core Y1 fragments are not observed, rather the loss of 445 (GlcNAc₂Fuc₁PA) from the parent in the case of core fucosylated glycans. For further illustrations of the multiple isomers of PC-modified N-glycans, refer to Figures 5 and 6 as well as Fig. S8.

spectra as shown by the loss of the m/z 718 fragments and the appearance of one at m/z 407 (Fig. 8, G–J). As some structures were initially considered to have three antennae (e.g., m/z 2404), one each of HexNAc₁, HexNAc₁Fuc₁, and HexNAc₂PC₁Fuc₁, they were treated with jack bean hexosaminidase, which removed only a single HexNAc residue, resulting in a shift in the B-ion fragment indicative of disubstitution of the α 1,6-mannose (m/z 1083; Hex₁HexNAc₃PC₁Fuc₁) to one at m/z 880 (Hex₁HexNAc₂PC₁Fuc₁; Fig. 8, L–O). Whereas 20 glycans with antennal fucose were found in the wildtype strain, only five were detected in the *hex-4* mutant.

For further information regarding the multiply fucosylated glycans, higher-energy collisional dissociation (HCD)-MS/MS with stepped normalized collision energy (NCE) was employed for analysis of the m/z 2550 glycan (Fig. 8I). When NCE was over 60%, intense fragment ions in the low-mass range were observed ($<m/z$ 500; Fig. 8K). These fragment ions were derived from either nonreducing or reducing end. Fragment ions at m/z 348 and 408 indicate two types of fucosylation, fucosylation of HexNAc and core fucosylation, respectively. Fragment ions at m/z 408 were annotated as ^{1,3}A cleavage of a mannose (usually the core α 1,3-mannose), which suggested that one fucosylated motif was most likely Fuc-GlcNAc β 1,2-Man α 1,3. Fragment ions at m/z 184 and 154 were assigned as Z/Z cleavage of internal GlcNAc and Z/Z-CH₂O. When NCE was increased over 70%, the dominant fragment ions were

those at m/z 154 (Fig. 8K, inset), which are possibly generated by double bonds forming between C4 and C5, resulting in loss of the C6 of the GlcNAc when this is either C3 or C4 substituted (25). The HCD data are thereby consistent with the conclusions based on MALDI-TOF MS/MS before and after hydrofluoric acid treatment. Therefore, we assume, also because of the substitution of C2 of a HexNAc by an N-acetyl moiety, that the antennal fucose residues are either α 1,3 or α 1,4 linked to GlcNAc and not α 1,2 or α 1,6 linked; in contrast to *T. suis* and two lepidopteran species (12, 23), the antennal fucose was not observed to cosubstitute a PC-modified GlcNAc.

Galactosylated N-glycans

Various nematodes express N-glycans decorated with galactose residues, and *C. elegans* has a rich repertoire of these, although none are related to the LacNAc antennal modifications found in vertebrates. Bisecting β -galactose, β -galactose attached to the core α 1,6-fucose, α -galactose attached to the α 1,3-mannose, and α -galactose modifications of core α 1,3-fucose have been reported by us (4, 7, 18, 26, 27). Some of these were detected in the water-eluted and methanol-eluted PNGase F-released glycan pools, including different isomers of m/z 1297 and 1459 (Hex₄₋₅HexNAc₂Fuc₁) with either fucosylated bisecting galactose or galactosylated core

Complex N-glycans in wildtype and mutant *Caenorhabditis*

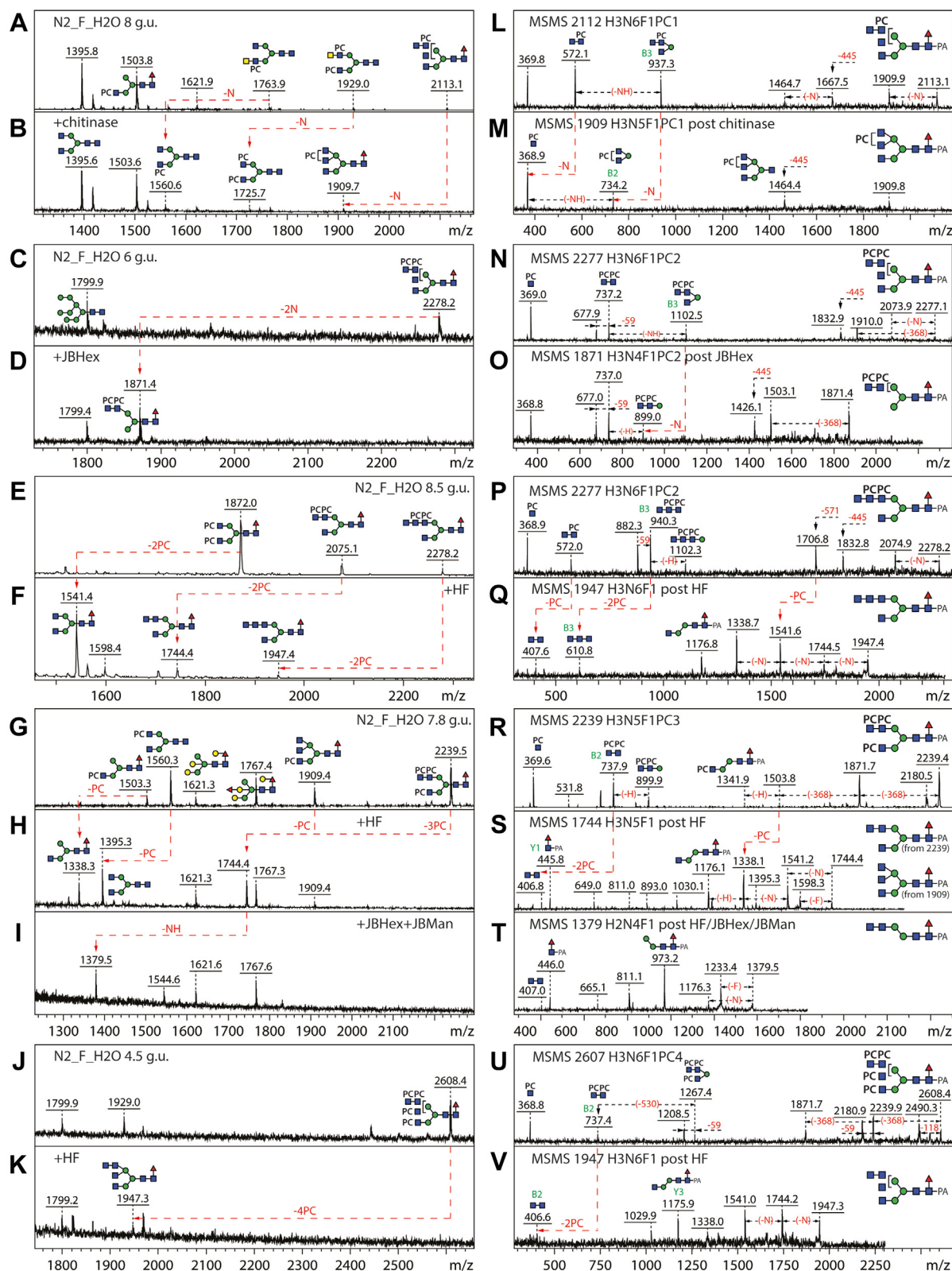


Figure 5. Enzymatic or chemical treatments of phosphorylcholine (PC)-modified N-glycans from the *Caenorhabditis elegans* N2 strain. A–K, positive mode MS of selected RP-amide fractions of water-eluted glycans (Fig. 1) before and after *Streptomyces* β 1,3/4-N-acetylhexosaminidase (chitinase; loss of 203 Da), jack bean β -N-acetylhexosaminidase (JBHEx; loss of 203 Da), hydrofluoric acid (HF; loss of 165 Da), or combined jack bean β -N-acetylhexosaminidase and α -mannosidase (JBHEx + JBMan; loss of 365 Da) treatments. L–V, corresponding MS/MS of selected original and product glycans, whereby the m/z 1744 product in panel S is partly derived from release of one PC from a coeluting m/z 1909 glycan. Losses of residues or differences in spectra are indicated with dashed lines (F, fucose; H, hexose; N, N-acetylhexosamine; PC); key B-ion fragments are also annotated. Unsubstituted antennae with a single GlcNAc could be digested with JBHEx, whereas unsubstituted terminal HexNAc residues in HexNAc_{2–3} motifs were sensitive to chitinase but generally not to HEX-4; this indicates that these motifs are most frequently GlcNAc_{2–3} in the wildtype.

