Dynamic Developmental Elaboration of N-Linked Glycan Complexity in the Drosophila melanogaster Embryo*

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The structural diversity of glycoprotein N-linked oligosaccharides is determined by the expression and regulation of glycosyltransferase activities and by the availability of the appropriate acceptor/donor substrates. Cells in different tissues and in different developmental stages utilize these control points to manifest unique glycan expression patterns in response to their surroundings. The activity of a Toll-like receptor, called Tollo/Toll-8, induces a pattern of incompletely defined, but neural specific, glycan expression in the Drosophila embryo. Understanding the full extent of the changes in glycan expression that result from altered Tollo/Toll-8 signaling requires characterization of the complete N-linked glycan profile of both wild-type and mutant embryos. N-Linked glycans harvested from wild-type or mutant embryos were subjected to direct structural analysis by analytic and preparative high pressure liquid chromatography, by multidimensional mass spectrometry, and by exoglycosidase digestion, revealing a predominance of high mannose and paucimannose glycans. Di-, mono-, and nonfucosylated forms of hybrid, complex biantennary, and triantennary glycans account for 12% of the total wild-type glycan profile. Two sialylated glycans bearing N-acetylneuraminic acid were detected, the first direct demonstration of this modification in Drosophila. Glycan profiles change during normal development consistent with increasing α-mannosidase II and core fucosyltransferase enzyme activities, and with decreasing activity of the Fused lobes processing hexosaminidase. In tollo/toll-8 mutants, a dramatic, expected loss of difucosylated glycans is accompanied by unexpected decreases in monofucosylated and nonfucosylated hybrid glycans and increases in some nonfucosylated paucimannose and biantennary glycans. Therefore, tollo/toll-8 signaling influences flux through several processing steps that affect the maturation of N-linked glycans.

Cell surface glycans mediate interactions between cells and define cellular identities within complex tissues at all stages of life (1–6). As embryonic cells differentiate and form organized tissues, glycan expression diversifies, generating glycosylation profiles that are specific for tissue and cell type (7–9). Mutations that affect oligosaccharide synthesis or processing result in neural deficits, skeletal/vascular tissue abnormalities, anemia, compromised immune response, muscular dystrophy, or generalized failure to thrive (10–14). The vital functions of cellular glycans and the pathophysiologic consequences of altered glycosylation emphasize the need for understanding the basic mechanisms that regulate glycan expression in intact organisms.

The expanding characterization of glycosyltransferases in Drosophila melanogaster has begun to define the bounds of structural diversity in the glycan portfolio of the organism and has also generated new opportunities for genetically dissecting the mechanisms that control glycosylation. Loss-of-function mutations have been described in a handful of key enzymes that are necessary for N-linked glycosylation (15–19). These studies have reinforced the predicted functions of the enzymes in vivo and verified the synthetic and compensatory pathways that lead to production of major N-linked glycans in Drosophila. However, relatively little is known about the extent to which glycan diversity is affected by mutations in genes that do not directly act on the substrates, precursors, or products of oligosaccharide synthesis pathways. Such genes are candidates for regulators of glycan expression.

We previously characterized a Drosophila Toll-like receptor protein, called Tollo/Toll-8, which is required for the induction of neural specific glycosylation in the embryo (20). Neurons in tollo/toll-8 mutant embryos fail to generate oligosaccharides that carry Fuc in α1–3 linkage to the internal GlcNAc residue of the chitobiose core (GlcNAcβ1–4GlcNAcβ-linked to Asn) of their N-linked glycans. This structure, known as the HRP3 epitope, constitutes a minor fraction of the total glycan.

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profile of the adult (21–23). The abundance of the HRP epitope has not been determined previously in wild-type embryos, and loss of the epitope in tollo/toll-8 mutant embryos has only been detected by staining with anti-HRP antibody. Thus, it has remained to be determined whether Tollo loss-of-function affects the expression of glycans other than those that bear the HRP epitope.

More broadly, the diversity of N-linked glycans in the wild-type Drosophila embryo has only been partially characterized (24). It has been appreciated for quite some time that the dominant class of N-linked glycans is of the high mannose type (24–28). However, considerable uncertainty has surrounded suggestions that Drosophila, at any stage of development, may be capable of synthesizing hybrid or complex glycans. Additional questions have been raised regarding the presence or absence of sialic acid (29–31). Therefore, to provide a basis for assessing the specificity of mutations that alter glycan expression, we have characterized the major and minor N-linked glycans of the wild-type Drosophila embryo. We demonstrate that complex glycans are present as a minor species, including structures capped with sialic acid. As development progresses, the wild-type glycan expression profile shifts toward increased structural complexity. Finally, changes in glycan expression in mutant embryos indicate that Tollo/Toll-8 regulates activities capped with sialic acid.

**Experimental Procedures**

**Materials**—PNGaseF (N-glycanase), α-sialidases (Arthrobacter ureafaciens and Streptococcus pneumoniae), and β-galactosidase (S. pneumoniae) were obtained from Prozyme (San Léandro, CA). Trypsin, chymotrypsin, α-fucosidase (bovine kidney), and PNGaseA were obtained from Sigma and Calbiochem. Other glycoprotein standards and fine chemicals were from standard sources. Standard media for propagation and growth of Drosophila were obtained from Genesee Scientific (San Diego, CA) or were prepared from commercially available agar and juices (apple or grape).

**Collection of Embryos and Preparation of Fly Embryo Powder**—Antibody staining was performed on embryos that were fixed, dechorionated, and devitellinized as described previously (20, 32). For the preparation of embryos for glycan analysis, large scale collections were harvested from grape juice agar plates placed into population cages that contained large scale collections were harvested from grape juice agar plates placed into population cages that contained Drosophila adults of the OreR strain. Overnight collections (0–18 h) generally yielded between 5 and 15 g wet weight of adults of the OreR strain. Overnight collections were removed from the plate after 18 h of incubation and embryos were then aged for an additional 6 h.

Collected embryos were de-lipidated as described previously (33, 34). Briefly, embryos were disrupted on ice by Dounce homogenization in ice-cold water. Lipids were extracted by adjusting the solvent mixture to give a final ratio of chloroform/methanol/water equal to 4:8:3. The extract was incubated for 18 h at room temperature with end-over-end agitation. The insoluble proteinaceous material was collected by centrifugation and re-extracted three times. The final pellet of insoluble protein powder was determined by BCA assay (Pierce) and by quantitative densitometry of SDS-polyacrylamide gels stained with Coomassie Blue. Protein recovery in fly embryo powder, relative to total protein in the homogenate, was determined to be 91–95%. By quantification of Western blots probed with anti-HRP antibody, fly embryo powder contained 95% of the total HRP-reactive material of the homogenate, and the profile of recognized glycoprotein bands was not detectably different (data not shown).

