Sugar-free frosting, a homolog of SAD kinase, drives neural-specific glycan expression in the Drosophila embryo

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SUMMARY
Precise glycan structures on specific glycoproteins impart functionalities essential for neural development. However, mechanisms controlling embryonic neural-specific glycosylation are unknown. A genetic screen for relevant mutations in Drosophila generated the sugar-free frosting (sff) mutant that reveals a new function for protein kinases in regulating substrate flux through specific Golgi processing pathways. Sff is the Drosophila homolog of SAD kinase, which regulates synaptic vesicle tethering and neuronal polarity in nematodes and vertebrates. Our Drosophila sff mutant phenotype has features in common with SAD kinase mutant phenotypes in these other organisms, but we detect altered neural glycosylation well before the initiation of embryonic synaptogenesis. Characterization of Golgi compartmentation markers indicates altered colocalization that is consistent with the detected shift in glycan complexity in sff mutant embryos. Therefore, in analogy to synaptic vesicle tethering, we propose that Sff regulates vesicle tethering at Golgi membranes in the developing Drosophila embryo. Furthermore, neuronal sff expression is dependent on transcellular signaling through a non-neural toll-like receptor, linking neural-specific glycan expression to a kinase activity that is induced in response to environmental cues.

KEY WORDS: Glycosylation, Golgi, Nervous system, Drosophila

INTRODUCTION
Essential aspects of neural development and function rely on the regulated expression of specific glycan structures. The involvement of glycans in neural cell function begins with neur ectoderm differentiation, in which the elaboration of specific glycan structures on the Notch glycoprotein facilitates the differential ligand activation that drives cell fate selection (Okajima and Irvine, 2002). Subsequently, glycans expressed in developing and mature neural tissues impinge on other important cell signaling, migration, adhesion and synaptic functions (Matani et al., 2007). Several underlying molecular mechanisms for glycan function are well characterized, including the influence of N-linked polysialic acid on neural cell adhesion molecule (NCAM) interactions, the engagement of axonal glycosphingolipid glycans by a carbohydrate-binding protein on oligodendrocytes, and the activity-dependent deposition of glycosaminoglycans into perineuronal nets (Acheson et al., 1991; Matthews et al., 2002; Vyas et al., 2002). These instances, and others, reveal the prevalence of temporally and/or spatially regulated glycan expression. This is further supported by the existence of many anti-glycan antibodies (HNK-1, CAT, Jones, A2B5, FORSE-1, anti-HRP) that delineate subpopulations of neuronal or glial cells at various stages of development (Allendoerfer et al., 1999; Constantine-Paton et al., 1986; Jan and Jan, 1982; Kruse et al., 1984; Matthews et al., 2002; Sanes et al., 1986; Schnitzer and Schachner, 1982; Snow et al., 1987). Furthermore, aberrant glycan expression in humans is associated with various mild to severe pathologies (Dennis et al., 2009; Freeze, 2002; Lee et al., 2007; Michele and Campbell, 2003; Zak et al., 2002). Despite the overwhelming evidence for regulated glycan expression and for the significant consequences of disregulated glycan expression, the mechanisms that govern tissue-specific glycosylation are unknown, particularly during embryonic development.

We have previously reported that a mutation in an ectodermally expressed Toll-like receptor, Tollo, alters N-linked glycosylation in the developing Drosophila embryonic nervous system (Seppo et al., 2003). This defect was detected as loss of staining with antibodies that recognize a family of structurally related N-glycans, known as HRP epitopes, which are normally expressed in a restricted set of embryonic tissues (Jan and Jan, 1982; Snow et al., 1987). The Tollo mutation specifically abolishes HRP-epitope expression in neural tissue although Tollo is not expressed in neural cells that carry HRP epitopes. Rather, it is expressed and functions within non-neural ectodermal cells that surround differentiating neurons, establishing the basis for a transcellular paracrine signaling pathway that drives neuron-specific glycosylation (Seppo et al., 2003). Such transcellular signaling might drive cell-specific glycan expression through altered transcription of glycan biosynthetic genes (glycosyltransferases, glycan processing enzymes, etc.) or through mechanisms that modify trafficking through specific glycoprotein processing pathways. The relative contribution of altered transcription and altered cellular organization to tissue-specific glycan expression is completely unresolved in any biological context. To address this lack of knowledge and to identify the unknown components of the Tollo transcellular signaling mechanism, we undertook a random mutagenesis screen for genes that specifically affect HRP-epitope expression in the Drosophila embryo. Here, we describe an informative mutation recovered from this screen called sugar-free frosting (sff), the phenotype of which reveals the importance of regulated Golgi dynamics for neuron-specific glycan expression. Our
sff mutation, which is the first described disruption of a Drosophila homolog of SAD kinase, interacts genetically with Tollo and modulates glycan complexity in neurons that are receptive to the Tollo transcellular signaling pathway (Crumpl et al., 2001; Inoue et al., 2006; Kishi et al., 2005). Our results lead us to propose a new paradigm in which tissue-specific glycan expression is sculpted by the relative activities of multiple protein kinases, each acting to facilitate flux through specific Golgi processing pathways.