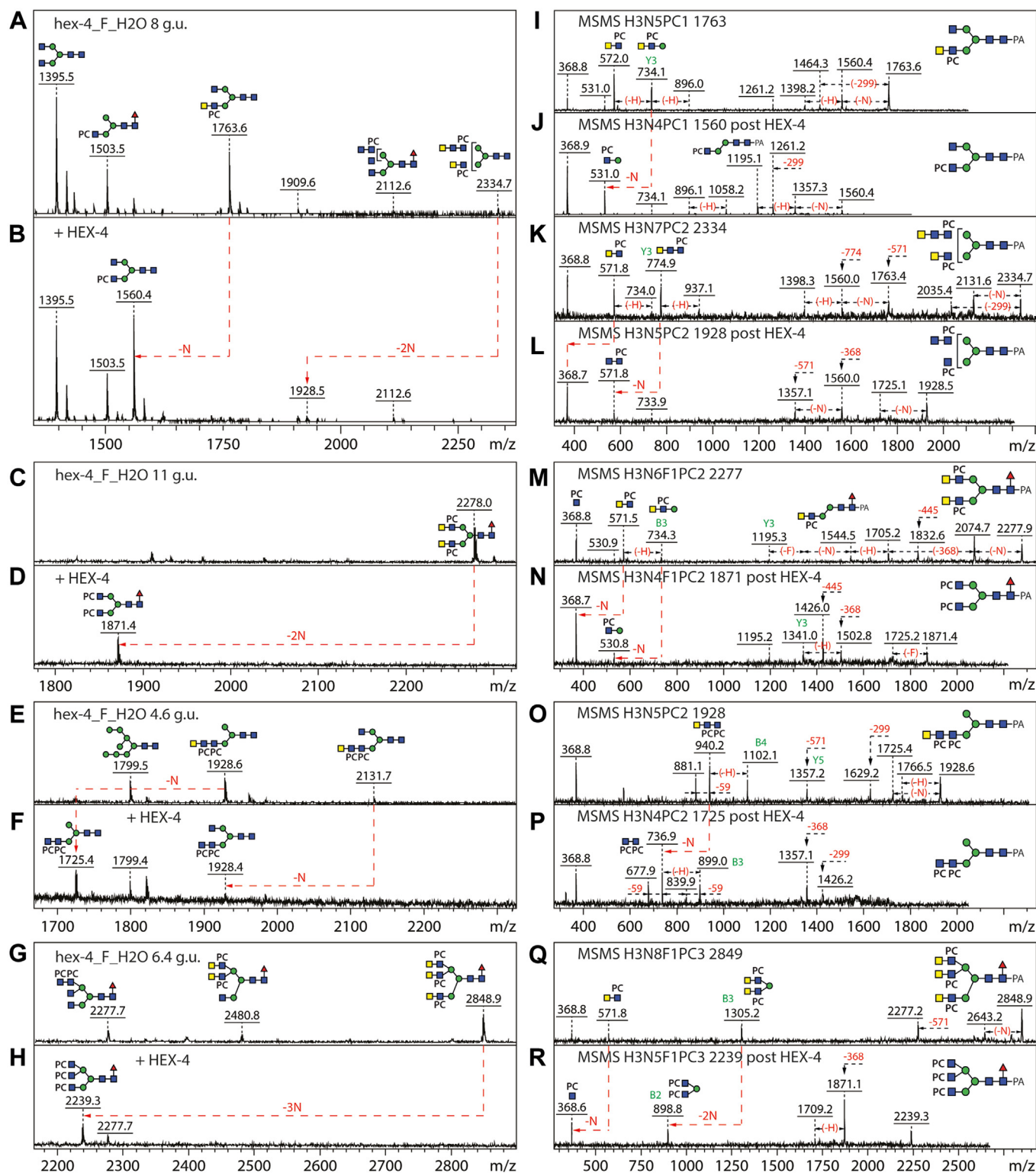


Figure 6. Enzymatic treatments of phosphorylcholine (PC)-modified N-glycans from the *Caenorhabditis elegans* hex-4 (cop1517) strain. A–H, positive ion MS of selected RP-amide fractions of water-eluted glycans (Fig. 1) before and after *Caenorhabditis* β 1,4-N-acetylgalactosaminidase (HEX-4) treatments. I–R, corresponding MS/MS of selected original and product glycans. Losses of residues or differences in spectra are indicated with dashed lines (F, fucose; H, hexose; N, N-acetylhexosamine; PC, phosphorylcholine); key B-ion fragments are also annotated. HEX-4 removal of terminal GalNAc residues from mutant strain glycans resulted in shifts in relevant PC-containing B fragments; note that HEX-4 removed two and not three HexNAc residues from the m/z 2334 glycan, indicative that only the terminal residue of the HexNAc₃PC₁ motif was GalNAc.

