Mass spectrometric comparison of N-glycan profiles from *Caenorhabditis elegans* mutant embryos

Hildegard Geyer · Martin Schmidt · Matthias Müller ·
Ralf Schnabel · Rudolf Geyer

Abstract The free-living nematode *Caenorhabditis elegans* is a well-characterized eukaryotic model organism. Recent glycomic analyses of the glycosylation potential of this worm revealed an extremely high structural variability of its N-glycans. Moreover, the glycan patterns of each developmental stage appeared to be unique. In this study we have determined the N-glycan profiles of wild-type embryos in comparison to mutant embryos arresting embryogenesis early before differentiation and causing extensive transformations of cell identities, which allows to follow the diversification of N-glycans during development using mass spectrometry. As a striking feature, wild-type embryos obtained from liquid culture expressed a less heterogeneous oligosaccharide pattern than embryos recovered from agar plates. N-glycan profiles of mutant embryos displayed, in part, distinct differences in comparison to wild-type embryos suggesting alterations in oligosaccharide trimming and processing, which may be linked to specific cell fate alterations in the embryos.

Keywords *Caenorhabditis elegans* · Mass spectrometry · N-glycan profiles · Mutant embryos

H. Geyer · M. Schmidt · R. Geyer (✉)
Institute of Biochemistry, Faculty of Medicine,
University of Giessen,
Friedrichstrasse 24,
35392 Giessen, Germany
e-mail: rudolf.geyer@biochemie.med.uni-giessen.de

M. Müller · R. Schnabel
Institut für Genetik,
Technische Universität Braunschweig Carolo Wilhelmina,
Spielmannstrasse 7,
38106 Braunschweig, Germany

Introduction

The free-living nematode *Caenorhabditis elegans* is a genetically and developmentally well characterized multicellular eukaryote. It is a simple organism comprising only 959 cells, the complete cell lineage of which has been mapped [1]. More recently, the glycome of *C. elegans* has been examined and characterized by several laboratories [2–11]. Considering the simple anatomy of *C. elegans*, its glycan composition is extensive with over 100 structures present in the wild-type N2 Bristol strain [8]. *C. elegans* glycans share similarities with vertebrate glycans in terms of their core structures, the most abundant N-glycans being those of the high mannose type ($Man_{5-9}GlcNAc_2$), whereas complex and hybrid N-glycans are either absent or present at low levels only [4, 6, 7]. Intriguingly, *C. elegans* contains large amounts of N-glycan structures, which are usually not expressed in vertebrates, such as paucimannose ($Man_{3-4}GlcNAc_2$), truncated complex ($Man_3GlcNAc_3$), highly fucosylated, *O*-methylated (Me), and phosphorylcholine (PC)-substituted glycans,while sialic acid residues have not been detected in *C. elegans* glycans [5, 6, 8, 12]. Furthermore, several O-glycan species expressing unusual features like novel core structures as well as glucose and glucuronic acid have been detected [13]. The importance of N-glycosylation in the development of metazoan animals, ranging from *Drosophila melanogaster* to mice and humans, is widely recognized (see [14, 15] and references therein). Detailed structural studies further revealed an increase in the prevalence of complex glycans during embryonic development of *Drosophila melanogaster* [16]. Likewise, the N-glycan profile of *C. elegans* is also unique for each developmental stage (L1–L4, Dauer, adult), similarly suggesting a role of these carbohydrates in worm development [3]. Glycans expressed in embryonic stages

of development, however, have not been investigated in this case. In order to provide a basis for future studies on putative functional roles of such glycans also during embryogenesis, we have compared the N-glycan patterns of *C. elegans* wild-type and mutant embryos. In two mutants, *cib-1* or *t1099*, development arrested around the 100-cell stage prior to the general cell diversification, which would allow to investigate whether indeed N-glycan diversification is coupled to cell complexity. In mutants *glp-1* or *lit-1* cell identities are extensively altered, which would allow to assess whether or not N-glycan diversity correlates with specific cell identities in the embryo. To this end, present N-glycans were enzymatically released and analyzed by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS). Resulting oligosaccharide profiles showed that glycan diversification obviously paralleled cell diversification and that alterations of cell identities may cause distinct variations in oligosaccharide trimming and processing.

Materials and methods

Worm culture and strains Methods for cultivation of wild-type *C. elegans* either in monoxenic agar plate culture or liquid culture, generation of wild-type embryos as well as isolation and purification of mixed-stage embryos were performed as described elsewhere [17, 18]. In brief, cultures were inoculated with synchronized L1 larvae grown at 15°C for approximately 70–80 h until they reached the L4 stage. The temperature was then raised to 25°C. Worms and eggs were harvested after about 20 h when the first terminal embryonic stages appeared to obtain a mixed population of all embryonic stages after bleaching.

The following mutant strains and alleles were used: N2 Bristol as standard wild-type strain [17], *glp-1 (e2144ts)* LGIII [19], *cib-1 (e2300)* [20], *lit-1 (t1512ts)* [21] and *t1099* (Ralf Schnabel, unpublished).

Glycan sample preparation Three individual preparations of wild-type and *glp-1, lit-1* and *t1099* mutant embryos were disintegrated by ultrasonication in an ice bath, whereas a single preparation was worked-up in the case of *cib-1*. Proteins were precipitated using ice-cold 80% aqueous acetone overnight at −20°C and harvested by centrifugation at 12,000×g for 15 min. After removal of the supernatant, the protein pellet was washed and vortexed with 2×1 ml cold acetone with centrifugation after each addition. The procedure was followed by protein determination using the microBCA assay kit (Pierce, Rockford, IL) demonstrating a total yield of 0.2–1 mg egg protein in each case. Precipitated proteins were suspended in 25 mM $(NH_4)HCO_3$, pH 8.5, and digested with trypsin overnight at 37°C. After boiling for 5 min the samples were lyophilized. Resultant (glyco)peptides were dissolved in