**Preparation of Glycopeptides and Release of N-Linked Glycans**—Fly embryo powder, generally 5 mg, was resuspended in 200 μl of trypsin buffer (0.1 M Tris-HCl, pH 8.2, containing 1 mM CaCl₂) by sonication and boiled for 5 min. After cooling to room temperature, 25 μl of trypsin solution (2 mg/ml in trypsin buffer) and 25 μl of chymotrypsin solution (2 mg/ml in trypsin buffer) were added. Digestion was allowed to proceed for 18 h at 37 °C before the mixture was boiled for 5 min. Insoluble material was removed by centrifugation, and the supernatant was removed and dried by vacuum centrifugation. The dried peptide mixture was resuspended in 250 μl of 5% acetic acid (v/v) and loaded onto a Sep-Pak C₁₈ cartridge (35). The cartridge was washed with 10 column volumes of 5% acetic acid. Glycopeptides were eluted stepwise, first with 3 volumes of 20% isopropyl alcohol in 5% acetic acid and then with 3 volumes of 40% isopropyl alcohol in 5% acetic acid. The 20 and 40% isopro pyl alcohol steps were pooled and evaporated to dryness. Dried glycopeptides were resuspended in 50 μl of 2 M sodium phosphate buffer, pH 7.5, for digestion with PNGaseF, or in 50 μl of 0.2 M citrate phosphate buffer, pH 5.0, for digestion with PNGaseA, or in 200 μl of anhydrous hydrazine. Following PNGase digestion for 18 h at 37 °C, released oligosaccharides were separated from peptide and enzyme by passage through a Sep-Pak C₁₈ cartridge. The digestion mixture was adjusted to 5% acetic acid and loaded onto the Sep-Pak. The column run-through and an additional wash with 3 column volumes of 5% acetic acid, containing released oligosaccharides, were collected together and evaporated to dryness. Glycan release by hydrazinolysis followed by re-N-acetylation and desalting over AG50-X8 were performed as described previously (36).

**Preparation and HPLC Analysis of Pyridylaminated Glycans**—Portions of released oligosaccharide mixtures were reductively aminated with twice-recrystallized 2-aminopyridine and sodium cyanoborohydride as described previously.
Pyridylamidated oligosaccharides were purified by passage through a Sep-Pak C₁₈ cartridge with stepwise elution using 100, 95, 90, and 50% acetonitrile in water. Oligosaccharides conjugated to 2-aminopyridine were quantitatively collected in the 50% step, dried, and stored at −80 °C. A standard of pyridylamidated isomaltooligosaccharides, ranging over 1–20 glucose units, was prepared from an acid hydrolysate of dextran. Pyridylamidated glycan mixtures were fractionated on TSK amide-80 (4.6 × 250 mm; Tosoh Bioscience). The column was equilibrated with 10% buffer A (3% acetic acid, pH adjusted to 7.3 with triethylamine) and 90% buffer B (80% acetonitrile, 3% acetic acid, pH adjusted to 7.3 with triethylamine) at a flow rate of 0.5 ml/min at ambient temperature (38). After sample injection, elution was performed with 100% buffer A for 10 min followed by a linear gradient from 0 to 50% of buffer B for 50 min. Fractions were collected every 2 min, and the elution of labeled glycans was monitored by fluorescence (excitation wavelength, 320 nm; emission wavelength, 400 nm). Fractionated glycans were analyzed by MALDI-TOF/MS and NSI-MS.

The elution position of pyridylamidated glycans was referenced to the elution of standard isomaltooligosaccharides and is reported in Glc units. For the assignment of candidate structures, elution positions of Drosophila glycans were compared with the known elution positions of standard glycans using the data base available through the Graduate School of Pharmaceutical Sciences, Nagoya University, Nagoya, Japan (39, 40).

Exoglycosidase Digestions—Exoglycosidase digestions were performed following protocols provided by the suppliers. For β-galactosidase and α-fucosidase digestions, the substrates were pyridylamidated glycans previously fractionated on a TSK-amide column. Following digestion, products were purified by applying the reaction mixture to a Sep-Pak C₁₈ column, washing the column with water, and eluting glycans with 10% acetonitrile in water. Eluted glycans were dried and resuspended for HPLC analysis on TSK-amide or permethylated for HPLC analysis on TSK-amide or permethylated for quantification. Glycans were identified by a combination of fluorescence and MALDI-TOF/MS. Glycan structures were assigned according to the systematic nomenclature of Domon and Costello (42) using a nanoelectrospray source at a syringe flow rate of 0.40–0.60 μl/min. The capillary temperature wasist to 210 °C, and MS analysis was performed in positive ion mode. CID MS/MS spectra were obtained for each glycan

Permethylation of Glycans—To facilitate analysis by mass spectrometry (MS), portions of released oligosaccharide mixtures were permethylated according to the method of Ciucanu and Kerek (41). To compare relative glycan abundances in different samples, released glycans were permethylated with [¹²C]methyl iodide or [¹³C]methyl iodide and combined before analysis by MALDI-TOF/MS or NSI-MS. The ratio of peak intensities for differentially labeled glycans, detected in the same spectra, defined the relative abundance of each glycan. To determine whether differences detected initially in relative abundance measurements were artifacts of permethylation efficiency or isotope effects, the methylation reagents were reversed in a second analysis. In these experiments, the same values for the relative abundances between glycan preparations were detected by [¹²C]/[¹³C] and by [¹³C]/[¹²C] ratios.

Matrix-assisted Laser Desorption-Ionization/Time-of-Flight Mass Spectrometry—MALDI-TOF/MS was performed on an Applied Biosystems Voyager System mass spectrometer in positive linear mode. Permethylated or 2-aminopyridine-labeled glycan solutions were mixed in a 1:1 ratio with matrix (1 μl of glycan solution with 1 μl of 2,5-dihydroxybenzoic acid as a 20 mg/ml solution in 50% methanol), and 1 μl of the mixture was spotted on the sample plate. The instrument was externally calibrated with permethylated authentic high mannose type glycans from M5N2 to M9N2 (see Fig. 3 and supplemental Tables 1–3 for glycan nomenclature) prepared from ribonuclease A or with a partial dextran hydrolysate in the size range of Glc3–12. Average masses were obtained from spectra summed over 100–200 shots with a 337 nm nitrogen laser. Ions corresponding to individual glycans, which gave signal intensities greater than 3-fold above background, were quantified relative to the sum of the intensities of all candidate glycan ions to give “% total profile” for each glycan.

Nanospray Ionization-Linear Ion Trap Mass Spectrometry—For mass analysis by NSI-MS, permethylated glycans were dissolved in 1 mM NaOH in 50% methanol and infused directly into a linear ion trap mass spectrometer (LTQ; Thermo Finnigan) using a nanoelectrospray source at a syringe flow rate of 0.40–0.60 μl/min. The capillary temperature was set to 210 °C, and MS analysis was performed in positive ion mode. For fragmentation by CID in MS/MS and MS² modes 28% collision energy was applied. Spectra generated in support of the reported structures are available upon request from the corresponding author. The systematic nomenclature of Domon and Costello (42) was used to guide the depiction of fragmentation derived from MS² spectra.

The total ion mapping (TIM) functionality of the XCalibur software package (version 2.0) was utilized to detect and quantify the prevalence of individual glycans in the total glycan profile. Through TIM, automated MS and MS/MS spectra (at 28% collision energy) were obtained in collection windows that were 2.8 mass units in width. Five scans, each 150 ms in duration, were averaged for each collection window. The m/z range from 500 to 2000 was scanned in successive 2.8 mass unit windows with a window-to-window overlap of 2 mass units. The 2.8 mass unit window allowed signals from the naturally occurring isotopes of individual glycans to be summed into a single response, increasing detection sensitivity for minor structures. The 2 mass unit overlap ensured that minor glycans, whose masses placed them at the edge of an individual window, would be sampled in a representative fashion. Although it is possible to obtain MS spectra up to m/z = 4000 on the LTQ, software limitations currently disallow TIM beyond m/z = 2000. Therefore, manual MS/MS was performed to determine whether ions detected in the range m/z = 2000–4000 arose from glycans.