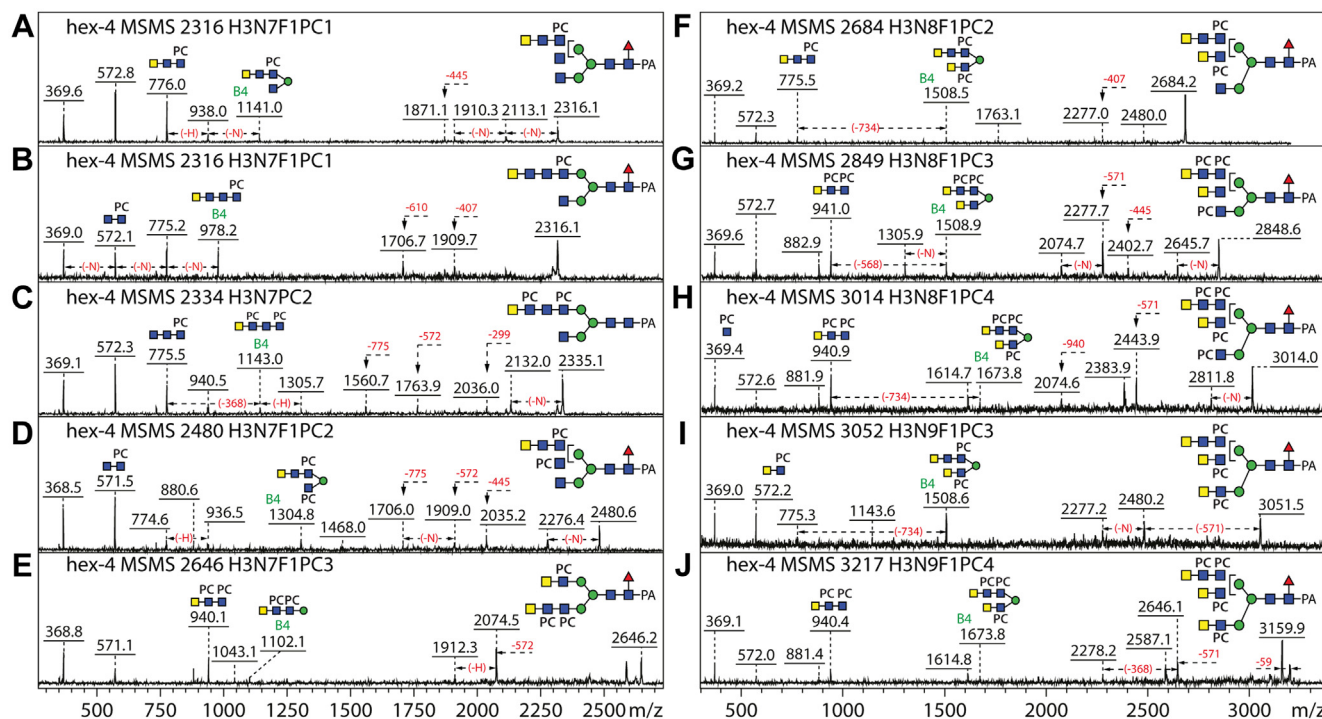


Figure 7. MS/MS of phosphorylcholine (PC)-modified N-glycans from *Caenorhabditis elegans* *hex-4* strains. A–J, positive-mode MS/MS of selected RP-amide fractions of water-eluted or methanol-eluted glycans (Figs. 1 and 2). Neutral losses of residues or differences in spectra are indicated with dashed lines (F, fucose; H, hexose; N, N-acetylhexosamine; PC); key B-ion fragments are also annotated, and brackets indicate antennal ambiguity. The *hex-4* strains were particularly rich in glycans containing both antennal GalNAc and PC residues in the context of LacdiNAc, “LactriNAc,” or “LactetraNAc” motifs (GalNAc β 1,4GlcNAc α 1–3). Terminal HexNAc antennae were defined on the basis of HEX-4 and chitinase sensitivity.

α 1,6-fucose—the contrasting retention times (on a classical C18 column) and fragmentation patterns of these isomers have been previously reported (18).

Enzymatic assays have shown that galactosylation of the core α 1,6-fucose by the GALT-1 to form the “GalFuc” epitope is optimal when there is also a nonreducing terminal N-acetylglucosamine on the α 1,3-mannose residue (28), thus it is not surprising that some GalFuc-containing glycans retain this residue; indeed we have previously detected Hex₄ HexNAc_{3–4}Fuc₁ (m/z 1500 and 1703) also modified with PC (m/z 1665 and 1868) in double fucosyltransferase mutants. In the current study, we show that the repertoire of glycans carrying both GalFuc and PC is indeed greater, including biantennary and hybrid structures with LacdiNAc or chitobiose-type antennae or even triantennary structures in either wildtype, *hex-4*, or *hex-5* mutant strains (Fig. 9). As the antennal PC-containing B-fragments dominate the MS/MS spectra (e.g., HexNAc₁PC, 369; HexNAc₂PC₁, 572; HexNAc₂PC₂, 737), the core GalFuc motif is only obvious by the neutral loss of fucose and hexose (–308 Da) or Hex₁HexNAc₁Fuc₁-PA (–607 Da). Only when the glycans are treated with hydrofluoric acid is the signature reducing terminal GalFuc Y-ion m/z 608 revealed, as shown by the biantennary isomer of m/z 2033 and its digestion product (Fig. 9, G and H).

N-glycans with core α 1,3-fucose

Those N-glycans not released by PNGase F were then isolated after digestion of residual glycopeptides with

PNGase Ar. A variable amount of noncore- α 1,3-fucosylated glycans were found in these pools because of incomplete PNGase F digestion in the first round of enzymatic release, but the vast majority of the glycans in these pools contained core α 1,3-fucose (Figs. 3 and S9 and S10). Here, the simplest of the modifications of the proximal core GlcNAc is double fucosylation as defined by the Y1 fragment of m/z 592 (HexNAc₁Fuc₂-PA; Figs. 10A and 11B). Some of the glycans differ only in the presence or the absence of a methyl modification (e.g., m/z 1735 and 1749, 1897 and 1911, 2059, 2073, 2219 and 2233; Figs. 10, A/B, D/E, G/H, and 11, B/C, D/E, I/J). Other glycans are isomeric varying in the position of a galactose residue (e.g., two wildtype versions of m/z 2235 or two *hex-4* versions of m/z 1913); here, the pattern of neutral losses and the intensity of characteristic Y1 fragments (m/z 754, 770, and 916; Hex_{1–2}HexNAc₁Fuc_{1–2}-PA; Figs. 10, I/J and 11, G/H) as well as digest information from previous studies make it possible to distinguish these structures. As judged by retention time and MS/MS, the range of postulated core modifications such as α -galactosylated α 1,3-fucose on the distal and proximal N-acetylglucosamine residues of the chitobiosyl core, β -galactosylated core α 1,6-fucose, α 1,2-fucosylated bisecting β -galactose, or α -galactosylation of the core α 1,3-mannose is similar to those found in our recent studies on *C. elegans* N-glycans (4, 7, 10, 18, 26). While the chromatograms for the N2 and *hex-5* strains are rather similar (Fig. 3) other than in terms of relative intensity of some peaks, the highest peak for the *hex-4* strains is not at 6.5 g.u. and the proportion of glycans with three or more