75–350 µl of 20 mM sodium phosphate buffer, pH 7.2, and N-glycans were released by treatment with 5–10 units peptide-*N*-glycosidase F (PNGase F; Roche, Mannheim, Germany) overnight at 37°C under shaking. After addition of the same amount of enzyme, incubation was repeated once. The resulting mixture was applied to a reversed-phase (RP) cartridge (25 mg, Thermo Fisher Scientific, Waltham, MA) and N-glycans were recovered in the flow-through with 0.1% aqueous trifluoroacetic acid (TFA). Residual glycopeptides were eluted by washing the cartridge with 3 ml of 0.1%TFA/acetonitrile (80:20, 60:40, 40:60; v/v) each, combined and lyophilized. For treatment with 0.25–0.5 mU peptide-*N*-glycosidase A (PNGase A; Roche) the samples were dissolved in 40–300 µl of 10 mM ammonium acetate buffer, pH 5.0, for 16 h at 37°C and released glycans were recovered as above. For desalting, glycans were applied to a porous graphitic-carbon (PGC) cartridge (Supelclean ENVI-Carb 25 mg, Supelco, Bellefonte, PA). The cartridges were washed with water and glycans were eluted with 25% (v/v) aqueous acetonitrile. For further purification, N-glycan samples were subjected to RP and PGC cartridges once again. In parallel, tryptic glycopeptides of wild-type embryos were also subjected to hydrazinolysis as described previously [22, 23].

Glycan analysis Mass spectrometric analyses were performed using an Ultraflex MALDI-TOF mass spectrometer (Bruker-Daltonik, Bremen, Germany) equipped with a nitrogen laser employing the methodology described previously [24]. The instrument was operated in the positive-ion reflectron mode throughout. Samples were spotted onto a 600 µm hydrophilic anchor of an AnchorChip[TM] MALDI sample plate (Bruker-Daltonik) using 6-aza-2-thiothymine (Sigma, Taufkirchen, Germany) solution in water (5 mg/ml) as matrix. 2-Aminopyridine-labeled isomaltosyl oligosaccharides were used for external mass calibration. From each sample spot 100–500 single spectra were accumulated. Assignment of oligosaccharide compositions was performed using the software tools GlycoWorkbench and Glyco-Peakfinder [25, 26]. From each sample 5 independent spectra were acquired. Relative intensities of the recorded signals were estimated as mean values of the individual spectra and typical representatives are shown. When necessary, samples were digested with α-glucosidase from rice (Serva, Heidelberg, Germany) directly on the target. To this end, samples were dissolved in 1 µl of 25 mM ammonium acetate buffer, pH 6.0, and 1 µl of enzyme equilibrated with the same buffer was added and incubated overnight [27].

For removal of Fuc residues by hydrofluoric acid (HF) hydrolysis [28], aliquots of glycan samples were evaporated to dryness, dissolved in 30 µl 48% HF and incubated for 16 h at 4°C. HF was removed by a stream of nitrogen and the sample was taken up twice with 30 µl of methanol and dried again [22].

Results and discussion

Obtained N-glycan patterns of C. elegans are dependent on the cultivation conditions Fine structure analyses performed by many laboratories have demonstrated that *C. elegans* has an extraordinarily diverse N-glycosylation repertoire [2–7, 29] with more than 100 individual glycan structures (for an overview, see [8]). Differences in glycan patterns were mainly observed in dependence on the developmental stage of the worms and the methods used for release of the glycan chains [3, 5, 8]. In our experiments, *C. elegans* was either grown on agar plates or in liquid cultures and embryos were recovered by hypochlorite treatment of worms obtained thereof [18]. Glycans were enzymatically released from worm proteins by treatment with PNGase F and PNGase A as PNGase F is inhibited in the case of N-linked glycans bearing (α1-3)-linked Fuc at the innermost GlcNAc of the chitobiose core, whereas PNGase A is not restricted by this type of core fucosylation [30]. As (α1-3)-fucosylated

species represent only a minor fraction of total *C. elegans* N-glycans [4, 12] almost similar oligosaccharide profiles were obtained with both enzymes. After purification, re-leased glycans were directly subjected to mass spectrometric profiling without any derivatization. Some glycan preparations, however, comprised significant amounts of hexose oligomers, which could be removed by on-target digestion with α-glucosidase from rice (data not shown). It may be, therefore, speculated that these oligosaccharides represented storage carbohydrates serving as food source. Similar oligo-hexose signals were observed in MALDI-TOF-MS N-glycan spectra of *Drosophila melanogaster* mutants, which were ascribed to contaminations by fly food [31].