**MATERIALS AND METHODS**

**Reagents**

Probes for immunohistochemistry and immunofluorescence used were: rabbit anti-HRP (1:2000 for embryos, 1:1000 for larvae), HRP-Concanavalin A (ConA; 1:100), HRP-conjugated goat anti-rabbit (1:1000) and goat anti-mouse (1:1000) antibodies from Jackson Laboratories; monoclonal antibodies CD4 (anti-Fas2; 1:3), nC82 (anti-Brp; 1:100) and 22C10 (1:5) from the Developmental Studies Hybridoma Bank (DHSB, University of Iowa, IA, USA); biotin-conjugated PNA (peanut lectin; 5 μg/ml) obtained from Vector Laboratories; anti-GM130 (1:1000) monoclonal antibody obtained from Abcam; Alexa-conjugated secondary antibodies (Alexa 488, 568 and 633; 1:500), rabbit anti-GFP (cross-reactive with YFP; 1:5000) and PROLON anti-fade obtained from Molecular Probes; TRITC-Phalloidin (1:100) obtained from Invitrogen. PNGaseA was from Calbiochem; trypsin and chymotrypsin were from Sigma.

**Drosophila mutagenesis and transgenese**

Males of genotype w^1118^; +; + were treated with 25 mM ethyl methanesulfonate and mated en masse to females of the genotype w^1118^; Kr/CyO; D/TM6b. Individual male progeny heterozygous for the second and third chromosome markers were backcrossed to w^1118^; Kr/CyO; D/TM6b females to create lines that carried mutagenized second or third chromosomes. Progeny from each individual line were sib-mated to give embryo collections that were stained with anti-HRP antibody. Lines showing altered HRP-epitope expression were subsequently stained with Concanavalin A. Lines that were deficient in anti-HRP staining but normal for Concanavalin A staining were from Sigma.

**Glycan analysis**

Total N-glycans were prepared from overnight embryo collections (25°C) by PNGaseA digestion of trypsin/chymotryptic peptides as previously described (Aoki et al., 2007). Following permethylation, total glycan profiles were acquired by mass spectrometry (MS) using nanospray ionization interfaced to a linear ion trap instrument (NSI-MS² on an LTQ Ion Trap, Thermo-Fisher). The Total Ion Mapping (TIM) functionality of the instrument control and data acquisition software (Xcalibur, v2.0) was used to acquire full MS and MS/MS spectra (~700 spectra per sample), which were manually interpreted to assign glycan structures and to quantify relative glycan prevalence. Ambiguities were resolved by acquisition of spectra at MS²-MS³ as needed.

**Proteomic analysis by liquid chromatography with tandem mass spectrometry (LC-MS/MS)**

Proteins from adult head lysates were reduced, alkylated, digested with sequencing grade trypsin (Promega) and subjected to proteomic analysis by LC-MS/MS (Lim et al., 2008). Two-dimensional RP/RP-HPLC (reverse phase/reverse phase high pressure liquid chromatography) was performed to separate the total peptide mixture into five fractions (F1-5) based on hydrophobicity. Fractionated peptides were loaded off-line onto a nanospray tapered capillary column emitter and LC-MS/MS analysis was performed on an LTQ Orbitrap mass spectrometer (Discovery, Thermo-Fisher) with a Surveyor MS pump plus (Thermo Scientific). As the five RP-fractions each contain peptide pools that possess different average hydrophobicities (increasing from F1-5), separate gradient elution conditions were employed for LC-MS/MS analysis of each fraction (see Fig. S6 in the supplementary material) in order to maximize peptide resolution. MS/MS spectra (36% normalized collision energy) were triggered upon detection of any mass-to-charge ratio (m/z) signal that corresponded to any entry into a parent mass list of theoretical CG6114 trypptic peptides, allowing for up to 2 missed cleavages, oxidized methionine, alkylated cysteine and charge states +2–4. MS/MS data was searched against the Fly database (Drosophila melanogaster, 8-31-09) from Swiss Prot to which CG6114 was added, and against the reverse of this.
linked fucose (Fuc) on the innermost N-acetylglucosamine (GlcNAc) arm of the third chromosome. Deletion mapping in the interval between \( sff^B22 \) and the left end of the chromosome identified a single deletion (\( \Delta brm^{11} \)) that failed to complement the \( sff \) mutation. Embryos of the genotype \( sff^{B22}/\Delta brm^{11} \) displayed a more severe HRP phenotype than did \( sff \) homozygotes, and \( \Delta brm^{11} \) deletion homozygous embryos lacked all HRP epitopes, indicating that the \( sff^{B22} \) allele is hypomorphic with regard to the induction of HRP-
neural driver, elav-Gal4. The UAS-CG6114 transgene was sufficient to rescue neuronal HRP-epitope expression in sff\textsuperscript{B22} embryos (Fig. 2G,H).