Most permethylated oligosaccharides were identified both as singly and as doubly charged species by NSI-MS. TIM peaks for all charge states of a given ion with m/z ≤ 2000 were summed together for quantification. The only singly charged ions detected at m/z > 2000 were assigned to the major structures M8N2, M9N2, and GlcM9N2, all of which were readily apparent and quantified as their doubly charged forms using TIM profiles collected over m/z = 500–2000. Signal intensity for the
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Full MS

Total Ion Mapping

Zoom

Filter scan for presence of signature fragment in MS/MS
singly charged forms of M8N2, M9N2, and GlcM9N2 were less than 5% of the signal intensity for their respective doubly charged ions. The singly charged forms of minor glycans with masses predicted to be >2000 Da were not detected above the threshold for quantification.

Peaks in TIM scans were quantified if 3-fold or greater above background. Glycan prevalence was calculated as the % total profile where the total profile was taken as the sum of the peak intensities for all quantified glycans. The MS/MS spectra for all TIM peaks were examined for the presence of daughter ions indicative of a glycan. A polyhexose detected in the starting material gave rise to a ladder of repeating ∆m/z = 204 fragments that overlapped with some N-linked glycan peaks detected by TIM. For peaks containing this contaminant, the MS signal intensity was apportioned to the relevant glycan based on the ratio of the first daughter ion of the glycan to the first contaminant ion measured in MS/MS.

Two parameters are used to express the relative contributions of individual glycans in embryo samples. The “prevalence” of a glycan, expressed as % total profile and calculated as described above, normalizes the signal intensity for an individual glycan to the total signal intensity for all glycans from a sample profile. Therefore, this parameter reports the change in the relative contribution a single glycan makes to the total glycan profile for a single sample. The “relative abundance” of a glycan is calculated based on the 12C/13C ratio for differentially permethylated glycans prepared from separate samples that are mixed together before analysis. The amount of each sample to be combined is determined by the recovery of protein in the starting material. Therefore, this parameter reports the fold difference in the absolute amount of a given glycan per mg of protein between two samples.

RESULTS

The N-Linked Glycan Profile of the Drosophila Embryo Is Dominated by High Mannose and Monofucosylated, Paucimannose-type Oligosaccharides—Glycans were released from Drosophila embryo proteins enzymatically, by treatment with PNGase, or chemically, by hydrazinolysis. Following enzymatic release and permethylation, mass spectra of unfractionated glycans prepared from separate samples that are mixed together before analysis. The amount of each sample to be combined is determined by the recovery of protein in the starting material. Therefore, this parameter reports the fold difference in the absolute amount of a given glycan per mg of protein between two samples.

For major glycans, the profiles detected by MALDI-TOF/MS or by NSI-MS are essentially identical (supplemental Fig. 1 and supplemental Table 1). Most permethylated oligosaccharides were identified both as singly and as doubly charged species by NSI-MS. All glycans detected by NSI-MS were subjected to fragmentation (MS^2^) to assign structure. Without fragmentation, MALDI-TOF/MS was able to detect a small subset of the minor glycans revealed by NSI-MS^2^ or by TIM. We utilized all three mass spectrometry methods, as needed, to confirm structural assignments. However, TIM analysis provided an unbiased approach for detecting minor glycans. By collecting MS and MS/MS spectra in discrete, overlapping windows, TIM effectively suppresses noise through base-line averaging and enhances sensitivity by summing signal from the naturally occurring isotopic forms of each glycan (compare the region around M5N2 in Fig. 1, A and B). TIM peaks provide landmarks behind which lie the MS/MS spectra that indicate whether MS signals arise from glycan. Examination of MS/MS spectra associated with major or minor peaks reveals diagnostic profiles of signature daughter ions (Fig. 1C). TIM also generates MS/MS spectra in regions where the MS signal is insufficient to generate a discernible peak. Examination of these spectra frequently reveals daughter ions diagnostic for glycan structures that fall below the quantification threshold (Fig. 1D).

To provide additional structural characterization, some preparations of released glycans were also derivatized with 2-aminoptyridine by reductive amination and then subjected to HPLC separation on TSK-amide. When referenced to Glc polymer standards, the retention of pyridylaminated glycans is predictive of oligosaccharide structure, especially in combination with subsequent MS analysis (39, 40). HPLC profiles for labeled

![Figure 1](https://example.com/figure1.png)
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FIGURE 2. HPLC separation of pyridylaminated glycans. Fluorescence detection of labeled glycans eluting from a TSK-amide column detects major glycan peaks as high mannose and paucimannose oligosaccharides. The profiles of major glycans released by PNGaseF and PNGaseA are very similar. Elution positions of pyridylaminated glycans are referenced to the elution positions of a Glc polymer ladder (G3–G12). The structural assignments presented in this figure are based on elution positions, relative to standard glycans, and were subsequently verified by MALDI-TOF/MS and NSI-MS.

FIGURE 3. Comparative quantification of pyridylaminated glycans by HPLC or of permethylated glycans by TIM or by MALDI-TOF/MS. A subset of glycans (indicated along the x axis) prepared from wild-type Drosophila embryos was quantifiable by TSK-amide HPLC of their pyridylaminated forms (see Fig. 2). The contribution of each glycan in this subset was determined by normalizing its signal intensity (ion current for MS or relative fluorescence for HPLC) to the summed signal for the entire subset. The mean ± S.D. for quantifications performed on three independent glycan preparations is shown for HPLC with fluorescence detection (dark gray bars), TIM (black bars), or MALDI-TOF/MS (light gray bars).

glycans again demonstrate the dominance of high mannose structures, whether PNGaseF or -A is used for release (Fig. 2). Preparative HPLC fractionation yielded pools containing major and minor pyridylaminated glycans that were subsequently analyzed by NSI-MS. Structural assignments for the glycans reported here are based on a combination of approaches, including susceptibility to release by PNGaseF or -A, elution from TSK-amide as pyridylaminated glycans, detection of parent ions by MALDI-TOF/MS, fragmentation of permethylated or pyridylaminated glycans by NSI-MS, and, as necessary, exoglycosidase digestion to distinguish isobaric glycans that were undifferentiated by CID fragmentation.

Recovery of N-Linked Glycans and Lower Limit of Detection—To assess our ability to characterize minor glycans, we determined the recovery, reproducibility, and sensitivity of our optimized methods. Bovine ribonuclease B was added to 5 mg of fly embryo powder, and pyridylaminated glycans were prepared from the mixture to assess recovery and reproducibility. The amount of ribonuclease B added as an internal spike was chosen to give glycan peaks by HPLC that were of approximately the same magnitude as those present in 5 mg of fly embryo powder. Recovery of glycans, calculated from peak height differences between spiked and unspiked material, was 100 ± 10% of expected, averaged across three different ribonuclease B glycans (M5N2, M6N2, and M8N2) from three independent determinations.

To assess the sensitivity of our glycan analysis by NSI-MS, we determined the lower limit of detection by MS, MS2, and MS3 for serially diluted glycans prepared from ribonuclease B or from fly embryo powder. The amount of glycan in the starting material was independently quantified by referencing the fluorescence intensity of released, pyridylaminated glycans to a labeled maltotetraose standard resolved by HPLC. The lower limit of detection at each MS level was taken as the lowest concentration of glycan at infusion that gave a signal-to-noise ratio of not less than 3-to-1. Following permethylation and release from ribonuclease B or from fly embryo powder, the lower limit of detection for the M5N2 glycan was 3 μM as parent ion [M + Na]⁺ for MS mode. For MS2 and MS3, permethylated M5N2 was detected as signature fragments at 3 pm and 3 nm, respectively. In almost all instances, MS3 was sufficient to define the structure of candidate glycans.