Mass spectra of wild-type embryo-derived N-glycans demonstrated an overwhelming dominance of high mannose type structures with compositions of $Hex_{5-10}GlcNAc_2$ with $Hex_5GlcNAc_2$ and $Hex_6GlcNAc_2$ as most prevalent structures (Fig. 1a). Intriguingly, considerable amounts of high mannose type components comprising only 1 HexNAc
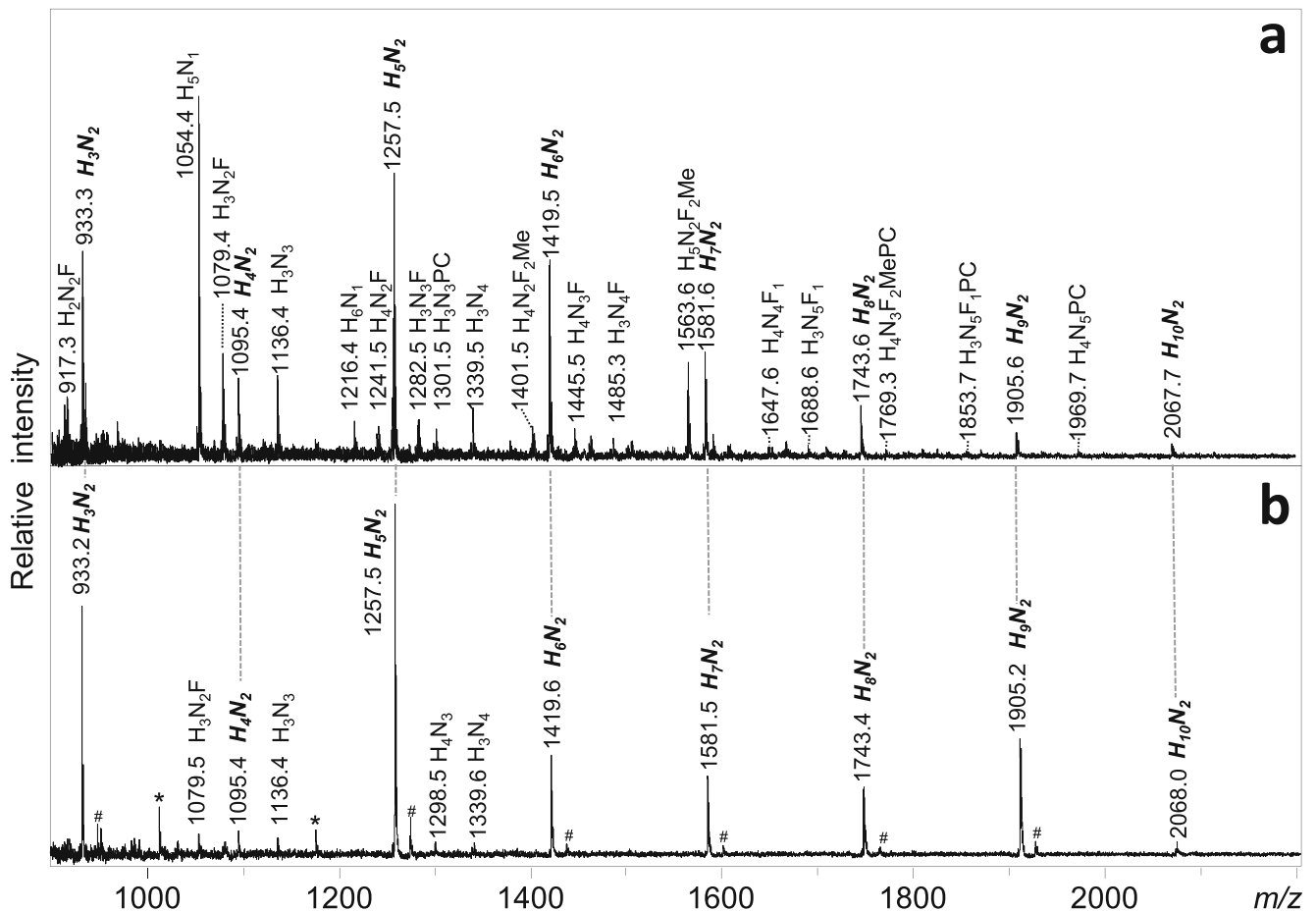
Fig. 1 Mass spectrometric N-glycan profiles of wild-type *C. elegans* embryos. N-glycans were released from *C. elegans* homogenates with PNGase A, purified and directly analyzed by MALDI-TOF-MS. **a** N-glycan pattern obtained from mixed embryos grown on agar plates, and **b** representative N-glycan profile obtained from mixed embryos grown in liquid culture. Compositions and respective *m/z* values of pseudo-molecular ions [M+Na]$^+$ are given for selected signals. H, hexose; N, *N*-acetylhexosamine; F, fucose; Me, *O*-methyl; PC, phosphorylcholine. Signals marked by # represent [M+K]$^+$adducts, whereas signals marked by asterisks (*) reflect hexose oligomers

residue were additionally detected in the profile spectra of wild-type *C. elegans* embryos grown on agar plates (see, for instance, signals at *m/z* 1054.4 ($Hex_5HexNAc_1$) and 1216.4 ($Hex_6HexNAc_1$) in Fig. 1a and Table 1). Most likely, these species result from the action of an endo-β-*N*-acetylhexosaminidase occurring in the cytosol of *C. elegans*, which hydrolyzes the chitobiose moiety of free oligosaccharides [32]. The question remains open as to whether species with a compostion of $Hex_2HexNAc_2PC$ are similarly formed by such an enzyme. In addition, a high amount of $Hex_3HexNAc_2$, a remarkable pattern of fucosylated *O*-methylated paucimannosidic and high mannose type glycans, truncated complex type oligosaccharides as well as minute amounts of phosphorylcholine (PC)-substituted species could be detected (*cf.* Fig. 1a and Table 1). The presence of fucosyl residues and PC substituents was verified by the disappearance of these signals after treatment with hydrofluoric acid (data not shown) which is known to rapidly remove (α1-3)-linked fucosyl residues [28, 33]. Such paucimannosidic glycan structures are typically found in insects, *C. elegans* and plants but are usually not seen in vertebrates [2, 10, 11, 34]. Hence, our data fully agree with earlier reports demonstrating the presence of highly fucosylated truncated N-glycans and *O*-methyl substituted Fuc or Man constituents in *C. elegans* [2, 8].

Analysis of oligosaccharide profiles obtained from wild-type *C. elegans* embryos grown in liquid culture, however, revealed a significant restriction in glycan expression. The spectrum was less heterogeneous and was dominated by the series of high mannose type glycans $Hex_{5-10}HexNAc_2$ with $Hex_5HexNAc_2$ as base peak. Likewise, the amount of $Hex_3HexNAc_2$, *i.e.*, the major paucimannosidic glycan, was increased whereas its fucosylated variant $Hex_3HexNAc_2Fuc$ was clearly decreased. A small subset of truncated complex glycans at *m/z* 1136.4, 1298.5 and 1339.6 was observed, whereas further fucosylated, *O*-methylated species were not detectable at all (see Fig. 1b and Table 1). N-glycan profiles obtained after enzymatic or chemical release of the oligosaccharides by hydrazinolysis have been shown to differ considerably due to the incapability of the used enzymes to liberate many of the core-substituted, elongated structures [5]. Therefore, we have used, in parallel, hydrazinolysis for the release of embryo-derived glycans. The obtained profiles, however, were in principle indistinguishable from those obtained after enzymatic treatment. Solely the fucosylated analogue of the truncated complex type species $Hex_3HexNAc_3$ could be detected at *m/z* 1282.5 as a minor additional component (data not shown). On the other hand, the obtained mass spectrum was dominated by unspecific background signals due to the formation of significant amounts of by-products during hydrazinolysis and the low amounts of material available. Hence, the conclusion that N-glycan patterns of *C. elegans* have to be

considered as stage-specific and unique [3] is confirmed by our study, showing for the first time the N-glycan pattern of *C. elegans* embryos. Obtained results demonstrated that glycan profiles of embryos are considerably less heterogeneous than those of most larval or mixed stages and adult worms, especially when embryos were harvested from liquid culture and not from agar plates. In agreement with our results all developmental stages analyzed so far were found to exhibit most abundant signal corresponding to $Hex_3HexNAc_2$, $Hex_{5-9}HexNAc_2$, $Hex_3HexNAc_2Fuc$, as well as $Hex_3HexNAc_3$ species [3] possibly indicating a substantial role or function of these glycans within the worm.