**Sff is expressed in embryonic neurons and is decreased in the sugar-free frosting mutant**

By in situ hybridization, mRNA for sff was detected in the embryonic ventral nerve cord and peripheral sensory clusters in the ventral and lateral ectoderm (Fig. 3A-E) (Kopczyński et al., 1996). If present in the hindgut or garland gland, sff mRNA was below the current detection threshold for our in situ analysis. Therefore, despite complete loss of HRP epitopes in A\textsuperscript{brm} homoygotes (see Fig. 2E,F), additional tissue-specific regulatory mechanisms are likely to contribute to HRP-epitope expression in non-neural tissues. Consistent with our genetic characterization of the sff\textsuperscript{B22} allele as a hypomorph, mRNA for sff was reduced, but not eliminated, in the mutant nervous system (Fig. 3B,E, quantified in Fig. 4). The cells expressing sff mRNA were neuronal as hybridization signal was detected within cells that were also positive for the neuron-specific mAb 22C10 (see Fig. S4 in the supplementary material). Expression of sff mRNA was first visible along the midline of the embryo at stage 11, prior to the initiation of synapse formation in the embryo and roughly coincident with the initial appearance of HRP epitopes and transcripts for FuCTA, the α\textsubscript{3}-fucosyltransferase that synthesizes HRP epitopes (see Fig. S5 in the supplementary material) (Rendic et al., 2006; Rendic et al., 2010). As neurons continued to differentiate and extend axons, sff mRNA levels increased across the maturing nerve cord. In peripheral sensory clusters, mRNA was detected as early as stage 13, prior to significant accumulation of HRP epitopes and before synaptic contact had been made between sensory afferents and their targets in the ventral nerve cord, which generally begins during stage 16 (Martin et al., 2008; Merritt and Whittington, 1995). Consistent with reduced sff mRNA expression in sff\textsuperscript{B22}, proteomic analysis by LC-MS/MS demonstrated reduced Sff protein levels in sff\textsuperscript{B22} adult heads, which also exhibited reduced HRP-epitope expression (see Table S3 and Fig. S6 in the supplementary material) (Lim et al., 2008).

**Sff Interacts with Tollo**

HRP epitope loss in sff\textsuperscript{B22} suggested that Sff might act in the transcellular signaling pathway defined by Tollo (Seppo et al., 2003). As heterozygous embryos, sff\textsuperscript{B22} or Tollo mutations (over a wild-type chromosome) exhibited normal HRP-epitope expression. As homozygous embryos, the sff phenotype was not identical to the Tollo phenotype. Hindgut staining was absent and garland gland staining was reduced in sff\textsuperscript{B22} but not in the Tollo mutant, whereas nerve cord staining was completely absent in Tollo mutants but still faintly present in sff\textsuperscript{B22}. Transheterozygous embryos of the genotype sff\textsuperscript{B22}, Tollo\textsuperscript{+/} sff\textsuperscript{B22}, Tollo \textsuperscript{+/} Tollo\textsuperscript{B22} possessed aspects of both phenotypes; faint residual nerve cord staining was seen along with rescue of hindgut HRP epitopes (Fig. 4A-D). This composite phenotype is consistent with an interaction in which reduction of either gene dose results in sensitization for both phenotypes. Thus, sff\textsuperscript{B22} enhanced partial loss of Tollo, and vice versa, indicating functional interactions between the two genes. Significantly, Tollo mutant embryos exhibited reduced sff transcripts, both in our previously described Tollo mutant (Fig. 4E,F) and in another mutant allele (tollo\textsuperscript{c5}) generated by FLP-FRT recombination (B. Charroux and J. Royet, personal communication), which also exhibits reduced HRP-epitope expression (Fig. 4G and see Fig. S7 in the supplementary material).
vertebrate glycan processing is the activity of an N-linked glycan biosynthesis and processing in Drosophila sff mutant embryos. Increased N-linked glycan complexity in sff mutant, similar to reduced SAD-1 in C. elegans mutant (Crump et al., 2001). To assess in a similar manner the integrity of the active zone scaffold in our Drosophila sff mutant, we examined the distribution of the Bruchpilot protein (Brp), which participates in vesicle tethering, the presynaptic specializations that make up the active zone complex. Despite deficient vesicle tethering, the presynaptic specializations that make up the active zone are well structured in the C. elegans mutant (Crump et al., 2001). To assess in a similar manner the integrity of the active zone scaffold in our Drosophila sff mutant, we examined the distribution of the Bruchpilot protein (Brp), which participates in the formation of T-bars (the electron-dense projections upon which synaptic vesicles are tethered) at the NMJ (Wagh et al., 2006). The distribution of Brp within individual boutons was unaltered in sff mutant NMJs compared with wild type (Fig. 5F,G). Therefore, reduced Sff in Drosophila, similar to reduced SAD-1 in C. elegans, did not affect the structural integrity of the presynaptic scaffold required for vesicle release.