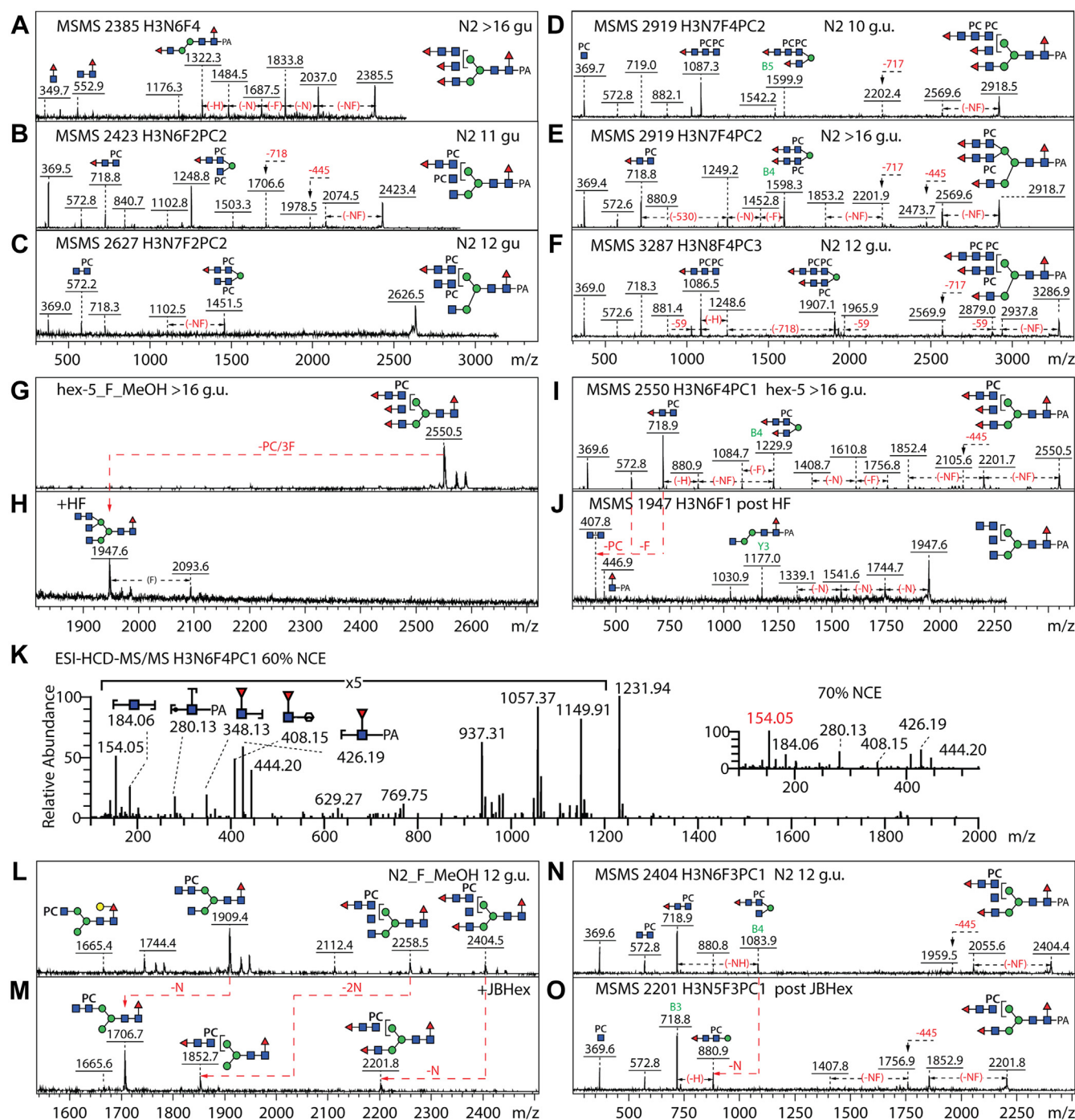


Figure 8. Analyses of *Caenorhabditis elegans* wildtype N-glycans with antennal fucose residues. A–F, MS/MS of selected methanol-eluted glycans with antennal fucose residues. Selected B-ion fragments are annotated. G–J, MS and MS/MS of the >16 g.u. fraction containing the Hex₃HexNAc₆Fuc₄PC₁ glycan with *m/z* 2550 before and after hydrofluoric acid treatment; the shift in the B-ion fragmentation pattern is indicated, whereas the Y1 and Y3 fragments are obvious only after treatment. K, higher-energy collisional dissociation (HCD)–MS/MS spectrum of the Hex₃HexNAc₆Fuc₄PC₁ glycan (*m/z* 1320.49 as [M–2H]^{2–} in negative-ion mode) with 60% NCE and 70% NCE (inset). These fragment ions were derived from either nonreducing or reducing end and are annotated with cartoon structures. When NCE increased over 70%, Z/Z–CH₂O fragment ions at *m/z* 154 (red) were dominant in the MS/MS spectrum. L–O, MS and MS/MS of the 12 g.u. fraction containing a glycan with *m/z* 2404 before and after jack bean hexosaminidase treatment; the shift in the B-ion fragmentation pattern indicates a loss of a single N-acetylglucosamine residue. Glycans containing both antennal fucose and phosphorylcholine residues have previously not been described in *C. elegans*; selected glycans were subject to hydrofluoric acid treatment, but underlying terminal HexNAc residues were only sensitive to chitinase. MS, mass spectrometry; NCE, normalized collision energy.

fucose residues and/or α -galactose on the α 1,3-mannose is reduced, whereas those with an α 1,6-mannose are increased (Fig. S11A and Table S2).

Mucin-type O-glycans

To assess whether there were major changes in the mucin-type O-glycome, we subjected worm lysates to β -elimination.

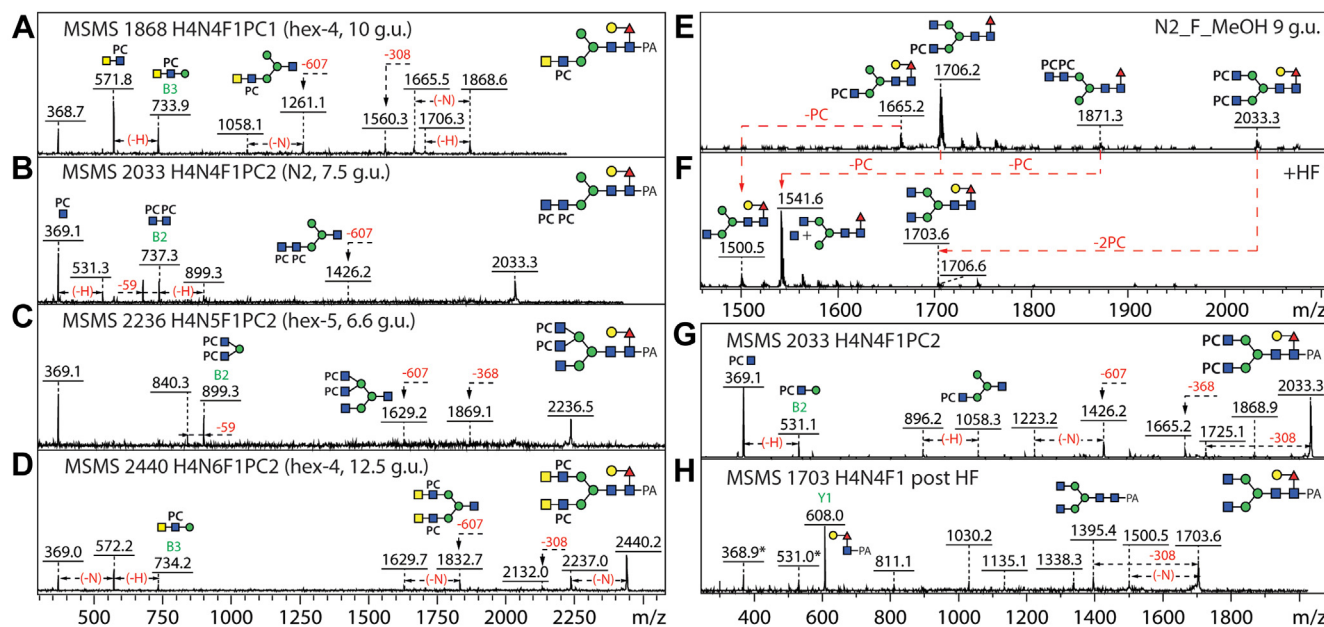


Figure 9. Analyses of *Caenorhabditis elegans* N-glycans with core galactosylated fucose motifs. A–D, MS/MS of selected N2, *hex-4*, or *hex-5* glycans carrying both “GalFuc” and phosphorylcholine epitopes. Selected B-ion fragments are annotated, and the difference of 59 Da is due to loss of choline; the loss of 607 Da from the parent is indicative of the presence of the galactosylated core fucose motif. E–H, MS and MS/MS of the 9 g.u. fraction containing a glycan with *m/z* 2033 before and after hydrofluoric acid (HF) treatment; the low-intensity *m/z* 369 and 531 B-ion fragments (H) derive from a cofragmenting residual phosphorylcholine-modified *m/z* 1706 structure, whereas the Y1 fragment at *m/z* 608, obvious only after HF treatment, is indicative for galactosylated core fucose (7, 27). MS, mass spectrometry.

The two major masses observed in both the wildtype and *hex-4* mutant (*Hex*_{3–4}*HexNAc*₁; Fig. S11, B–E) correspond to those detected in permethylated form in other studies (29), whereas the pentasaccharide was also analyzed by NMR (30); we conclude that ablation of the *hex-4* gene does not cause a major shift in the O-glycome.

Localization of HEX-4

Considering our data indicating that HEX-4 plays a role in N-glycan maturation, the *C. elegans hex-4* gene was edited to contain an in-frame enhanced GFP (eGFP)-coding sequence. The resulting fusion protein was expressed in larval stages as well as young adults of *C. elegans* (Figs. 12A and S12). Punctate HEX-4::eGFP signals were observed, coincident with those for the Golgi stain BODIPY TR Ceramide (31); as compared with studies on *C. elegans* AMAN-2 (32), this is indicative of Golgi localization of HEX-4.

Discussion

Despite some 2 decades of analyses by various research groups, the N-glycome of the model nematode *C. elegans* continues to surprise, and the role of its many glycosylation-relevant genes is still unclear. In this study, we compared N-glycans of the wildtype N2 strain with those of three hexosaminidase mutants, two with a deletion in the *hex-4* gene and one with a deletion in *hex-5* gene. While both HEX-4 and HEX-5 utilize substrates with β -linked N-acetylgalactosamine, they have contrasting pH optima (pH 6.5 and 4.5, respectively) suggestive of a different intracellular localization (6); as the luminal pH Golgi cisternae has been estimated to be in the pH

range of 6.2 to 6.7 as compared with a lysosomal pH of 4.8 (33–35), we supposed that HEX-4 is a Golgi enzyme, whereas HEX-5 may have a typical lysosomal degradative function. Indeed, our data indicate that worms lacking HEX-4 have an altered set of N-glycans enriched in structures with LacdiNAc motifs and fewer with longer antennae and/or antennal fucose, whereas the N-glycome of the *hex-5* strain is almost identical to the wildtype. In addition, fluorescence microscopy indicates colocalization of a HEX-4::eGFP fusion protein with a Golgi tracking probe (Fig. 12A).