Comparison of N-glycan profiles from C. elegans wild type and mutant embryos One major aim of this investigation was to assess as to whether the N-glycan patterns of *C. elegans* mutant embryos were specific for certain blastomere, *i.e.*, regional identities and/or tissues in the embryo including the terminal stages [35, 36]. The mutant *glp-1 (e2144)* reduces the complexity of the mostly ectodermal AB lineage from normally eight different identities at the 12-cell stage to only two. These four ABala and four ABarp blastomeres produce almost exclusively cells with neuronal and hypodermal fates. The P1 derived cells are essentially normal, only 20 mesodermal(MS)-lineage derived muscles of the normally 81 body wall muscles are missing [19]. The mutant *lit-1 (t1512ts)* causes variable posterior to anterior transformation in all lineages but the D lineage, which produces 20 body wall muscles. This causes a strong reduction of body wall muscles to approximately 30%. The intestine is eliminated since the endodermal E lineage is transformed into the MS lineage. This duplication of the MS lineage together with the internal transformation of posterior body wall muscles into pharyngeal cells cause a duplication of pharyngeal structures [21]. In addition, two mutants *t1099* and *cib-1 (e2300)* were chosen, which arrest around the 50–100 cell-stage and do show very little, if any, tissue differentiation [20].

C. elegans mutant embryos were prepared from synchronized populations propagated in liquid cultures. To determine as to whether the synthesis of high mannose, paucimannosidic and truncated complex type N-glycans was differently affected in the mutant embryos, we have examined the mass profiles of N-glycans obtained after release by PNGase A (Fig. 2). In all embryonic mutants, N-glycan profiling revealed that high mannose glycans with compositions of $Hex_{5-9}HexNAc_2$ and the paucimannosidic species $Hex_3HexNAc_2$ clearly represented the most abundant types of glycans. Oligosaccharide profiles of mutant embryos were distinguished from those of wild-type embryos by different relative proportions of these components and by the extent of additional, further processed glycans. Most striking differences were observed with regard to the

Table 1 N-glycan compositions of *C. elegans* wild-type (wt) and mutant embryos deduced from MALDI-TOF-MS data after enzymatic release using PNGase A

| *m/z* [M+Na]$^+$	Composition	wt embryos[a]	wt embryos[b]	Mutant embryos[b]			
				glp-1	*lit-1*	*t1099*	*cib-1*
892.4	H4N1	+					
917.3	H2N2F1	+[c]					
933.4	***H3N2***	+	+	+++	++	++	++
936.3	H2N2PC	+[c]					
1054.4	H5N1	+	+				
1077.3	H2N2F2Me	(+)[c]					
1079.5	H3N2F1	+[c]	+	+	+	+	
1093.4	H3N2F1Me	+[c]					
1095.5	***H4N2***	+	+	+	+	+	
1136.5	H3N3	+	+	+	+	+	+
1216.4	H6N1	+					
1239.6	H3N2F2Me	+[c]					
1241.6	H4N2F1	+[c]		(+)		(+)	
1255.6	H4N2F1Me	+[c]	+				
1257.6	***H5N2***	+	+	++	+	++	++
1282.6	H3N3F1	+[c]	(+)[d]	(+)			
1298.5	H4N3	+	+	(+)			
1301.5	H3N3PC	+[c]			(+)		
1339.6	H3N4	+	+	(+)	+		
1378.5	H7N1	+					
1401.6	H4N2F2Me	+[c]					
1403.6	H5N2F1	+[c]		(+)			
1417.6	H5N2F1Me	+[c]	+				
1419.6	***H6N2***	+	+	+	+	+	+
1445.5	H4N3F1	+[c]					
1460.6	H5N3	+					
1463.6	H4N3PC	(+)[c]					
1485.3	H3N4F1	+[c]					
1501.6	H4N4	+[c]					
1504.6	H3N4PC	+[c]			(+)		
1542.6	H3N5	(+)					
1547.4	H4N2F3Me	(+)[c]					
1549.8	H5N2F2	(+)[c]					
1561.5	H4N2F3Me2	+[c]					
1563.6	H5N2F2Me	+[c]		(+)			
1579.5	H6N2F1Me	+[c]	+			(+)	
1581.7	***H7N2***	+	+	+	+	+	+
1606.6	H5N3F1	+[c]					
1647.6	H4N4F1	+[c]					
1650.5	H3N4F1PC	+[c]					
1688.6	H3N5F1	+[c]					
1702.6	H9N1				(+)	(+)	(+)
1707.7	H3N5PC/H4N2F4Me2	+[c]					
1725.6	H6N2F2Me	(+)[c]		(+)			
1741.6	H7N2F1Me		+	(+)		(+)	
1743.7	***H8N2***	+	+	+	+	++	+
1769.3	H4N3F2MePC	+[c]					
1807.6	H4N4F2Me	+[c]					
1853.7	H3N5F1PC	+[c]					
1868.6	H4N5PC	+[c]					
1903.4	H8N2F1Me		+	+	(+)	+	
1905.6	***H9N2***	+	+	++	+++	+++	+++
1969.7	H5N4F2Me	+[c]					
2067.5	***H10N2***	+	+	+	+	+	+

Monoisotopic masses [M+Na]$^+$ are given. H, hexose; N, *N*-acetylhexosamine; F, fucose; Me, *O*-methyl; PC, phosphorylcholine. Hex$_{3-10}$HexNAc$_2$ species are printed in bold and italics. Glycan species of mutant embryos were determined from 5 individual mass profiles of representative glycan preparations and semi-quantified as follows: (+), <1% of total peak areas; +, 1–10%; ++, >10%; +++, main component