**Increased N-linked glycan complexity in sugar-free frosting mutant embryos**

N-linked glycan biosynthesis and processing in Drosophila proceeds through steps that are well conserved across the animal kingdom (Fig. 6A). A major difference between Drosophila and vertebrate glycan processing is the activity of an N-acetylhexosaminidase, known as Fused Lobes (Fdl), which removes the GlcNAc residue added by N-acetylglucosaminyltransferase-I (GlcNAcT1). The Fdl enzyme

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**Behavioral and neuromuscular deficits in the sugar-free frosting mutant**

Homzygous sff mutants were reluctant to climb up the side of their culture vials, a phenotype previously described for Tolll mutants (Seppo et al., 2003). In general, sff adults righted themselves when knocked to the bottom of an empty culture vial and exhibited reasonable locomotion once movement was initiated, but failed to move in a directed fashion away from gravity (see Movie 1 in the supplementary material). We quantified this negative geotaxis defect by measuring the time required for individual adults to climb to a pre-designated height (Fig. 5A). Wild-type adults quickly reached the target height; 81% succeeded within 15 seconds at 25°C. However, sff mutants tarried and often never reached the designated height before the trial was ended at 2 minutes; only 36% succeeded within 15 seconds at 25°C. As with ant-HRP antibody staining, the geotaxis phenotype was more pronounced at reduced temperature.

Because motility requires a functional NMJ, we investigated the status of the NMJ in third instar larvae. In sff mutants, the NMJ was considerably less complex (Fig. 5B-E), with a significant reduction in both bouton and primary branch number. NMJ morphology is also disrupted in the C. elegans SAD-1 mutant, with poor alignment of synaptic vesicles at the active zone complex. Despite deficient vesicle tethering, the presynaptic specializations that make up the active zone are well structured in the C. elegans mutant (Crump et al., 2001). To assess in a similar manner the integrity of the active zone scaffold in our Drosophila sff mutant, we examined the distribution of the Bruchpilot protein (Brp), which participates in the formation of T-bars (the electron-dense projections upon which synaptic vesicles are tethered) at the NMJ (Wagh et al., 2006). The distribution of Brp within individual boutons was unaltered in sff mutant NMJs compared with wild type (Fig. 5F,G). Therefore, reduced Sff in Drosophila, similar to reduced SAD-1 in C. elegans, did not affect the structural integrity of the presynaptic scaffold required for vesicle release.

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**Fig. 3. Drosophila sff mRNA is expressed in the embryonic nervous system and is reduced in sff mutant.** (A-E) In situ hybridization (ISH) with anti-sense probe for CG6114 (sff) to late stage 13 wild-type embryo (A,D) reveals prominent message expression in the ventral nerve cord and in sensory neurons in the PNS (arrows in D). ISH on a late stage 13 sff embryo shows decreased mRNA levels in the ventral nerve cord (B) and PNS (E). Arrow indicates low staining in sensory neurons of the PNS. No hybridization was observed with the sense probe (C; late stage 13 wild-type embryo). Anterior is to the left. Embryos presented in all panels were processed in parallel. Scale bar: 60 μm for A-C; 15 μm for D,E.