Our recovery and sensitivity measurements predict that we can detect glycans in fly embryo powder if they are present at a minimum concentration of 1 fmol per mg of protein. This abundance is equivalent to ~30 attomol per mg of embryo wet weight or ~2 × 10⁵ oligosaccharide molecules per embryo (one embryo weighs ~10 μg).

Quantification of pyridylaminated glycans separated by HPLC demonstrates that the M5N2 glycan is present at 97.4 ± 4.3 pmol per mg of embryo wet weight in overnight collections of wild-type embryos (mean ± S.D. for five independent glycan preparations). The sensitivity and selectivity of MS allows detection of permethylated glycans at a prevalence of almost 6 orders of magnitude below that of M5N2. However, the signal-to-noise ratio of MS for glycans present at less than 0.1% of M5N2 is below 3-to-1, precluding accurate quantification of extremely rare glycans. Nonetheless, relative quantification by MS compares well with absolute quantification by HPLC of pyridylaminated glycans (Fig. 3). Relative quantification of permethylated glycans by TIM reports composition similar to HPLC for the major, well resolved pyridylaminated glycans across a broad mass range, from the relatively small paucimannosidic glycan M2N2F (1142 Da) to the large high mannose-type glycan M9N2 (2398 Da).

The Minor N-Linked Glycans of the Drosophila Embryo Include Hybrid and Complex Structures That Are Sialylated or Mono- or Difucosylated—The predominance of high mannose and paucimannose glycans in the Drosophila embryo overwhelm attempts to detect minor glycans as pyridylaminated derivatives by HPLC, precluding the characterization of N-linked glycan diversity by this method alone. Furthermore, the relatively low TOF signals generated by minor Drosophila
Glycans impart an unacceptable level of uncertainty for structural assignment by one-dimensional mass spectrometry. The sensitivity and selectivity of multidimensional MS analysis provide a method to systematically screen for minor glycan structures.

Permethylated, unfractinated glycans were analyzed by TIM. For each collection window (2.8 mass units wide) in the TIM profile, MS² spectra were manually inspected for loss of characteristic masses. Loss of the reducing terminal monosaccharide as GlcNAc (Δm/z = −277) or as substituted GlcNAc (Δm/z = −451 for monofucosylated GlcNAc, Δm/z = −625 for difucosylated GlcNAc), or loss of nonreducing terminal GlcNAc from complex glycans (Δm/z = −259) generated strong daughter ions that were diagnostic for the presence of a glycan within the mass window. Further analyses of candidate glycans by subsequent MS³ or MS⁴ fragmentation of identified parent ion masses were performed as needed to determine structure (supplemental Figs. 2–5).

The structures of minor glycans were assigned based on multiple lines of evidence as follows: parent ion mass determined by TIM (and MALDI-TOF/MS in some cases); fragmentation of permethylated glycans by NSI-MS² up to MS⁴, HPLC elution, MS of pyridylaminated glycans, and sensitivity to endo- or exoglycosidase digestion. In addition, previously described specificities of Drosophila N-linked glycan synthetic enzymes place important limits on structural diversities. Glycans bearing one nonreducing terminal GlcNAc were assigned as products of GlcNAcT-I, placing the GlcNAc on the Manα1–3 arm of the trimannosyl core (15, 16, 47). Therefore, subsequent extension and capping reactions onto hybrid and nascent complex glycans were also assigned to the Manα1–3 arm. A GlcNAcT-V homologue capable of adding GlcNAc to the 6-position of the Man on the α1–6 arm does not exist in the Drosophila genome (48). Therefore, additional nonreducing terminal GlcNAc residues were assigned as products of GlcNAcT-II and/or GlcNAcT-IV enzymes; homologues of both are predicted in the genome sequence, and isobaric glycans indicative of both activities were detected. Trisubstituted Hex, diagnostic for the activity of the bisecting GlcNAcT-III enzyme, was not detected in fragmentation profiles for any of the permethylated glycans. Taken together, the direct detection of diagnostic fragments and known biosynthetic specificities allowed 38 of 42 structures to be confidently assigned (Fig. 4 and supplemental Table 1). For structures 19, 25, 40, and 42, extensions cannot currently be assigned to a particular arm.

The total glycan profile of the Drosophila embryo includes a series of non-, mono-, and difucosylated glycans that include paucimannose, hybrid, and complex structures (Fig. 4). The family of fucosylated, complex glycans include biantennary species capped with nonreducing terminal Gal residues, as well as triantennary glycans that terminate with GlcNAc. Biantennary glycans bearing a single GlcNAc on both the Manα1–3 and Manα1–6 arms of the trimannosyl core or bearing two GlcNAc residues on the Manα1–3 arm were detected. The relative prevalence of these isobaric species can be approximated by the ratio between the intensity of daughter ions that represent loss of disubstituted (Δm/z = −190) or monosubstituted (Δm/z = −204) Manα1–3 (see relative ratio of parent ions at m/z = 676 and 662 in supplemental Fig. 3). Nonfucosylated biantennary glycans were predominantly disubstituted on the Manα1–3 arm, whereas the difucosylated biantennary glycan (N2M3N2F2) was predominantly monosubstituted on both arms. Nonfucosylated biantennary glycans were mixed (Fig. 4).

Of particular interest to this study, seven glycans were identified that carry Fuc in α1–3 linkage to core GlcNAc and are therefore HRP epitopes. The diversity of HRP epitopes in the embryo includes not only the expected difucosylated, paucimannose glycan M3N2F2.6 (structure 27), but also the complex and extended hybrid, difucosylated glycans, NM3N2F2.6 (structure 28), GalNM3N2F2.6 (structure 29), and N2M3N2F2.6 (structure 30). The M3N2F2.6 glycan is the dominant HRP epitope in the embryo (Fig. 4). Two minor monofucosylated, paucimannose glycans carry α1–3-linked Fuc without α1–6-linked Fuc. The fucosylation positions of these monofucosylated species, M2N2F3 (isobaric with structure 10, see supplemental Table 1) and M3N2F3, (isobaric with structure 11, see supplemental Table 1) were verified by HPLC elution and by resistance to bovine kidney α1–6 fucosidase (49). Both the M2N2F3 and M3N2F3 Drosophila glycans were susceptible to digestion with this fucosidase (data not shown). Interestingly, the M2N2F3 glycan was released by PNGaseF, albeit with ~40-fold lower efficiency than by PNGaseA. As expected, however, the M3N2F3 glycan was resistant to PNGaseF. Together, the five difucosylated glycans account for 0.7% of the total glycan profile in the Drosophila embryo.

The hybrid glycan GalNM3N2 (structure 35) was detected in the following four forms: nonfucosylated, monofucosylated, difucosylated, or monosialylated (Fig. 4 and supplemental Table 1). Hybrid structures extended with Gal have not been described previously in Drosophila. Therefore, the linkage position and anomeric configuration of the nonreducing terminal Gal residue was probed in greater detail. Digestion of released glycans with S. pneumoniae β-galactosidase, which is specific for Gal linked β1–4 to HexNAc, resulted in the disappearance of the GalNM3N2 glycan, as detected by MS of the permethylated digest products (50). The result of the exoglycosidase digestion is consistent with the observed HPLC elution position of the pyridylaminated glycan (supplemental Table 1) and with the detection of a daughter ion derived from ring fragmentation at m/z = 329 (data not shown) by MS³, which distinguishes β1–4 from β1–3-linked Gal (51).