[a] Obtained from cultures on agar plates

[b] Grown in liquid culture

[c] Not detectable after HF treatment

[d] Only detected after glycan release by hydrazinolysis
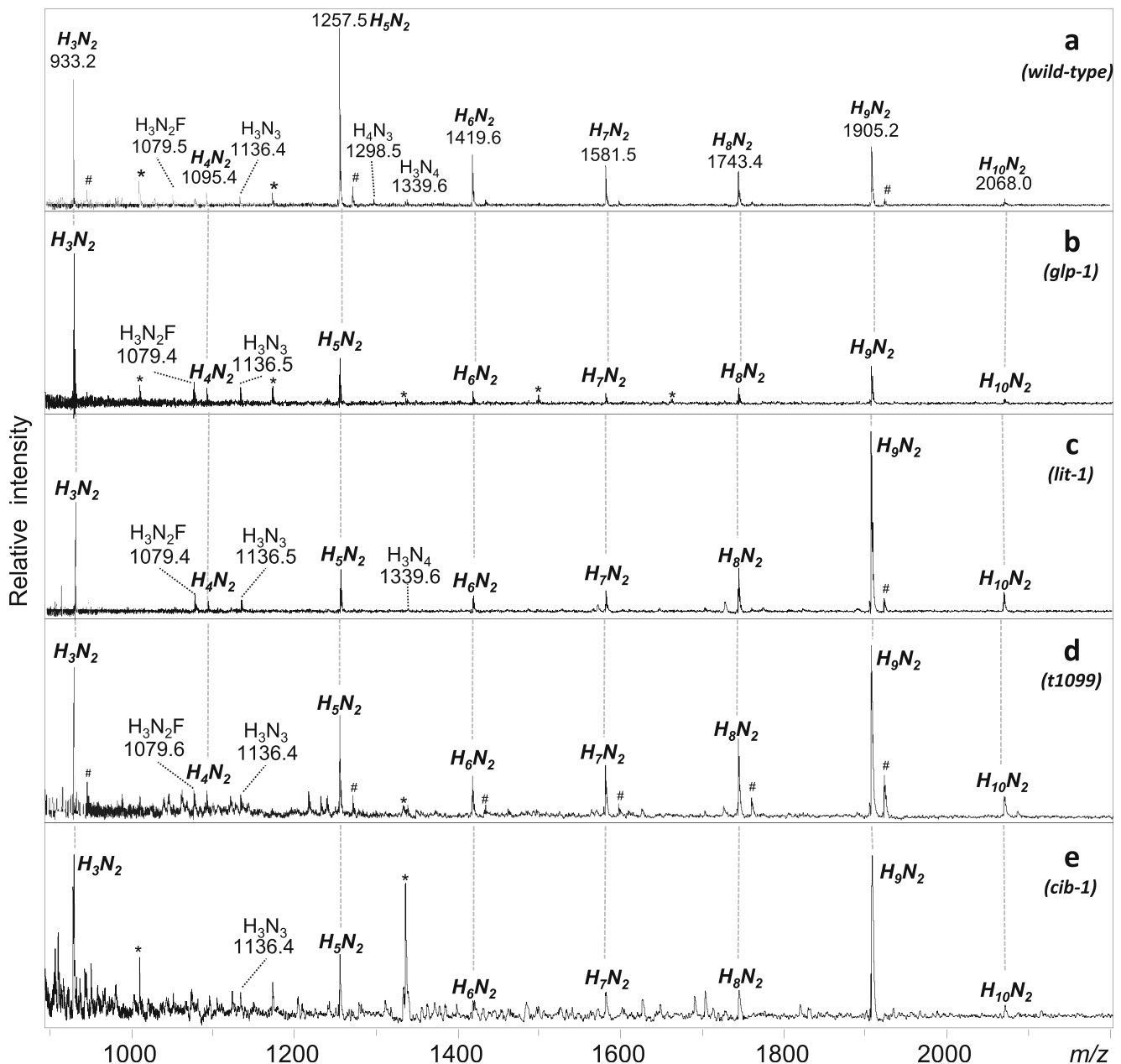
Fig. 2 N-glycan patterns of *C. elegans* mutant embryos. Embryos were cultivated in liquid culture, and N-glycans were enzymatically released from wild-type and mutant embryos using PNGase A, purified and directly analyzed by MALDI-TOF-MS. Typical profiles are shown for wild-type (**a**), *glp-1* (**b**), *lit-1* (**c**), *t1099* (**d**), and *cib-1* mutant embryos (**e**). Compositions and respective *m/z* values for pseudomolecular ions [M+Na]$^+$ are given for selected signals. Only major signals are assigned. H, hexose; N, *N*-acetylhexosamine; F, fucose. Signals marked by # represent [M+K]$^+$ adducts, whereas signals marked by asterisks (*) correspond to hexose polymers which were insensitive to α-glucosidase treatment

abundance of Hex$_3$HexNAc$_2$, Hex$_5$HexNAc$_2$ and Hex$_9$HexNAc$_2$ species which varied considerably. Whereas in wild-type embryos Hex$_5$HexNAc$_2$ glycans at *m/z* 1257.5 showed the highest abundance (Fig. 2a), N-glycan profiles of mutant embryos were either dominated by Hex$_3$HexNAc$_2$ or Hex$_9$HexNAc$_2$ species at *m/z* 933.3 or *m/z* 1905.6, respectively (Fig. 2b–e). In order to assess more thoroughly differences in the N-glycan patterns, respective peak areas

were quantified and displayed as bar chart (Fig. 3). To this end, mean peak areas of glycan signals were determined from five individual spectra and either normalized to the sum of peak areas for each measurement (Fig. 3a) or normalized to the respective wild-type compositional species set to 100% (Fig. 3b).