**Fig. 4. sff interacts with Tolll and expression of sff mRNA is dependent on Tolll.** (A) Wild-type embryos and embryos heterozygous for either Tolll or sff are identical in appearance, with strong staining of the ventral nerve cord (vnc), garland gland (gg) and hindgut (hg). (B) Embryos homozygous for the sff mutation exhibit reduced vnc and hg staining. (C) Embryos homozygous for loss of Tolll lack vnc staining but retain gg and hg expression of the HRP epitope. (D) Embryos that are transheterozygous for both sff and Tolll have a composite staining phenotype, indicating functional interactions between the two genes. (E,F) In situ hybridization of antisense probe for sff in wild-type (E) and Tolll mutants (F) indicates that Tolll signaling induces neuronal expression of sff mRNA. Scale bar: 90 μm for A-F. (G) qRT-PCR of sff transcript levels in sff and Tolll mutant embryos, relative to wild type. *P<0.05, ***P<0.01; mean ± s.e.m. for three independent analyses.
drives the predominance of paucimannose structures in the wild-type *Drosophila* glycan profile by converting NM3N2 glycans to M3N2, thereby eliminating the precursor pool for hybrid or complex structures (Leonard et al., 2006). Limited expression of branching and terminal glycosyltransferases, such as GlcNAcT2, GlcNAcT4, GalT, GalNAcT or SiaT, also restrict the capacity for generating complex structures (Haines and Irvine, 2005; Koles et al., 2004; Repnikova et al., 2010; Sarkar et al., 2006). HRP epitopes represent less than 1% of all N-linked glycans in the *Drosophila* embryo (Aoki et al., 2007). Therefore, anti-HRP antibody staining reports the status of a limited set of N-glycan processing pathways.

To comprehensively identify glycan expression changes induced by the sff mutation, total N-linked glycan profiles of sff and wild-type embryos were analyzed by mass spectrometry. High mannosine and paucimannose glycans together accounted for almost 90% of the total profile in wild-type (89.5%) and sff mutant (85.5%) embryos, indicating that the dominant glycan processing pathways are largely unaffected by the sff mutation. (see Table S4, structures 1-13, in the supplementary material). However, the prevalence of the M5N2 structure, an essential precursor for paucimannose, hybrid and complex glycan production, was reduced in the sff mutant (Fig. 6B). The decrease in M5N2 was mirrored by an increase in M3N2, a paucimannose glycan production of which requires Fdl, indicating robust activity of the processing hexosaminidase in sff embryos. Hybrid and complex glycans together made up 9.6% of the total profile in wild-type and 11.1% in sff embryos (see Table S4, structures 14-25 and 31-42, in the supplementary material). This modest increase in the prevalence of processed glycans, equivalent to a shift of 1.5% of the total glycan profile in the sff mutant, was more dramatic for a subset of individual complex structures (Fig. 6C). For example, the N2M3N2 glycan, a biantennary non-fucosylated structure, was increased by 385% in the sff mutant. As expected, glycans bearing α1-3-linked Fuc were significantly reduced in sff embryos (54% reduction overall), accounting for 0.5% less of the total glycan profile than in wild type (Fig. 6D). However, one HRP epitope, the complex biantennary N2M3N2F2 glycan, was more prevalent in sff than in wild-type embryos. This difucosylated structure, like the non-HRP complex structures that were also increased in sff mutants, requires the activity of GlcNAcT2 or GlcNAcT4 (Fig. 6A), indicating that the sff mutation increases the flux of glycan processing towards greater complexity. Our previous structural characterization of the glycans expressed in *Tollo* mutant embryos also demonstrated increased prevalence of complex non-fucosylated glycans in addition to reduced expression of HRP epitopes, consistent with both genes acting within an intersecting pathway (Aoki et al., 2007).

To determine if the shifted glycan profile in sff results from altered transcription of genes encoding glycosyltransferases or glycan processing enzymes, we performed qRT-PCR in order to quantify transcript levels for genes encoding Fdl, FucTA, GlcNAcT1, GlcNAcT2 and a GlcNAcT4 candidate (CG9384) relative to *GAPDH1* (Fig. 6E). *FucTA* transcription was modestly reduced in sff B22 (30%), although the reduction in message was...
disproportionate to the larger reduction in HRP-epitope expression (54%). Furthermore, the increase in Fdl transcripts and the decrease in GlcNAcT2 transcripts were inconsistent with the shift in the total glycan profile towards greater complexity and reduced HRP-epitope production. Similar transcript abundances were detected in Tollo mutant embryos (see Fig. S7 in the supplementary material). The inability of transcriptional changes to account for the nature of the shift in glycan expression indicates that modulation of enzyme production is not the primary mechanism by which sff alters N-glycosylation.