The hybrid and complex structures that we have detected are novel for Drosophila, but together account for only 12% of the total glycan pool (Fig. 4 and supplemental Table 1). Therefore, we analyzed a series of blank samples to demonstrate that the oligosaccharides reported here actually derive from the embryonic tissue from which they were prepared. Glycan analysis was performed on fly embryo powder without trypsin/PNGase treatment, on trypsin/PNGase reactions without fly embryo powder, and on preparations of the fly food matrix that were not inoculated with embryos. Analysis was performed by HPLC of pyridylaminated blank material, by MALDI-TOF/MS, by NSI-MS², and by TIM on pyridylaminated or permethylated blank material. The blank reactions failed to yield fluorescent peaks or parent or daughter ions attributable to any of the

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structures reported here. A ladder of hexose oligomer was detected in some samples, but this polyhexose is easily distinguished from N-linked glycan by fragmentation. We also observed the consistent presence of a non-glycan contaminant at m/z = 1452, which we were able to generate by performing blank permethylation reactions in the absence of any Drosophila-derived material.

Sialic Acid Is Linked α2–6 to a Type II LacNAc Acceptor—Two sialylated structures, SA-GalNM3N2 and SA-GalN2M3N2, were detected among the nonfucosylated, hybrid, or complex glycans in the Drosophila embryo (Fig. 4 and supplemental Table 1). Parent ions of the expected mass were detected and fragmented as singly charged ions following permethylation with [12C]methyl iodide (supplemental Fig. 5) or as doubly charged ions following permethylation with [13C]methyl iodide (Fig. 5). Fragmentation of the 12C-permethylated, singly charged parent at m/z = 1983 or of the 13C-permethylated, doubly charged parent ion of SA-GalNM3N2 at m/z = 1017 yielded daughter ions that demonstrate loss of sialic acid, as N-acetylneuraminic acid (m/z = 382 for 13C and 375 for 12C) from the nonreducing terminal (52). After loss of sialic acid, the remaining glycan fragments as the hybrid structure GalNM3N2. Masses consistent with other forms of sialic acid, such as N-glycolylneuraminic acid or ketodeoxynonulosonic acid, were not detected. The high pH associated with the permethylation reaction would be expected to strip O-acetyl groups from sialic acid, precluding the detection of this modification by our current methods.

Fly embryo powder was subjected to sialidase digestion prior to protease digestion and glycan release. Following digestion with the broad specificity sialidase from A. ureafaciens, which cleaves sialic acid-linked α2–3 or α2–6 to Gal (53), TIM analysis revealed a dramatic loss in the signature fragment ion for SA-GalNM3N2 (Fig. 6). The recovery of other nonsialylated glycans was unaffected. Incubation with the sialidase of S. pneumoniae, which is specific for α2–3-linked sialic acid, did not affect recovery of the SA-GalNM3N2 glycan (54). Therefore, considered along with our characterization of the GalNM3N2 core (see above), the sialylated glycans that we have detected in the Drosophila embryo carry sialic acid that is, at least predominantly if not exclusively, linked α2–6 to oligosaccharides terminated with Galβ1–4GlcNAc (type II LacNAc).

FIGURE 4. The N-linked glycan profile of wild-type Drosophila embryos. Glycans were released by PNGaseA from fly embryo powder glycopeptides that were prepared from overnight collections of wild-type (OreR) embryos. Following permethylation, glycan profiles were quantified by TIM. The prevalence of each major glycan (A) and minor glycan (B) is expressed as a percent of the total pool of detected glycans. The dashed line in B indicates the threshold for quantification (0.1% of total profile which corresponds to three times the background for TIM profiles; see Fig. 1). Numbers next to each bar are used consistently throughout all figures and tables to facilitate rapid comparison and identification of the same glycan structure. For instances in which isobaric components were quantified together (structures 21, 25, 30, 36, and 40), the approximate relative contribution of each species is indicated (<or >), as determined by the ratio of signature daughter ions detected in MS^n analysis (see supplemental Fig. 2). Insufficient signal was generated to distinguish between the isobaric species shown for structure 42. The data represent the mean ± S.D. for determinations performed on three independent glycan preparations. Difucosylated glycans recognized as HRP epitopes are indicated by the orange box. Glycan structures are assigned based on parent ion mass, MS^n fragmentation, HPLC elution position, and exoglycosidase digestion.
Four independent lines of evidence derived from our results support the presence of sialylated glycans in the *Drosophila* embryo. First, the elution times of pyridylaminated candidate glycans from TSK-amide are consistent with the proposed structures. Second, by NSI-MS the m/z values observed for charge states and 12C or 13C differentially permethylated forms are consistent with the proposed structures. Third, fragmentation by CID demonstrates both the loss of sialic acid alone from the parent ion, and also the generation of daughter ions that are consistent with loss of sialic acid in combination with other expected residues. Fourth, the sialic acid is sensitive to linkage-specific removal by exoglycosidase digestion.

The Prevalence of Complex Glycans Increases during Embryonic Development—Expression of the HRP epitope in the *Drosophila* embryo is first apparent as faint anti-HRP antibody staining early in stage 11. At this stage, neural precursor cells have just begun to differentiate and segregate from the ectoderm in which they originate. Therefore, the temporal boundary between late stage 10 and early stage 11 is potentially of interest for investigating the ontogeny of tissue-specific glycan expression. The stage 10/11 boundary occurs, on average, at 6 h after egg laying for embryos raised at 25 °C. We collected embryos from 0 to 6 h after egg laying (early) and from 6 to 24 h after egg laying (late) for glycan analysis (Fig. 7, A and B). To facilitate relative quantification of glycan differences between the two developmental periods, glycans released from early embryos were permethylated with [12C]methyl iodide and glycans released from late embryos were permethylated with [13C]methyl iodide. Following permethylation, the labeled glycans were mixed together on an equal protein basis and analyzed by NSI-MS and by TIM (Fig. 7C).

Glycan profiles of early embryos are distinguished from those of late embryos by four major differences (Fig. 9, A–C, and supplemental Tables 2 and 3). The first major difference is that early embryos contain less total glycan per mg of protein than late embryos. Among the predominant high mannose glycans,
the difference in abundance ($^{12}$C/$^{13}$C ratio) is generally between 2- and 3-fold. However, the M9N2 glycan is reduced by almost 5-fold in early embryos. The general increase in glycan abundance in later developmental stages may reflect a depletion of maternal components during early embryogenesis that is reversed by the induction of zygotic glycosylation in later embryonic stages. Three additional differences between early and young embryos are apparent upon comparing the prevalence of individual glycans expressed as a percent of the total profile. The glycan profiles of early embryos are enriched in high mannose glycans (structures 4, 5, and 7 in Fig. 9A) and in glycans that bear nonreducing terminal GlcNAc residues without further extension (structures 33, 34, 36, and 38 in Fig. 9B and structures 20 and 22 in Fig. 9C). Late embryos are enriched in glycans that have undergone further processing, such as mono- and difucosylation (structures 11 and 12 in Fig. 9, A and B, respectively, as well as most glycans lying above the normal line in Fig. 9C), Gal extension of hybrid or biantennary acceptors (structures 18, 19, and 40 in Fig. 9C), or additional branching to form triantennary structures (structure 39 in Fig. 9C). Although the prevalence of the two sialylated glycans (structures 41 and 42) is very low in young and old embryos, quantification of the relative abundance by $^{12}$C/$^{13}$C ratio indicates that both increase during normal development.