The results revealed that all mutant embryos expressed higher levels of less processed Hex$_{8-10}$HexNAc$_2$ glycans

Fig. 3 Quantitative evaluation of N-glycan compositions in *C. elegans* wild-type and mutant embryos. **a** Mean relative peak areas (%) were calculated from 5 individual MALDI-TOF-MS profiles of each N-glycan preparation. Signals were normalized to the sum of peak areas for each measurement. Individual compositional species are grouped as follows: high mannose (including respective $H_{5-9}N_1$) species, modified (*i.e.*, fucosylated and/or *O*-methylated) high mannose species as well as paucimannose, hybrid and truncated complex glycans. **b** In order to illustrate differences in N-glycan compositions, peak areas of wild-type high mannose, paucimannose and hybrid N-glycan species were used as reference values and set to 100%, respectively
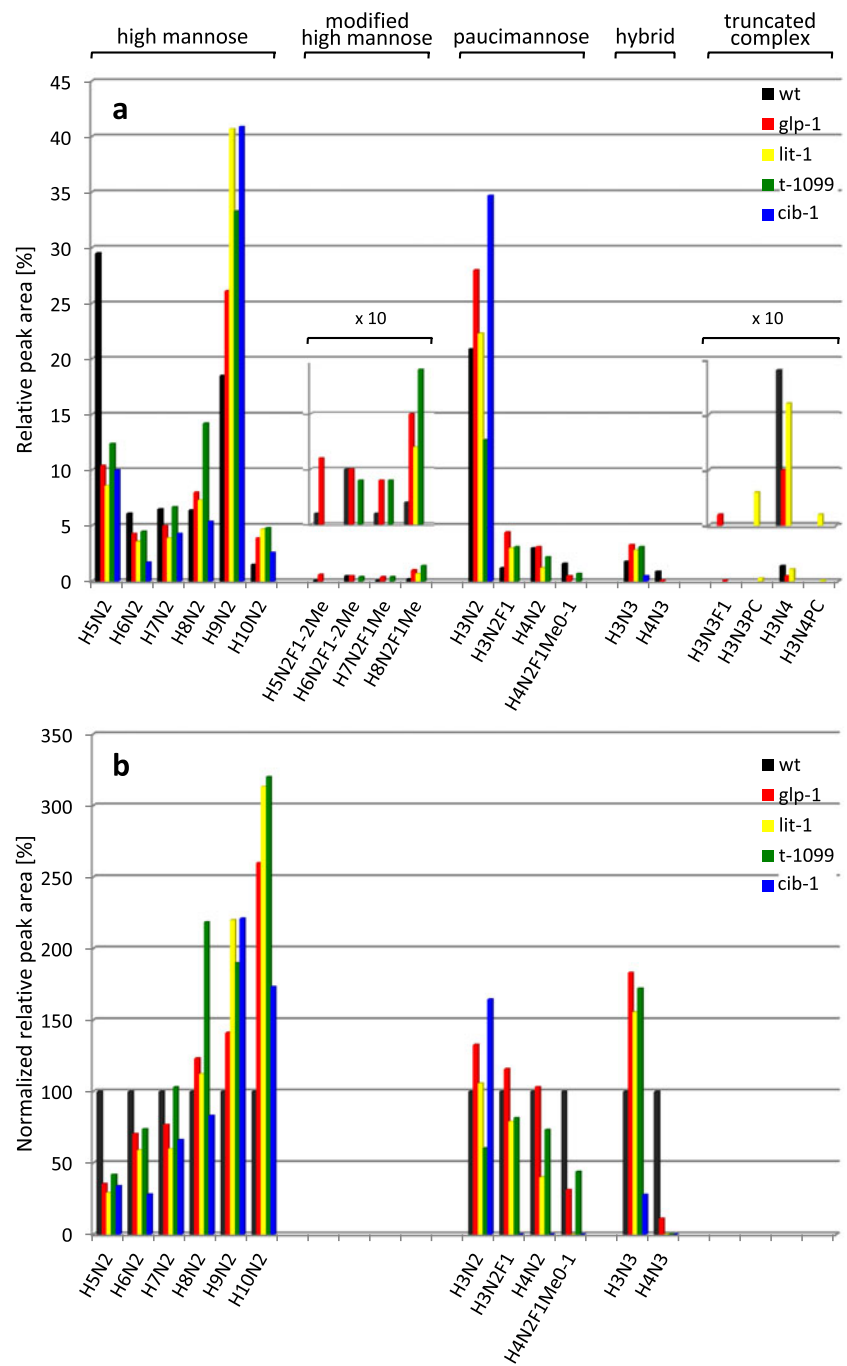
and lower levels of $Hex_{5-7}HexNAc_2$ species than wild-type embryos (Fig. 3b). In contrast, remaining classes of N-glycans, *i.e.*, modified high mannose, paucimannose, hybrid and truncated complex sugar chains clearly displayed differences between the individual mutant embryos. The *glp-1* mutant N-glycan pattern (Fig. 2b) resembled mostly that of wild-type embryos with respect to the presence of minor fucosylated, *O*-methylated high mannose glycans ($Hex_{5-8}HexNAc_2Fuc_{1-2}Me_{0-1}$), paucimannosidic ($Hex_{2-4}HexNAc_2Fuc_{0-1}Me_{0-1}$) and truncated complex type ($Hex_3HexNAc_{3-4}Fuc_{0-1}$) species (*cf.* Fig. 3 and Table 1). The profile of

lit-1 mutant N-glycans (Fig. 2c) was characterized by a dominant $Hex_9HexNAc_2$ signal at *m/z* 1905.6, the presence of partially fucosylated paucimannosidic ($Hex_{3-4}HexNAc_2Fuc_{0-1}$) glycans, hybrid or truncated complex type ($Hex_3HexNAc_{3-4}$) sugar chains as well as a reduced pattern of modified high mannose (only $Hex_8HexNAc_2Fuc_1Me_1$) species and the presence of minute amounts of phosphorylcholine (PC) substituted oligosaccharides ($Hex_3HexNAc_{3-4}PC_1$) (Fig. 3a and Table 1). In *t1099* mutant embryos, $Hex_{9,8\ or\ 5}HexNAc_2$ and $Hex_3HexNAc_2$ species (*m/z* 1905.6, 1743,2, 1257,5 and 933.3) were again the most prominent glycan components

(Fig. 2d). In addition to prototypic high mannose structures, weak signals were recovered reflecting the presence of fucosylated, O-methylated high mannose glycans ($Hex_{6-8}Hex\text{-}NAc_2Fuc_1Me_1$), paucimannose as well as $Hex_3HexNAc_3$ species. Intriguingly, truncated complex type species were not registered at all (Fig. 3 and Table 1). Finally, the cib-1 mutant expressed the most restricted N-glycan profile which was dominated by $Hex_9HexNAc_2$ species together with other high mannose glycans as well as paucimannosidic $Hex_3Hex\text{-}NAc_2$ oligosaccharides (Fig. 2e). Apart from trace signals indicative for the presence of $Hex_3HexNAc_3$ species, other glycans were not detected in this case (Fig. 3 and Table 1), thus indicating a reduced processing of glycoprotein-N-glycans in this mutant. Some mutants additionally exhibited trace levels of $Man_9GlcNAc_1$ species which might reflect the action of an intracellular endo-N-acetylhexosaminidase. Such compounds, however, were only detected when $Man_9GlcNAc_2$ represented the main high mannose glycan.