**Sff modulates Golgi compartmentation in neurons**

Protein glycosylation reflects Golgi dynamics. Therefore, we hypothesized that Sff participates in the establishment of or trafficking through Golgi compartments. The Drosophila Golgi apparatus is striking for its lack of morphological similarity to classic conceptualizations of the ribbon-like stacked cisternae evident in many vertebrate cell types. Instead, the Drosophila Golgi exists as distributed puncta, forming multiple organelles within a single cell (Kondylis and Rabouille, 2009; Stanley et al., 1997). As the major change in sff N-glycan expression is a shift towards greater complexity, we focused first on late Golgi compartments using markers described previously to delineate the medial/trans and trans-Golgi in Drosophila (LaJeunesse et al., 2004; Yano et al., 2005). We assessed the relative distributions of Golgi-YFP (a medial/trans marker, YFP fused to the transmembrane localization domain of human β4GalT), Arachis hypogaea lectin (PNA) binding (a trans marker, Galβ3GalNAc specific lectin) and a neuronal protein (Fasciclin 2, Fas2) that bears an HRP epitope (Desai et al., 1994; Lin et al., 1994).

In triple-stained preparations (Golgi-YFP, PNA, Fas2), Golgi puncta were distributed throughout the nerve cord (see Fig. S9 in the supplementary material). Colocalized fluorescence intensity for each marker was quantified across all of the objects imaged within regions of interest in the ventral nerve cord (Fig. 7A-I and see Fig. S10 in the supplementary material). Colocalization of Fas2 with the trans marker demonstrates a striking change in the association of the HRP-bearing glycoprotein with this compartment of the Golgi (compare Fig. 7A with 7B, quantified in 7C). Essentially, all of the quantified Fas2 fluorescence intensity colocalized with the trans marker in sff mutants. The differential colocalization of Fas2 in sff

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**Fig. 6. The N-linked glycan profile of the sffB22 mutant is deficient in HRP epitopes and shifted towards greater complexity.** (A) N-linked glycan processing in Drosophila proceeds through steps that are well conserved across animal species. See Fig. 1 for key to graphical representation of structures. GlcNAcTransferase-1 (GlcNAcT1) transfers a GlcNAc residue to the trimmed high-mannose structure M5N2. Subsequent removal of Man residues by Golgi mannosidases produces NM3N2, a structural node that feeds into three separate pathways. The Fused Lobes hexosaminidase (Fdl) can remove the GlcNAc added by GlcNAcT1 to generate the paucimannose glycan M3N2. Alternatively, NM3N2 can be branched by the action of another GlcNAcTransferase (GlcNAcT2 or GlcNAcT4) to produce complex glycans that are resistant to degradation by Fdl. The NM3N2 glycan is also a substrate for α6-fucosylation (FucT6) and subsequent branching by GlcNAcT2 or GlcNAcT4. Addition of α3-Fuc residues (FucTA) generates HRP epitopes. (B-D) The prevalences of the indicated glycans are given as the percent that they contribute to the total profile of N-linked glycans (% Total Profile, see Table S4 in the supplementary material for full glycan profiles). (E) qRT-PCR of glycogene transcript levels in sff mutant embryos relative to wild type *P<0.05, **P<0.02, ***P<0.01. Values are the mean ± s.e.m. for three independent analyses in panels B-E.
Fig. 7. Neuronal Golgi compartmentation is altered in sffB22. (A-F) Representative three-dimensional reconstructions are shown of portions of regions of interest that were used to quantify Golgi marker colocalization in the ventral nerve cord of wild-type (A,D,G) and sffB22 (B,E,H) Drosophila embryos. Preparations were triple stained with PNA lectin (a trans Golgi marker; green), mAb 1D4 (Fas2, an HRP protein; blue) and anti-YFP (a medial/trans Golgi marker; red). Objects labeled for Fas2 and the trans marker are closely apposed (arrows in A) but exhibit less overlap in the wild-type nerve cord (arrowheads in A) than in the sffB22 mutant (B). Almost all the Fas2 signal overlaps with the trans marker in the mutant (arrows in B). Fluorescence intensity colocalization was quantified (C) within regions of interest for wild-type and sffB22 mutant embryos (mean ± s.e.m. for n=24 segments for each genotype). Fas2 signal is tightly associated with the trans marker in sffB22 (mean Pearson’s correlation coefficient (PCC)=0.62±0.14), demonstrating a statistically significant increase over the colocalization of these markers in wild type (PCC=0.45±0.12). Consistent with increased polarization between Golgi compartments, trans and medial/trans markers display less overlap in sffB22 mutant embryos (arrows in D and E, quantified in F, PCC=0.19±0.03 and 0.31±0.05 in sffB22 and wild type, respectively, n=24 segments for each genotype). (G-I) Preparations were double stained (G,H) with PNA lectin (green) and anti-GM130 (a Golgin localized to the cis Golgi; red). Unlike the medial/trans marker, the cis marker GM130 displays increased overlap with the trans compartment in sffB22 mutant embryos (arrows in G and H, quantified in I; PCC=0.46±0.14 and 0.05±0.03 in sffB22 and wild type, respectively, n=12 segments for wild-type and n=7 segments for sffB22). Regions of interest were selected to eliminate any contribution of axon staining to the final quantification (see Figs S9, S10 in the supplementary material). Gridlines correspond to 1 μm in all reconstructions. ***P<0.002.