Our glycomic analyses also reveal structures with up to three antennae and four PC residues in the wildtype N2 strain (see also summary in Fig. 12B); thus, the PC-modified N-glycome repertoire of the model nematode is actually akin to the complexity of some parasitic species. Chito-oligomers decorated with PC and/or fucose are known in filarial worms such as *Onchocerca volvulus* or *Dirofilaria immitis* (24, 36), whereas antennal fucosylation of terminal GlcNAc is found in a cestode, *Taenia crassiceps* (37). The maximal detected length of the antennae was a series of four N-acetylhexosamine residues in the wildtype, which is the same as that found in the pig parasite *O. dentatum* (22), but less than the possibly six or more in filarial nematodes (36), including *D. immitis* (24). In the latter, we could find GalNAc β 1,4GlcNAc β 1,4GlcNAc “LactriNAc” motifs, detected here also in the *hex-4* mutants, in addition to “uncapped” longer chains consisting of serial N-acetylglucosamine residues. Indeed, in these studies, the specific HEX-4 enzyme has proven a valuable tool to verify the absence or the presence of nonreducing terminal N-acetylglactosamine (24). The relative lack of terminal GalNAc in N2 would correlate with the inability of GalNAc to inhibit binding

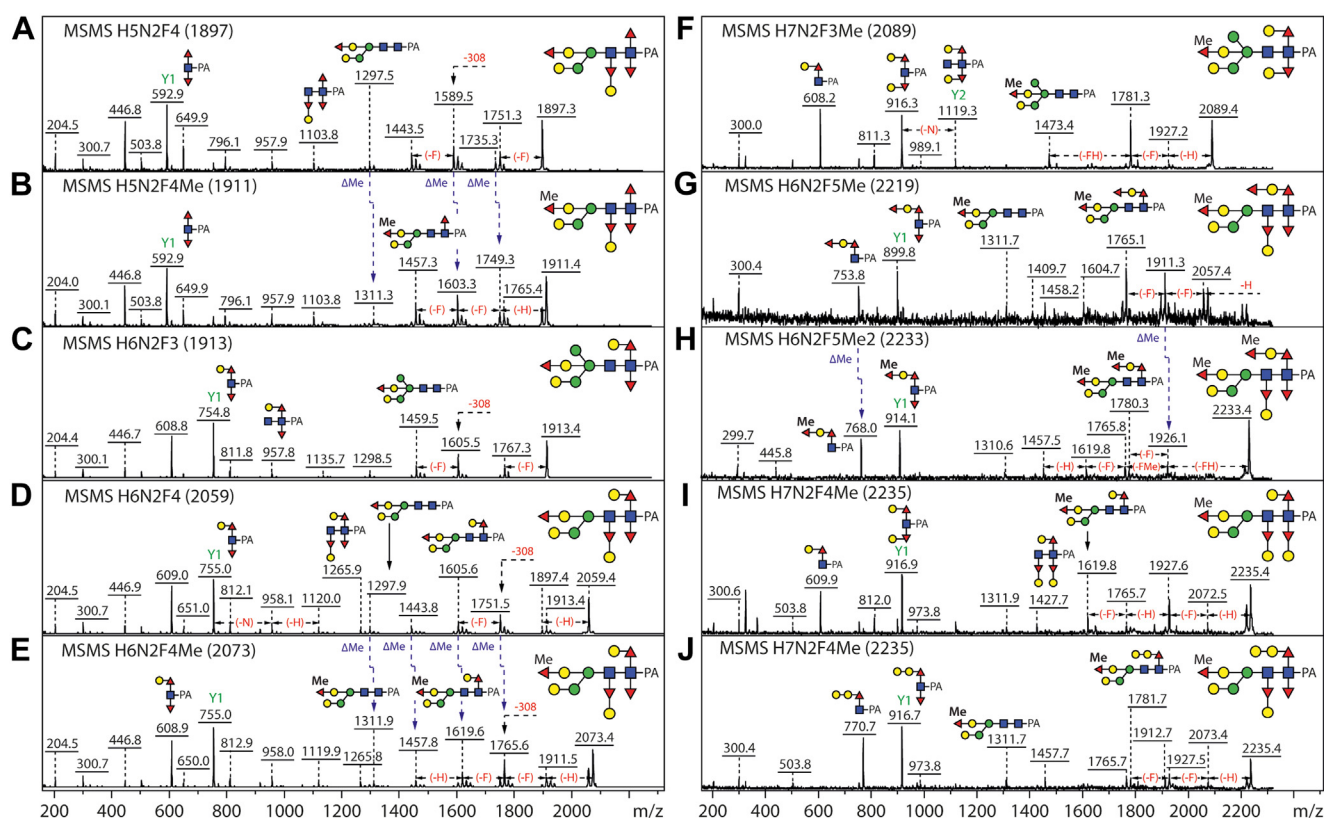


Figure 10. MS/MS of core α 1,3-fucosylated N-glycans from wildtype *Caenorhabditis elegans*. A–J, positive mode MS/MS of selected PNGase Ar-released glycans (Fig. 3); key Y fragments as well as neutral losses are indicated as are differences between nonmethylated and methylated N-glycans (A/B, D/E, and G/H). Differences in the occurrence or intensity of Y-ions are indicative of the various core modifications with fucose and galactose residues, also for isomeric glycans eluting at different retention times (I/J). Isomers were also distinguished on the basis of α -galactosidase, β -galactosidase, α -mannosidase, and/or hydrofluoric acid (HF) treatments and in comparison to previously published data on core modifications of *C. elegans* N-glycans (4, 18). The maximal detected degree of core modification in the wildtype (Hex₇HexNAc₂Fuc₄Me₁, see I) reflects also α -galactosylation of the core α 1,3-mannose and core α 1,3-fucose residues, which only occur to a low extent in the *hex-4* mutant.

of wildtype worm proteins to *Wisteria floribunda* agglutinin, in contrast to other tested helminths (38). However, even in the *hex-4* mutants, there is no fucosylated LacdiNAc; thereby, *C. elegans* lacks a motif found in *T. suis*, *D. immitis*, *Haemonchus contortus*, and *Schistosoma mansoni* (12, 24, 39, 40). Such similarities and differences between *C. elegans* and parasitic nematodes do offer perspectives for the discovery of enzymes making novel epitopes relevant to the design of diagnostics or vaccines.

In terms of the genetic basis for the occurrence of LacdiNAc and fucosylated chito-oligomer modifications, *C. elegans* has a proven GT7 β 1,4-N-acetylgalactosaminyl-transferase, BRE-4 which also has a role in glycolipid biosynthesis (41); this enzyme can also generate poly-LacdiNAc chains when overexpressed in mammalian cells (42), whereas the enzymatic function of a GT7 paralog, NGAT-1, remains to be proven (43). *C. elegans* also has five GT10 α 1,3-fucosyltransferases (8, 44); however, while FUT-1 and FUT-6 actually modify *C. elegans* N-glycan cores, *in vivo* glycan targets remain to be identified for FUT-3, FUT-4, and FUT-5. The accurate prediction of glycosyltransferase and glycosidases homologs in other nematodes is more difficult because of incomplete genome annotations. However, there are multiple clade B hexosaminidase genes including potential HEX-4 ortholog in *O. dentatum*, which has chito-

oligomer but not LacdiNAc modifications (22), in *D. immitis*, which has both types of antennae (24), and in *P. pacificus*, which has only minute amounts of complex glycans with more than one HexNAc per antenna (17); on the other hand, *T. suis* has LacdiNAc-modified N-glycans (12) but lacks HEX-4. All these species possess BRE-4 orthologs.