General discussion and conclusion

N-glycan synthesis in C. elegans has been extensively studied by various groups [2–11]. In wild-type worms, conversion of high mannose glycans to paucimannose species by the sequential action of class I α-mannosidases and N-acetylglucosaminyltransferase I (GnTI) is followed by the action of α-mannosidase II together with a specific Golgi β-N-acetylhexosaminidase that removes GlcNAc to form the typical $Man_4GlcNAc_2$ and $Man_3GlcNAc_2$ paucimannosidic structures (Fig. 4a). To a minor extent, truncated complex type oligosaccharides (e.g., $GlcNAc_2Man_3GlcNAc_2$) can be formed by retaining the first GlcNAc residue and transferring a second GlcNAc by N-acetylglucosaminyltransferase II (GnTII) (for detailed reviews see [8–11, 34]). Moreover, glycans can be further modified by the action of different fucosyltransferases (FTs), including also GnTI-independent FTs, and a specific, GnTI-dependent phosphorylcholine-transferase (PCT). In addition, glycans may be further substituted by O-methyl groups in close association with fucosylation [8].

Based on the observed N-glycan patterns, conclusions could be drawn on the glycosylation potential of C. elegans wild-type and mutant embryos under study. As a striking feature, wild-type embryos obtained from liquid culture expressed a less heterogeneous oligosaccharide pattern than embryos recovered from an agar plate (cf. Fig. 4a,b and Table 1). Present oligosaccharides appeared to be less processed and exhibited reduced amounts of Fuc, O-methyl groups, and complex type species. It has been speculated that heterogeneous N-glycan patterns of certain developmental

stages of C. elegans might reflect "significant lifestyle changes" of the worm during these stages [3]. Here, we find that also embryos vary in glycosylation depending on their environment as their glycosylation pattern is more complex on solid agar plates than in liquid culture. Unpublished observations of N. Memar and R. Schnabel suggest that glycosylation in the hypodermal seam cells is required for proper crawling of worms on plates, which may be dispensable while swimming. It is an interesting question how embryos within the egg shell would "know" about their environment. This could be either sensed directly or may be passed on by the hermaphrodite during egg production. The simplicity of the environmental cue swimming versus crawling may offer an interesting opportunity to study the influence of the environment on glycosylation in the future.

It was the aim of this study to investigate whether the complexity of glycosylation patterns reflects the complexities of the cell fates and tissues in the embryo and thus could, for example, guide cell sorting in embryogenesis [35]. For a first investigation we used two genes, glp-1 or lit-1, which have significantly different consequences on cell fates in the embryo. The analysis of glycosylation requires, however, significant amounts of homozygous embryos, which can be only obtained from liquid culture. This is possible using the two temperature sensitive mutations (t2144, t1512) available for these genes.

The hallmark of glp-1 embryos is that the number of founder cell fates derived from the AB blastomere is reduced from eight to two fates [19, 35]. This causes an elimination of the anterior pharynx and a significant hypertrophy of neuronal tissues. Moreover, about 30% of the body wall muscle is missing. The glycosylation profile of glp-1 (Fig. 4c) resembled to some extent the one of wild-type embryos from liquid culture (Fig. 4b) as these mutants exhibited an almost similar overall degree of fucosylation of high mannose glycans. Since the amounts were insufficient for further analysis, such as, MS/MS experiments of derivatized glycans we could neither determine the position nor the linkage of these fucosyl residues. Thus, the observed similar overall fucosylation patterns might still reflect products of different fucosyltransferases. In addition, however, distinct high mannose ($Man_5GlcNAc_2$), paucimannose ($Man_4GlcNAc_2Fuc$), hybrid ($Man_4GlcNAc_3$) and truncated complex type ($Man_3GlcNAc_4$) species appeared to be expressed with lower relative abundance in comparison to wild-type embryos (Fig. 4c). This reduced level of N-glycan processing might correlate with the fate transformations in glp-1 mutant embryos. The glycosylation pattern of mutant lit-1 (Fig. 4d) was characterized by a strongly reduced expression of fucosylated high mannose type and paucimannose glycans as well as reduced levels of $Man_5GlcNAc_2$, paucimannose ($Man_4GlcNAc_2$) and hybrid type species.