DISCUSSION

Tissue-specific protein glycosylation requires coordinated trafficking of substrates and modifying enzymes through Golgi compartments

We have presented data demonstrating that a mutation in Drosophila Sff alters glycan expression in the embryonic nervous system. The neural-specific N-linked glycans known as HRP epitopes are reduced in sff mutant embryos and the expression of a subset of complex glycans is increased. Protein kinases have previously been linked to altered glycosylation, based primarily on pharmacological manipulations in cultured cells (Yu and Bieberich, 2001). For example, treatment of NRK cells with activators of protein kinase A alters vesicular stomatitis virus G protein glycosylation in infected cells (Muniz et al., 1996; Muniz et al., 1997). Furthermore, protein kinase C activity modulates sialylation of glycosphingolipids in NG108-15 and of glycoproteins in SH-SY5, as well as polysialylation of NCAM in neuro-2A and PC-12 cells (Bieberich et al., 1998; Breen and Georgopoulou, 2003; Gallagher et al., 2000). However, the functional consequences of these pharmacological effects have not been validated in vivo, nor have molecular or cellular mechanisms underlying altered glycosylation been identified.

The expression of Drosophila HRP epitopes is not only tissue-restricted, it is also protein-restricted in wild-type embryos; only a subset of neuronal proteins bears α3-fucosylated glycans (Desai et al., 1994). The influence of α3-fucosylation on the function of any of the individual proteins that carry this specific post-translational modification is currently unknown; the glycan might influence protein stability or protein interactions, or serve as a recognition marker independent of the protein on which it resides. Regardless of its function, the tissue-specificity of HRP-epitope expression in the Drosophila embryo provides a platform for assessing the regulatory mechanisms that control glycosylation in vivo. In general, the regulation of glycosylation, and particularly of glycan diversity on specific proteins, is multi-dimensional. The coordinated expression of appropriate glycosyltransferases and target proteins alone is insufficient for achieving specific glycosylation. Target and enzyme must also be brought together within a permissive Golgi domain, which must contain other supporting activities, such as appropriate nucleotide sugar transporters and trimming exoglycosidases.

The multidimensionality of regulated glycan expression means that SFF might influence protein glycosylation at any of several control points. The kinase might modulate the expression of glycan modifying enzymes, but our analysis of glycogene transcript levels in the sff mutant indicates that the expression of glycosyltransferases and glycan processing enzymes cannot account for the observed shifts in glycan prevalence. Differential lysosomal degradation of specific protein glycoforms could also generate a shift in glycan profiles, but altered catabolism cannot...
explain the increase in complex glycans observed in the sff mutant. Finally, glycosyltransferase phosphorylation has been described in vitro, but evidence for a functional consequence of this modification is currently lacking (Gallagher et al., 2001; Hathaway et al., 2003; Ma et al., 1999). The hypothesis most consistent with our results is that Sff modulates glycan processing by influencing the compartmentation of glycan processing within the Golgi apparatus.

Very little is known regarding the coordinated Golgi dynamics that produce the confluence of components necessary for specific glycan expression in embryos or in mature tissues (Gerardy-Schahn et al., 2001; Nilsson et al., 2009), but a specific role for protein phosphorylation in controlling Golgi function is not without precedent. The cyclin-dependent kinase Cdk1-cyclin B initiates the process of Golgi disassembly during mitosis by phosphorylating Rab1 and Golgin GM130, which together recruit an ER-derived vesicle tethering factor (p115) to cis-Golgi membranes (Misteli and Warren, 1995). Other kinases, including polo-like kinases, extracellular signal-regulated kinases and mitogen-activated protein kinases, function along with Cdk1-cyclin B to dismantle the Golgi during mitosis, and additional kinases contribute to bulk Golgi trafficking during interphase or modulate vesicle coat assembly (Dirac-Svejstrup et al., 2000; Doray et al., 2002; Preisinger and Barr, 2005). In these examples of full Golgi collapse, Golgi partition during mitosis and vesicle coat disruption, kinase activities initiate dramatic Golgi remodeling events. The Sff function that we have described is more restricted, impacting a subset of relatively minor glycan structures primarily associated with the late Golgi. Thus, the relative dosage of specific kinases, within a larger ensemble of phosphorylating enzymes, might sculpt unique glycan expression profiles in specific cell types through targeted modulation of Golgi compartmentation.