**Loss of tollo/toll-8 Affects Core Fucosylation and the Generation of Minor Complex Glycans**—By anti-HRP antibody staining, tollo/toll-8 mutant embryos lack glycans bearing $\alpha$-3-linked Fuc in neural tissue. A small number of non-neural tissues that normally express the HRP epitope continue to do so in tollo/toll-8 mutants (Fig. 8, A and B). However, the extent to which loss of Tollo/Toll-8 function might impact the expression of other glycans has been unknown. Differential labeling with light and heavy methyl iodide was employed to investigate the differences in glycan expression between wild-type and tollo/toll-8 mutant embryos (Fig. 8C).

Analysis of glycan profiles reveals five significant changes in glycan expression in tollo/toll-8 mutants (Fig. 9, D–F, and supplemental Tables 2 and 3). First, tollo mutant embryos contain less total glycan per mg of protein than wild-type embryos ($^{12}$C/$^{13}$C ratio). However, the difference in total glycan content is not as great as between young and old embryos. Second, as predicted by anti-HRP antibody staining, difucosylated glycans are dramatically decreased (structures 26, 27, 28, and 30 in Fig. 9F), whether expressed as relative abundance ($^{12}$C/$^{13}$C ratio) or as prevalence (percent of total profile). Third, the prevalence of many of the minor glycans that bear $\alpha$-1–6-linked Fuc is reduced (structures 12 and 15 in Fig. 9E and structures 13, 14 and 18 in Fig. 9F). Despite this reduction in minor $\alpha$-1–6 fucosylated glycans, the prevalence of M9N2 is decreased (structure 11 in Fig. 9D), the predominant $\alpha$-1–6 fucosylated glycan in the embryo, is relatively unaffected in the tollo mutant. Fourth, M3N2 (structure 2 in Fig. 9D), a major paucimannose glycan, is increased in prevalence, along with the levels of a subset of minor nonfucosylated biantennary glycans (structures 36, 37, and 38 in Fig. 9E). Fifth, similar to young embryos, lower levels of nonfucosylated hybrid and triantennary glycans were detected in tollo mutants (structures 35 and 39 in Fig. 9F). In contrast to the glycan profile of young embryos, a smaller sub-
FIGURE 7. The relative abundance of N-linked glycans in early and late embryos. N-Linked glycans were prepared from populations of embryos aged between 0 and 6 h after egg laying (A) or from embryos aged 6 to 24 h after egg laying (B). Representative morphologies for embryos at the beginning (left embryo) or near the end (right embryo) of each age range are presented. The early population spans stages from cellularization to the completion of gastrulation. The late population begins with early neurogenesis in the extended germ band stage and concludes with fully formed embryos ready for hatching. Glycans prepared from early or late embryos were permethylated with [12C]- or [13C]methyl iodide, respectively. Before analysis by NSI-MS or by TIM, the permethylated glycans were mixed together in amounts that were determined by the content of protein in the starting material. The relative abundance of each glycan in early and late embryo preparations is quantified by the peak heights of the respective 12C- or 13C-permethylated forms. Early embryos possess significantly less of all the major glycan species per mg of protein. The asterisks indicate contaminants at m/z = 1452 for 12C-methylation and at m/z = 1472 for 13C-methylation that are not derived from Drosophila material. Structural assignments shown in the figure are based on MS² fragmentation.

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set of GlcNAc-terminated glycans was observed to be enriched (compare structures 33 and 34 in Fig. 9, B and E).

DISCUSSION

Previous studies of Drosophila N-linked glycans have documented the abundance of the high mannose oligosaccharides, M3N2-M9N2, and a single fucosylated paucimannose glycan, M3N2F, in adult and larval tissues (15, 17, 25, 27, 55). Minor paucimannose glycans with additional core fucosylation have also been characterized (16, 21). We have focused on determining the full diversity of major and minor glycans in the embryo. The results presented here demonstrate that the Drosophila embryo is capable of expressing extended hybrid and complex N-linked glycans, in addition to the expected family of abundant high mannose and fucosylated paucimannose oligosaccharides. Most notably, the presence of sialic acid, both on a hybrid and on a complex core, demonstrates that the Drosophila embryo possesses the biosynthetic capacity to generate complex biantennary glycans that are almost as diverse as the analogous vertebrate structural class. A recent publication corroborates the existence of complex glycans in the Drosophila embryo (24).

At least two differences distinguish the total N-linked glycan diversity of Drosophila from that of most vertebrates. First, compared with their prevalence in vertebrates, complex Drosophila glycans are minor components of the total glycan pool (8, 56). The relative under-representation of these structures agrees with the expression patterns of Drosophila glycosyltransferases that have been demonstrated or predicted to synthesize complex N- or O-linked glycans (57–59). For example, the only characterized sialyltransferase in Drosophila is expressed in a very small subpopulation of neurons, beginning in the late embryo, consistent with the very low levels of sialylated N-linked glycan that we detect (29). Drosophila embryos appear to restrict the elaboration of complex glycans to limited temporal and spatial domains, where they may fulfill very specific developmental purposes. Second, we were unable to detect tetra- or penta-antennary complex glycans in Drosophila embryos. This deficit may reflect a true difference between Drosophila and vertebrates. The CAZy data base suggests the existence of Drosophila proteins with structural similarity to the GlcNAcT-III bisecting enzyme (CAZy family GT17), but a corresponding enzyme activity has not been identified, and there is, as yet, no clear Drosophila GlcNAcT-V candidate (48). If these activities exist in Drosophila, they generate glycans at levels that fall below our detection threshold.

Recently, the analysis of partially methylated alditol acetates (PMMA) derived from Drosophila embryo N-linked glycans has reported the presence of 2,6-substituted Man, indicative of GlcNAcT-II and GlcNAcT-V activity (24). In the same analysis, 2,4-substituted Man, indicative of GlcNAcT-I and GlcNAcT-IV activity, was not detected. This restrictive profile of terminal GlcNAc substitutions, preferentially transferred onto the α1–6 arm of the trimannosyl core, disagrees with our data in two respects. First, our fragmentation demonstrates that a subset of the hybrid glycans in Drosophila embryos can be extended with two terminal GlcNAc residues on a single arm (structures 22, 23, 37, and 38). The presence of a disubstituted Man on a hybrid structure requires that GlcNAcT-I and GlcNAcT-IV must add onto the α1–3 arm, generating the 2,4-substitution. Second, our fragmentation data also report the
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FIGURE 8. The relative abundance of N-linked glycans in tollo/toll-8 mutant and wild-type embryos. Glycans recognized by anti-HRP antisera are missing from neural tissue in tollo/toll-8 mutants (A) but clearly define the ventral nerve cord in wild-type embryos (B). The deficiency is first apparent shortly after neurogenesis is underway, ~6 h after egg laying (early stage 11, left embryos in A and B). By early stage 14 (right embryos in A and B), the distinct and well formed ventral nerve cord lacks the HRP epitope in the mutant. Following differential isotopic labeling, 12C-labeled glycans from tollo/toll-8 mutants were mixed with 13C-labeled glycans from wild-type (OreR) embryos based on protein content of the starting material. Among the major glycans, almost all are slightly reduced per mg of protein in the mutant. However, many minor mono- and difucosylated glycans are reduced substantially (see Fig. 9, D–F, and supplemental Tables 2 and 3). Asterisks indicate contaminants as in Fig. 7. Structural assignments shown in the figure are based on MS n fragmentation.

presence of biantennary, complex glycans with two GlcNAc termini on one arm (structures 21, 30, 36, 40, and 42). To generate a complex, biantennary glycan with both GlcNAc extensions on the α1–6 arm, the glycan must begin as a triantennary product of GlcNAcT-I, -II, and V. Subsequent removal of the GlcNAc on the α1–3 arm would generate a glycan with α2,6-substituted Man. This triantennary degradation route requires an additional synthetic step and the existence of an enzyme apparently missing from the genome (GlcNAcT-V), in contrast to the straightforward production of a biantennary glycan with 2,4-substituted Man on the α1–3 arm.