a

b *(wild-type)*

c *(glp-1)*

d *(lit-1)*

e *(t1099)*

f *(cib-1)*
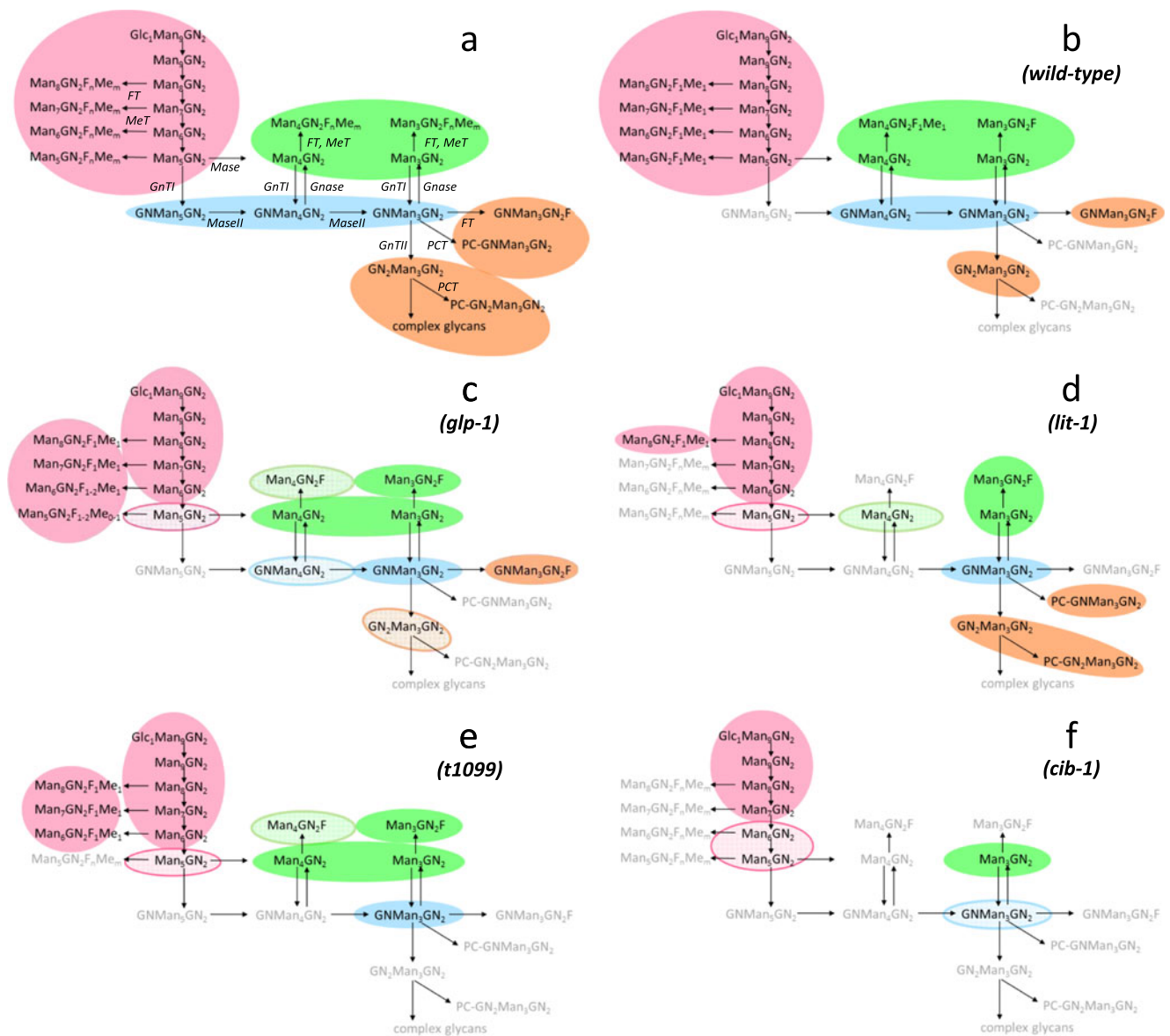
Fig. 4 Schematic outline of N-glycan biosynthesis in *C. elegans* (modified from [10]). **a** General scheme of N-glycan synthesis in wild-type *C. elegans* embryos obtained from cultures on agar plates. Structural details of the species shown were not elucidated in this publication but are assumed to be as published [3–8, 11]. The different classes of oligosaccharides, *i.e.* high mannose ($Man_{5-9}GlcNAc_2$), paucimannose ($Man_{3-4}GlcNAc_2Fuc_{0-1}$), hybrid ($GlcNAc_1Man_{3-5}GlcNAc_2$) and truncated complex type glycans ($PC_{0-1}GlcNAc_{1-2}Man_3GlcNAc_2Fuc_{0-1}$) plus further processed complex glycans, are colored in pink, green, blue and orange, respectively. **b–f** Proposed routes of N-glycosylation in wild-type embryos grown in liquid culture (**b**) as well as *glp-1* (**c**), *lit-1* (**d**), *t1099* (**e**), and *cib-1* (**f**) mutant

embryos were deduced from MALDI-TOF-MS data. Glycans exhibiting normalized relative peak areas of less than 50% in comparison to wild-type embryos from liquid culture (*cf.* Fig. 3) are shaded in the respective color, whereas species which were not detected in the respective mutant embryo are marked in grey. FT, fucosyltransferase; GnTI or GnTII, *N*-acetylglucosaminyltransferase I or II; Gnase, β-*N*-acetylhexosaminidase; Mase, GnTI-independent α-mannosidase; MaseII, α-mannosidase II; MeT, methyltransferase; PCT, phosphorylcholinetransferase. GN, *N*-acetylglucosamine; F, fucose; Me, *O*-methyl; PC, phosphorylcholine. Numbers of Fuc residues (n) and *O*-methyl groups (m) present in high mannose type glycans range between 1–4 and 0–2, respectively

Intriguingly, only this mutant expressed small amounts of truncated complex, PC-substituted oligosaccharides (Fig. 4d). This finding may be in so far relevant as this mutant expresses initially all cell identities till founder cell formation. However, after this all cell lineages, but D, show posterior to anterior transformations resulting in the elimination of the

intestine and reduction of body wall muscles and hypodermis [21].

Data obtained in the case of *t1099* (Fig. 4e), and *cib-1* (Fig. 4f) mutant embryos reflected a restricted (*t1099*) or strongly restricted (*cib-1*) oligosaccharide pattern, which is consistent with the strongly limited cell and tissue

differentiation occurring in these mutant embryos, which arrest at the 50–100 cell-stage. Whereas mutant *t1099* glycans (Fig. 4e) were characterized by a reduced expression of fucosylated high mannose species, fucosylation was completely absent in *cib-1* mutants. Likewise, the patterns of paucimannosidic ($Man_{3-4}GlcNAc_2Fuc_{0-1}$) and hybrid type glycans were strongly reduced in these mutants, in particular, in the *cib-1* mutant. As a striking feature, truncated or PC-substituted complex type species were also not detected in this case (Fig. 4e,f). This might indicate that the production of complex glycosylation patterns depends on the specification of cell identities in later embryogenesis. However, all analyzed mutants seem to express GnTI, MaseII and Gnase activities as evidenced by the presence of $Man_3GlcNAc_2$ species, which may point to a maternal contribution for these components.

In conclusion, our results suggest that the development of specific glycosylation patterns may be coupled to the differentiation of cells after initiation of gastrulation. The cell fate mutants additionally insinuate that specific cells during the ongoing embryogenesis or later tissues may display specific N-glycan profiles. This is in agreement with the observation that the complexity of the N-glycan profiles is increased during development to adulthood [3]. In this context, it has to be pointed out, however, that O-glycosylation might be similarly involved in these processes as suggested recently [13]. The observation that *C. elegans* expresses a panel of unusual O-linked glycans, which mediate, at least in part, resistance towards infection by *Mycobacterium nematophilum* or *Bacillus thuringiensis* toxin [37, 38] clearly underlines the functional relevance of this type of glycans, which needs to be further addressed in future studies.

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