Based on the function of SAD kinase at the mature synapse, where it facilitates tethering of vesicles to membrane-associated protein complexes, and on the narrowly distributed changes in the N-glycan profile of sff mutant embryos, we propose that Sff kinase activity modulates Golgi vesicle interactions within a subset of cisternal membranes (Fig. 8). Our glycan characterization indicates that appropriate targeting of these vesicles normally limits the expansion of N-linked glycan complexity and facilitates the generation of HRP epitopes in neural tissue. When sff activity is reduced, some glycoprotein substrates gain access to alternative Golgi processing enzymes and the loss in tethering specificity generates a shift in Golgi vesicle trafficking.

**Molecular targets for Sff at the Golgi apparatus and signaling pathways that impinge upon tissue-specific glycosylation**

Vesicular capture at presynaptic membranes requires the interaction of specific Rab GTPase proteins and scaffolding/tethering factors (Inoue et al., 2006; Schoch et al., 2002; Sinka et al., 2008). Vesicular transport through the Golgi apparatus is also modulated by interactions between Rab proteins and tethering factors (Ren et al., 2009; Sztul and Lupashin, 2009). The Golgins, which exhibit compartment-specific distributions across the Golgi, are a component of the mechanism that imparts specificity to vesicle-target recognition (Short et al., 2005). Structural analysis predicts that the Golgins are extended molecules that reach into the surrounding cytoplasm from the Golgi membranes to which they are attached, providing a matrix for interaction and tethering of transport vesicles. The glycosylation phenotype that we have quantified in our *Drosophila* sff mutant indicates that the stability or functional integrity of Golgin-Rab-vesicle complexes might be differentially influenced by the activity of specific kinases. Therefore, Golgins, Rabs and, possibly, components of the

![Fig. 8. Parallels between known functions for Sff/SAD at the presynaptic membrane of the neuromuscular junction (NMJ) and proposed functions for Sff/SAD at cisternal membranes of the Golgi apparatus. At the NMJ, SAD kinase facilitates the tethering of synaptic vesicles at presynaptic active zones. The tethering complex brings together molecular components necessary for priming and fusion of synaptic vesicles. Presynaptic tethering complexes comprise spatially extended tethering factors such as Bruchpilot (a *Drosophila* protein that combines the signature domains of the vertebrate Bassoon/Piccolo and ERKs/CAST family members) and other scaffold or tethering components. In the absence of SAD kinase, synaptic vesicles are inefficiently associated with the active zone. Altered glycan expression in the *Drosophila* sff mutant indicates that Sff/SAD kinase serves an analogous function at Golgi cisternal membranes, ensuring that specific Golgi transport vesicles associate with appropriate stack-specific tethering factors such as Golgin family members. In the sff mutant, Golgi vesicular transport is not halted because secondary cisternal targets are available and competent for fusion. But, the resulting aberrant trafficking shifts glycan profiles by allowing access of glycoprotein substrates to alternative ensembles of processing enzymes (Golgi glycosyltransferases). In sff222, decreased activity reduces HRP-epitope expression (orange processing pathway) and increases complex glycan production (green processing pathway).](image-url)
retrograde transport machinery [conserved oligomeric Golgi (COG) and Golgi associated retrograde protein (GARP) complexes], are prime candidate substrates for phosphorylation by Sff. The modestly expanded distribution of a Golgin (GM130) in the nervous system of the sffB22 mutant is consistent with the hypothesis that Sff modulates tethering factor function and could suggest that GM130 is a substrate for the enzyme. However, altered GM130 distribution might also result from aberrant modulation of Rabs or downstream tethering factors. Further genetic, biochemical and proteomic analysis will be necessary to ascertain the identity of the Sff substrates most relevant for controlling the Golgi dynamics that regulate tissue-specific glycosylation.


microenvironments and culture conditions on cellular protein mechanism for reinterpreting many years of empirical observations describing the impact of endocrine factors, tissue microenvironments and culture conditions on cellular protein glycosylation (Knezevic et al., 2009; Varki, 1993).
heritability and environmental determinants of human plasma N-glycome. J. Proteome Res. 8, 694-701.