Considering both the glycomic data on nematodes and their glycogenomic potential, we speculate that BRE-4 transfers N-acetylgalactosamine to nonreducing termini of N-glycans in *C. elegans* and that HEX-4 can remove this residue (Fig. 12C); in the absence of the glycosidase, these transfer-removal steps do not take place, which would correlate with the similar tissue-specific transcriptomic expression of *hex-4* and *bre-4* (Fig. S1). A Golgi localization of HEX-4 would be compatible with a role in biosynthesis, rather than degradation, as the number of N-glycans of over 2500 Da is greater in the wildtype than the *hex-4* mutant. Thereby, it can be hypothesized that N-acetylgalactosamine may act as a cap preventing the formation of longer chito-oligomer chains (HexNAc₄ or higher) type and limits the antennal length to HexNAc_{2–3} motifs in *hex-4* mutants. An alternative cap in *C. elegans* would be fucose, as found especially in relatively late-eluting N-glycans of the wildtype (Figs. 2 and 8). Such a concept of capping units controlling the biosynthesis of oligosaccharide chains is known

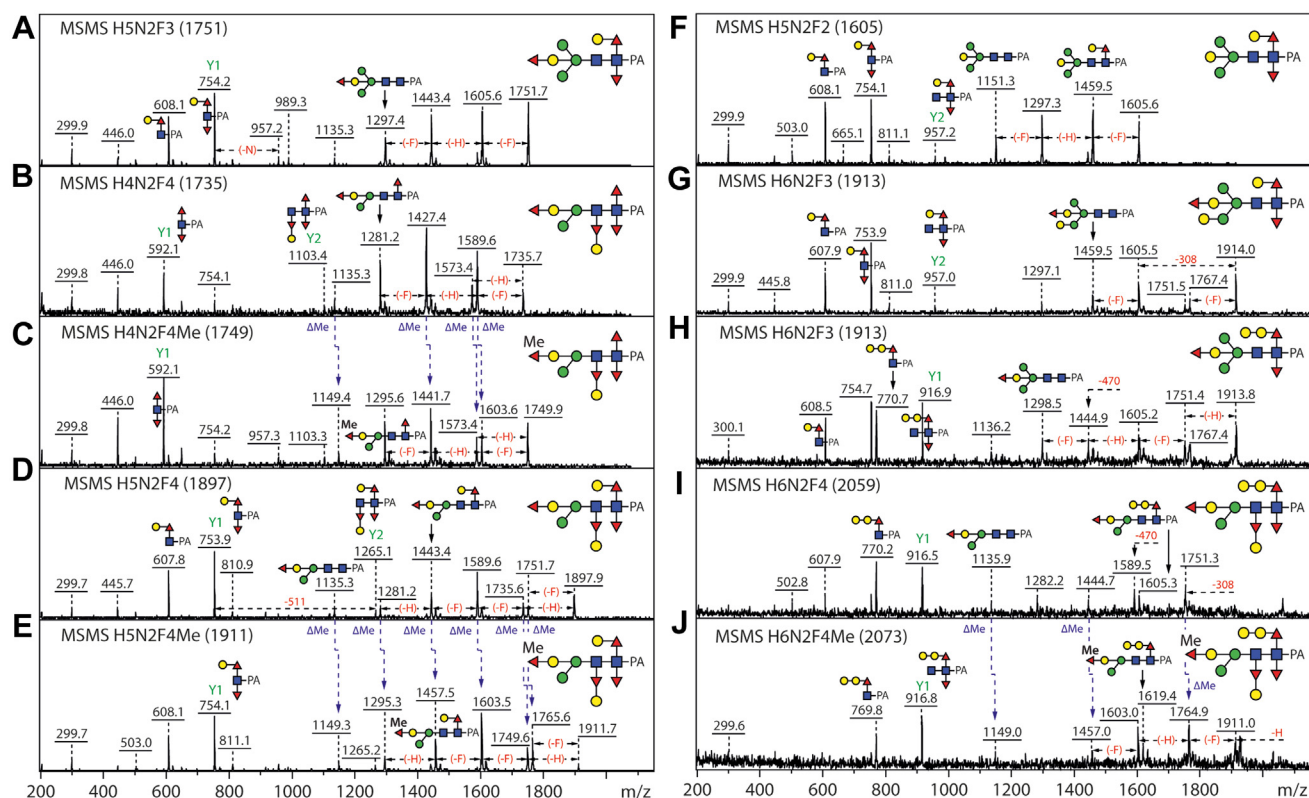


Figure 11. MS/MS of core α 1,3-fucosylated N-glycans from the *Caenorhabditis elegans* *hex-4* strain. A–J, positive mode MS/MS of selected PNGase Ar-released glycans (Fig. 3); key Y fragments as well as neutral losses are indicated as are differences between nonmethylated and methylated N-glycans (B/C, D/E, and I/J). Differences in the occurrence or intensity of Y-ions are indicative of the various core modifications with fucose and galactose residues, also for isomeric glycans eluting at different retention times (G/H). Isomers were also distinguished on the basis of glycosidase or HF treatments and in comparison to previously published data on core modifications of *C. elegans* N-glycans (4). HF, hydrofluoric acid; MS, mass spectrometry.

in many species. In bacteria, methylphosphate or *N*-acetylmuramic acid is chain termination motif (45, 46), whereas in plants, developmentally relevant Golgi β 1,3-galactosidases seemingly remove previously transferred galactose during arabinogalactan synthesis (47); other termination signals may include sulphation of the terminal sialic acid of polysialylated glycolipids in sea urchins (48) and sialylation of mammalian keratan sulphate (49). However, in terms of hexosaminidase-mediated alteration of protein-linked N-glycans in mammals, it appears that “acidic” glycosidases mediate removal of nonreducing terminal GlcNAc in compartments such as azurophilic vesicles of neutrophils rather than *via* processes within the Golgi (50).

A surprising result of our study is that the “core chitobiose modifications” (see also summary in Fig. 12B) are also affected in both *hex-4* strains resulting in a large shift in the N-glycome released by PNGase Ar. This subpool is enriched in N-glycans modified with bisecting galactose and/or multiple fucose residues and represents structures unique to *C. elegans* (4); for biosynthetic reasons related to the specificity of the FUT-1 core α 1,3-fucosyltransferase (5), glycans with LacdiNAc- or chito-oligomer motifs are not expected to be present in this pool. Thus, little impact if HEX-4 is absent was expected. However, it seems that a processing step (removal of the core α 1,6-mannose) required for transfer of fucose by FUT-6 (8) to the distal GlcNAc may be reduced in the *hex-4* mutant. Fewer

core α 1,3-fucosylated glycans, including those also modified by bisecting galactose, are α -galactosylated as compared with the wildtype, and no penta-fucosylated glycans are observed in the *cop1517* strain (Table S2). Such a shift to a lower degree of core fucosylation in *hex-4* strains offers a parallel to the respective 30% and 90% reduction in the abundance of trifucosylated and tetrafucosylated glycans observed for the *cogc-1* mutant, which has defective Golgi function (51). Interestingly, the *hex-4* gene neighbors *trpp-10*, which encodes an ortholog of mammalian TRPPC10, a protein with a role in Golgi trafficking (52); therefore, it is possible that the precise deletion of *hex-4* affects expression of the downstream *trpp-10*. Alternatively, knocking out a glycoenzyme can alter the Golgi per se; for instance, an absence of GlcNAc-TV is associated with a reduction in volume of this organelle in mammalian cells (53). Certainly, it can be stated that knocking out one glycosylation-relevant gene can have both explicable and less explicable effects on the glycome.