The reported fractional recovery of PMAA as 2,6-substituted Man implies that a major portion, perhaps 20%, of the total N-linked glycan profile of the Drosophila embryo should carry disubstituted Man on the α1–6 arm of the tri-mannosyl core (24). Our analysis indicates that these structures account at most for 2.1% of the total profile, even if all of the biantennary or triantennary candidates were exclusively 2,6-substituted. Furthermore, we note that 2,6-substituted Man is an expected component of the high mannose glycans of bakers' yeast, a common food source contaminant of Drosophila embryo preparations that are not subjected to dechorionation with hypochlorite before extraction (60). Therefore, the lack of detected 2,4-substituted Man in the previously reported PMAA analysis likely reflects nonrepresentative recovery of the full diversity of monosaccharides that constitute Drosophila embryo glycans.

We characterized the sensitivity of our analyses by determining the minimum amount of glycan required to give informative MS n fragmentation. Given the signal-to-noise ratios obtainable by NSI-MS n, we determined our lower limit of detection to be 3 pm for a single glycan at infusion. Considering our standard extraction and sample work-up volumes, this corresponds to ~2 × 10^5 molecules of a single oligosaccharide per embryo. By mid- to late-development, the Drosophila embryo consists of 33,000–65,000 cells (61). Therefore, our lower limit of sensitivity predicts that we should be able to detect glycans that are found at ≥5 copies/cell if expressed uniformly throughout the embryo and at ≥10,000 copies/cell if expressed on as few as 20 cells. Although exceedingly minor species may still remain to be detected, we propose that our analysis has laid out the general boundaries for the full diversity of N-linked glycans in the Drosophila embryo.

Genetic, biochemical, and immunochemical data have demonstrated the presence of GlcNAcT-I in Drosophila, a minimal requirement for the synthesis of paucimannose and complex glycans (15, 47). GlcNAcT-I activity is prerequisite for core α1–6 Fuc addition and our analysis of glycan expression in embryos indicates the presence of M3N2F6 early in development. The fucosyltransferase that catalyzes the core addition of α1–3 Fuc (FucTA) also requires the previous action of GlcNAcT-I (16, 21, 62, 63). Embryos that lack GlcNAcT-I fail to make the HRP epitope, which first becomes apparent early in stage 12 concomitant with neural differentiation (15). The early presence of 6-linked Fuc and the subsequent appearance of 3-linked Fuc indicate that GlcNAcT-I activity is expressed throughout embryogenesis, providing the appropriate substrates for subsequent elaboration of complex glycans.

The relative paucity of complex glycans in Drosophila has been attributed, at least partially, to the presence of an active hexosaminidase in the secretory pathway of many arthropod cells (64, 65). Encoded by the fused lobes (fdl) gene in Drosophila, the enzyme removes GlcNAc added by GlcNAcT-I in β1–2 linkage to the Mano1–3 arm of M5N2 core glycans (16). Removal of terminal GlcNAc by Fdl requires processing of the NM5N2 glycan to NM3N2 by α-mannosidase II and blocks
Changes in glycan prevalence and abundance during normal development and in tollo/toll-8 mutant embryos. The prevalences of individual glycans were calculated as a percent of the total glycan profile for wild-type (OreR) embryos harvested from early (0 – 6 h) or late (6 – 24 h) stages in development (A–C) and for overnight collections (0 – 18 h) of tollo/toll-8 mutant or wild-type embryos (D–F, and supplemental Tables 1 and 2). For each glycan, the prevalence in early embryos is plotted relative to its prevalence in late embryos (A–C), or the prevalence in tollo/toll-8 mutants is plotted relative to its prevalence in OreR wild-type embryos (D–F). For both comparisons, the full profile of glycans is presented in three ranges as follows: major glycans (A and D), less abundant glycans (B and E), and minor glycans (C and F). Data represents the mean ± S.D. for determinations performed on three independent glycan preparations for each sample. Numbers associated with data points refer to structures illustrated in Fig. 4. The gray zone in each panel describes the average error of the data set plotted in that panel as a range above or below the diagonal (dotted line indicates no difference in prevalence between preparations). C and F, the gray square bounded by 0.1% total profile on both axes indicates the lower limit of quantification for the method. Glycans that lie within this box were detectable and fully characterized by MS but could not be quantified because their peak responses by TIM were ≤3 times background. To improve the clarity of the data presentation, the prevalence of such glycans has been allowed an additional significant figure, which serves to spread these points within the gray box. The prevalence data set indicates changes in the distribution of glycans within the total glycan profile. The relative abundance of each glycan was determined by differential isotopic labeling (see Figs. 7 and 8 and supplemental Table 3) and is coded by the color of the data point referenced to the 12C/13C ratio scale at the bottom of the figure. Relative abundance was calculated from 12C-early/13C-late or from 12C-tollo/13C-wild-type preparations normalized to the protein content of each embryo preparation. Therefore, color values below 1.0 on the ratio scale indicate less glycan per mg of protein in early (A–C) or tollo embryos (D–F) relative to late or wild-type, respectively. Total glycan per mg of protein is lower in early embryos than in late and, to a lesser extent, in tollo than in wild-type, resulting in many color-coded data points that indicate relative abundances less than 1.0. In general, glycans with the lowest relative abundance are also above the diagonal line, and glycans with relative abundances approaching or exceeding 1.0 are at or below the diagonal line. For minor glycans whose TIM peaks are below the threshold of quantification (within the gray box in C and F), relative abundances were determined by the ratio of signature MS/MS fragments. The enhanced signal-to-noise in MS/MS allows the relative abundance measurement to be significant even though the prevalence is below threshold. Arrows (C and F) indicate glycans that are recognized by anti-HRP antibody. These difucosylated glycans exhibit the greatest increase in prevalence and abundance during development and the greatest decrease in the tollo/toll-8 mutant.
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subsequent extension reactions, leaving a predominance of paucimannose glycans on glycoproteins (66). *Drosophila* adults with reduced Fdl activity exhibit glycan profiles that are shifted toward the production of more complex structures, primarily NM3N2 and N2M3N2 with or without core fucosylation (16).

Although Fdl activity has not been investigated in the embryo, progressive down-regulation of this enzyme would permit the elaboration of complex glycans as *Drosophila* embryogenesis proceeds. In fact, the ratio of predicted Fdl products (the sum of the percent of total profile for structures 1, 2, 10, 11, 26, and 27) to Fdl substrates (the sum of the percent of total profile for structures 14, 15, 28, 31, and 32) is 50% higher in young embryos than in old embryos, consistent with decreasing Fdl activity during development. Some GlcNAc-terminated glycans are enriched in young embryos, but these glycans are not expected substrates for Fdl. Instead, they predict that α-mannosidase II activity may be lower in early development (structures 22, 33, 34, and 38) and that GlcNAcT-II/IV activities are present (structures 20, 22, 36, and 38) in the early embryos. Therefore, the accumulation of complex, extended glycans during *Drosophila* development requires coordinated changes in early processing steps that generate NM3N2 from M5N2, in addition to the up-regulation of glycosyltransferases that captures GlcNAcT-I products for further elaboration.