Experimental procedures

Nematode strains

Wildtype *C. elegans* (N2) was obtained from the *Caenorhabditis* Genetics Centre, University of Minnesota. The *hex-4* (*cop1517* and *cop1518*; prepared by CRISPR–Cas9 technology) and *hex-5* (*tm3307*; prepared by trimethylpsoralen-induced

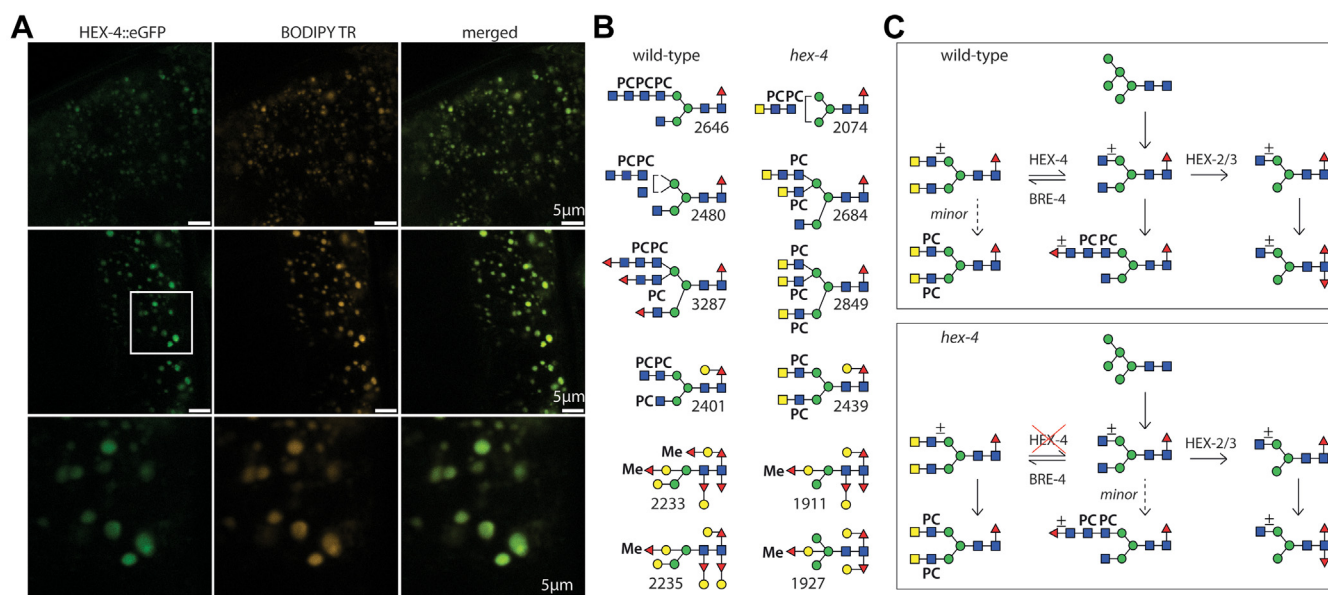


Figure 12. Localization of HEX-4 and summary of unusual N-glycan structures found in *Caenorhabditis elegans* wildtype and *hex-4* mutant strains.

A, fluorescence microscopy of L1 larvae at two levels of magnification showing perinuclear colocalization of the HEX-4::enhanced GFP (eGFP) fusion protein (green) with the BODIPY TR Ceramide Golgi marker (gold); for further images, refer to Fig. S12. B, example of N-glycans, together with *m/z* values, particularly found in either the wildtype N2 or *hex-4* mutant strains representative of the diversity of most highly processed motifs in *C. elegans* are depicted (see also Fig. S8 for a depiction of multiple isomers of *m/z* 2277). Striking is the tendency to GalNAc capping and simpler core modifications if the *hex-4* gene is ablated. C, the shift in the antennal modifications can be explained if there is GalNAc capping (potentially via the BRE-4 β 4-*N*-acetylgalactosaminyl-transferase) in near equilibrium with GalNAc removal (via HEX-4) in compartments of the Golgi apparatus. The relative lack of GalNAc capping in the wildtype or *hex-5* strains reveals GlcNAc residues, which are available for extension by chito-oligomer synthesizing/modifying enzymes. On the other hand, in both wildtype and *hex-4* strains, HEX-2 and HEX-3 mediate removal of the GlcNAc attached to the “lower arm” α 1,3-linked mannose, resulting in paucimannosidic and pseudohybrid structures, which can be core α 1,3-fucosylated. Only indirect effects can explain the relative lack of tetra-fucosylated glycans in the *hex-4* strains. See Figures 1–3 for summaries of all N-glycans found in this study, including the highly abundant oligomannosidic and paucimannosidic structures.

mutagenesis) strains were respectively obtained from Knudra Transgenics, and the National Bioresource Project for the Experimental Animal Nematode *C. elegans*, Tokyo Women’s Medical University. The homozygous cop1517 and cop1518 strains were constructed based on the use of two sgRNAs targeting the entire *hex-4* open reading frame on chromosome I (aagatactctacatggacgg and cacacactctgacatcacgt corresponding to nucleotides 12,592–12,573 and 20,352–20,333 of cosmid Y51F10). The homozygous tm3307 strain has a 1047 bp deletion resulting in loss of four exons in the middle of the *hex-5* gene on chromosome X (specifically 2276–3322 of cosmid Y70D2A). The *hex-4::gfp* transgenic line (strain PHX5035; allele *syb5035*) was generated using CRISPR–Cas9 technology by SunyBiotech; the natural stop codon was replaced by a *gfp* sequence, and the localization of the resulting HEX-4::eGFP fusion protein was observed by confocal microscopy.

Confocal microscopy

N2 wildtype and *hex-4::gfp* worms were harvested from nematode growth media plates and washed in M9 buffer prior to 1 h incubation with BODIPY TR Ceramide complexed to bovine serum albumin (5 μ M in M9 buffer) at room temperature. After washing in M9 buffer (three times), worms were paralyzed using 20 mM levamisole hydrochloride and transferred to poly-L-lysine-coated glass slides. Images were recorded using a Leica TCS SP5 laser scanning confocal microscope (Wetzlar).

Transcriptomic reanalyses

RNA-Seq and sci-RNA-Seq data used for stage and tissue expression analysis of selected genes (14, 54) were downloaded from GExplore on an open access basis and visualized using the plot method of the R graphics library (stage-specific data) and the ggplot function of the tidyverse library (L2 tissue-specific data).

Glycosidase assays

Recombinant HEX-4 and HEX-5 were produced in *Pichia pastoris* as previously described. The His-tagged version of HEX-4 was purified by nickel chelation chromatography, whereas the untagged HEX-5 was enriched by ammonium sulphate precipitation from culture supernatants (6, 16). The remodeled fibrin dabsylated glycopeptide (two LacdiNAc antennae) and pyridylaminated glycans from either royal jelly (hybrid N-glycan with a single LacdiNAc) or *C. elegans* (glycosaminoglycan type) were isolated during previous studies (16, 55, 56). Aliquots of purified HEX-4 or enriched HEX-5 were incubated overnight at 37 °C with the glycopeptide or glycans at either pH 4.5 or 6.5 (ammonium acetate) prior to MALDI-TOF-MS as described later.

N-glycan preparation

C. elegans were grown in liquid culture with *Escherichia coli* OP50 in standard S complete medium, mixed stages were

Complex N-glycans in wildtype and mutant *Caenorhabditis*

harvested after cultivation at 20 °C (160 rpm) for 4 to 6 days, and purified by sucrose density centrifugation (57, 58). Harvested worms (2 g) were boiled, homogenized; after adjusting the pH with ammonium carbonate buffer to 8.2, CaCl₂ was added to a final concentration of 0.5 mM prior to addition of 2 mg thermolysin (Promega). Proteolysis was allowed to proceed for 2 h at 70 °C.

We adapted our glycomic workflow to facilitate the detection of low abundance glycans, especially considering that glycans carrying PC residues can be enriched by reversed phase-based solid phase extraction; previously, we found that glycans with multiple PC residues elute late on standard reversed-phase HPLC, but early if using fused core columns (17). Thus, similarly as for glycans from a marine snail (59), prefractionation on standard C18 material was performed by serially eluting with water and 15% methanol, but HPLC separation after fluorescent labeling with 2-aminopyridine was done on a fused core RP-amide column.

Specifically, the glycopeptides were purified using standard laboratory protocols (60) prior to serial release using two different peptide:N-glycosidases: recombinant bacterial PNGase F (from *Flavobacterium [Elizabethkingia] meningosepticum*, Roche; at pH 8.0) and recombinant rice PNGase Ar (from *Oryza sativa*, expressed in *Pichia pastoris [Komagataella phaffii]* and Endo H treated, New England Biolabs; at pH 5.0). First, the glycopeptides were treated with PNGase F overnight at 37 °C. After cation exchange chromatography on Dowex, the unbound material was subject to solid phase extraction on nonporous graphitised carbon (ENVicarb, Supelco) and eluted with 40% acetonitrile. These fractions were subject to a further solid phase extraction step on a C18 reversed phase resin (LiChroprep; Merck), and the glycans were eluted with water and with stepwise increases in the methanol concentration (15%, 30%, and 100% [v/v]). Glycan-containing fractions, as judged by MALDI-TOF MS, were fluorescently labeled with 2-aminopyridine (61). The remaining glycopeptides bound to the Dowex resin were gel filtrated (Sephadex G25) prior to incubation with PNGase Ar overnight at 37 °C. The glycans released with this enzyme were then no longer bound by Dowex and were subject to the same purification and fluorescent-labeling steps as for the PNGase F-released ones. See also the glycomic workflow summarized in the scheme in the [supporting information](#).

N-glycan fractionation

Pyridylaminated N-glycome pools were fractionated by reversed-phase HPLC (Ascentis Express RP-amide; 150 × 4.6 mm, 2.7 µm; Sigma–Aldrich) and a gradient of 30% (v/v) methanol (buffer B) in 100 mM ammonium acetate, pH 4 (buffer A) was applied at a flow rate of 0.8 ml/min as follows: 0 to 4 min, 0% B; 4 to 14 min, 0 to 5% B; 14 to 24 min, 5 to 15% B; 24 to 34 min, 15 to 35% B; 34 to 35 min, return to starting conditions (62). Lyophilized HPLC fractions were dissolved in water and subject to MALDI-TOF MS. The RP-HPLC column was calibrated daily in terms of glucose units using a pyridylaminated dextran hydrolyzate, and the degree of

polymerization of single standards was verified by MALDI-TOF MS (63); the masses of selected peaks of this linear glucose polymer were verified by MALDI-TOF MS after RP-HPLC (e.g., 6. g.u. eluting at 11 min; m/z 1069.6 as $[M + H]^+$).

MALDI-TOF MS

Free glycans and pyridylaminated glycans were analyzed in positive ion mode using a Bruker Autoflex Speed instrument (1000 Hz Smartbeam-II laser) and 6-aza-2-thiothymine as matrix; calibration was performed using a Bruker peptide standard. MS/MS was performed by laser-induced dissociation (precursor ion selector was generally $\pm 0.6\%$). The detector voltage was normally set at 1977 V for MS and 2133 V for MS/MS; 1000 to 2000 shots from different regions of the sample spots were summed. Spectra were processed with the manufacturer's software (Bruker Flexanalysis 3.3.80) using the SNAP algorithm with a signal/noise threshold of 6 for MS (unsmoothed) and 3 for MS/MS (four-times smoothed). Glycan MS and MS/MS spectra (approximately 5500 in total) were manually interpreted on the basis of the masses of the predicted component monosaccharides, the differences of mass in glycan series, fragmentation patterns, and results of enzymatic and chemical treatments. For the 300 structures (see [Table S1](#) for a list of compositions), the minimum criterion for inclusion in the [Supporting Tables](#) was an interpretable MALDI-TOF MS/MS spectrum (see also mzxml raw data files). Furthermore, examples for each core and antennal motif were verified by digestion data; comparison was also made to elution, in terms of glucose units, with previous data. Calculated theoretical masses were verified using GlycoWorkbench 2.0. The deviation between calculated and observed m/z values was typically 0.1 to 0.2 Da.

Exoglycosidase and hydrofluoric acid treatment

Aliquots of the isolated HPLC fractions were, based on the results of HPLC elution and MALDI-TOF MS and MS/MS data, subject to targeted exoglycosidase digestion and chemical treatment (62). Either α -mannosidase (jack bean from Sigma), α -galactosidase (coffee bean from Sigma), β -galactosidase (recombinant *Aspergillus niger* LacA prepared in house (64)), or β -hexosaminidases (recombinant *C. elegans* HEX-4 prepared in-house (16), *Streptomyces plicatus* chitinase from New England Biolabs or jack bean hexosaminidase from Sigma) was used for further treatment of the sample in 25 mM ammonium acetate, pH 5 (pH 6.5 in the case of HEX-4), at 37 °C for 24 h. For removal of PC or α 1,3-fucose residues, selected fractions were dried and incubated for 48 h at 0 °C with 3 µl 48% (v/v) hydrofluoric acid prior to evaporation in a centrifugal concentrator. The samples were diluted in water and re-evaporated, before redissolving once again. The chemically or enzymatically treated fractions were subject to MALDI-TOF MS and MS/MS (as aforementioned) without further purification.

LC-HCD MS/MS analysis

An HPLC-purified glycan (2549 Da) was analyzed using an Easy-nLC 1200 LC system (Thermo Fisher Scientific) coupled to an LTQ Orbitrap Elite mass spectrometer (Thermo Fisher

Scientific). The analytes were trapped on a 2 cm × 100 µm Acclaim PepMap C18 precolumn (particle size 5 µm; Thermo Fisher Scientific) and separated on a 30 cm × 75 µm analytical column packed in-house with 3 µm Reprosil-Pur C18 material (Dr Maisch) at 300 nl/min flow using a stepwise elution profile (solvent A was 5 mM di-*n*-butylamine and 8 mM acetic acid in water, solvent B was 70% methanol in solvent A): B buffer increases 20% to 70% in 32 min; and then to 100% in 3 min and keep it for another 10 min (65). The column oven temperature was 50 °C. Nano-Flex ion source (Thermo Fisher Scientific) was operated in negative ionization mode at 1.8 kV with the ion transfer capillary temperature 325 °C. The LTQ Orbitrap Elite mass spectrometer was operated in the data-dependent mode, collecting a full MS scan in the range *m/z* 350 to 2000 at 120 K resolution. The samples were analyzed at 50, 60, and 70% stepped NCE for five most intensive precursors from each full MS scan. For all settings, the automatic gain control target in the full MS spectra was 10⁶, precursor isolation window was 5 Da, and the HCD spectra were recorded at 15 K resolution with the first *m/z* 100 and an automatic gain control target of 10⁵; precursor ions with unassigned charged states were rejected.

O-glycan analysis

O-glycans were released from *C. elegans* N2 or *hex-4* glycoproteins (extracted by methanol–chloroform precipitation of fresh lysates) via ammonium-based β-elimination (66) resulting in nonreduced O-glycans. In brief, extracted glycoproteins were dried and incubated with a mixture of 16 µl H₂O, 16 µl hydroxylamine, and 32 µl 1,8-diazabicyclo [5.4.0]undec-7-ene (an organic superbases) at 50 °C for 30 min. Released O-glycans were purified using serial small columns of Dowex AG50, C18, and nonporous graphitized carbon prior to labeling with 2-aminopyridine and MALDI-TOF MS/MS as described for N-glycans.

Data availability

Data described in the article are shown in the figures; mzxml files have been submitted to Glycopost: <https://glycopost.glycosmos.org/entry/GPST000200>.

Supporting information—This article contains supporting information including further information regarding the glycomic analyses, [Supplementary Figures S1–S12](#), and [Supplementary Tables S1–S2](#) (14, 15, 29, 30, 54–56).

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W. writing—original draft; K. P., F. W., D. M., and I. B. H. W. visualization; K. P. and I. B. H. W. supervision.

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Conflict of interest—The authors declare that they have no conflicts of interest with the contents of this article.

Abbreviations—The abbreviations used are: EC, Enzyme Commission; eGFP, enhanced GFP; HCD, higher-energy collisional dissociation; MS, mass spectrometry; NCE, normalized collision energy; PC, phosphorylcholine.

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