Following commitment to the complex pathway, glycan extension usually proceeds through the addition of Gal residues to GlcNAc-terminated cores. A total of 10 *Drosophila* genes, which fall into three CAZY families (GT7, -31, and -32), are candidate enzymes for catalyzing Gal addition in β3/4 linkage to GlcNAc. Three candidates add Gal in β3 linkage to elongate mucin type, O-linked core-1 or glycosphingolipid glycans (67). Five additional βGal transferase genes predict enzymes with greater similarity to the β3Gal transferase family than to the β4 family, perhaps predicting the presence of multiple synthetic pathways for O-linked or glycosphingolipid extension (34, 68). Expressed as recombinant proteins, two other Gal transferase candidates (β4GalNAcTα and β4GalNAcTβ) transfer GalNac rather than Gal in β4 linkage to a GlcNAcβ1-p-nitrophenyl acceptor, despite their sequence similarity with vertebrate β4Gal transferases (57). However, the *Trichoplusia ni* (lepidopteran) β4GalNAc transferase that is homologous to the *Drosophila* β4GalNAcTα/B enzymes can utilize UDP-Gal as a donor *in vitro*, albeit with reduced efficiency compared with UDP-GalNac (68).

Transfer of GalNac to nonreducing terminal GlcNAc residues would generate the LacdiNac structure (GalNacβ1–4GlcNAc), which we are unable to detect in embryo preparations. Our analysis of HPLC elution times (as pyridylaminated conjugates separated on TSK-amide) and fragmentation of per- methylated glycans demonstrate that all of the distal HexNAc residues exist either as a nonreducing terminal residue linked to subterminal hexose (Man) or as a subterminal residue capped by hexose (Gal). Furthermore, our exoglycosidase digestions assigned the terminal Gal linkage on *Drosophila* embryo N-linked glycans as β1–4. Therefore, the insect β4GalNAcT enzymes may be bifunctional with respect to preferred donor nucleotide sugar. This bifunctionality may be regulated in favor of a specific donor *in vivo*, perhaps through interactions with as yet unidentified allosteric factors that function analogously to mammalian lactalbumin and its ability to alter mammalian βGalT1 specificity (69). Confident assignment of specific Gal transferase(s) to the synthesis of the glycans that we have detected in embryos will require further characterization of enzyme specificities.

The absence of the LacdiNac structure among the glycans that we have detected in embryos is interesting in light of the specificity previously determined for the single known *Drosophila* sialyltransferase (dSiaT). Recombinant dSiaT transfers N-acetylmuramidic acid in α2 – 6 linkage to a LacdiNac acceptor with ~2-fold greater activity than to the Gal-terminated type II LacNac structure, Galβ1–4GlcNAc (29). Consistent with the linkage specificity described for dSiaT, the sialylated glycans that we detect in embryos are sensitive to the broad specificity sialidase of *A. ureafaciens* but resistant to the α2–3-specific sialidase of *S. pneumoniae*. For dSiaT, the efficiency of transfer to LacdiNac, type II LacNac, and lacto-N-neotetraose differed by less than 3-fold, indicating that structures terminated with Galβ1–4GlcNAc are likely to be viable substrates for dSiaT. The *in vitro* preference of dSiaT for LacdiNac acceptors may be better reflected in the sialylation of larval, pupal, or adult glycans, perhaps in concert with a coordinated shift in the donor substrate specificity of the β4GalNAcTα/B enzymes in these later developmental stages.

The total profile of N-linked glycans in the wild-type embryo exhibits an inverse correlation between extent of fucosylation and degree of complexity. The nonfucosylated glycans include hybrid, complex, and triantennary structures; both of the sialylated glycans were also determined to lack core Fuc. On the other hand, difucosylated glycans (or any monofucosylated glycans bearing only α1–3-linked Fuc) were predominantly paucimannose or hybrid. The structural diversity of the fucosylated and nonfucosylated glycans is consistent with a model in which sequential fucosylation reactions limit the accessibility of the NM3N2 core for subsequent elongation (Fig. 10). Such limits may be imposed stochastically, rather than determined structurally, perhaps reflecting the sorting of acceptors or sequestration of transferases into unique Golgi compartments (70). The apparent correlation between fucosylation and glycan complexity might also reflect tissue-specific or temporal regulation of fucosylation that is not resolved by our whole embryo analysis.

In individual embryonic tissues, multiple regulatory mechanisms contribute to the tissue-specific expression of various glycan classes during development. Our differential glycan analysis indicates that the activity of the Tollo/Toll-8 protein increases α1–3 and α1–6 core fucosylation and the expression of hybrid glycans. Based on anti-HRP antibody staining, it was expected that α1–3 fucosylation would be deficient in *tollo/toll-8* mutants, but it was not expected that the prevalence of glycans bearing α1–6-linked Fuc would also be affected. Therefore, Tollo/Toll-8 may coordinate multiple activities that broadly impact N-linked core fucosylation, including the synthesis and transport of GDP-Fuc or the expression of appropriate Fuc transferases. Loss of the HRP epitope in *tollo/toll-8* mutants may be, at least in part, a secondary effect that arises from a neural specific decrease in α1–6 fucosylation. This
interpretation is consistent with the reported specificity of Drosophila FucTA, an α1–3-fucosyltransferase that can generate the HRP epitope (21). Recombinant FucTA exhibits a 4-fold higher acceptor preference in vitro for N2M3N2F6 over N2M3N2, suggesting that α1–6-linked Fuc may facilitate subsequent α1–3 fucosylation. Alternatively, tissue-specific increases in Fdl hexosaminidase activity could also contribute to local reductions in fucosylated, hybrid, and complex glycans. In fact, the ratio of predicted Fdl products to Fdl substrates is 40% higher in tolo/toll-8 mutants than in wild-type embryos, consistent with the interpretation that tolo/toll-8 activity influences multiple steps leading to tissue-specific glycosylation (Fig. 10).

It has long been debated whether Drosophila, and other insects, possess the capacity for synthesizing glycans other than high mannose or paucimannose type (30, 71). The identification and characterization of key synthetic and processing enzymes in Drosophila (GlcNAcT-I, Fdl, dSiaT, N-acetylneuraminic acid phosphate synthase, and CMP-sialic acid synthase), coupled with the expanding genomic annotation of relevant Drosophila genes, have built an increasingly convincing argument that complex glycans should be present in this organism (72, 73). Also, extensive efforts to bioengineer mammalian-like glycosylation into insect cell lines have demonstrated that dipteran and lepidopteran cells can accommodate the components necessary for complex glycan synthesis (74, 75). Several earlier reports presented indirect evidence for sialylated glycans in insects by antibody and lectin binding, but the data presented here provide the first direct demonstration of complex, sialylated N-linked glycans in Drosophila (31, 76–79). Our results demonstrate that characterization of the expressed glycans isolated from a small collection of mutant embryos (<50 mg wet weight) can indicate potential targets for regulation by developmentally relevant genes. With a clear understanding of the range of N-linked glycan diversity in the Drosophila embryo, it will now be possible to assess whether many interesting mutations exert phenotypic effects through altered glycan expression.

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