GENETIC AND MOLECULAR MECHANISMS REGULATING GLYCOPROTEIN GLYCOSYLATION IN THE *DROSOPHILA* EMBRYO

by

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(Under the Direction of Michael Tiemeyer)

ABSTRACT

Appropriate glycosylation on biological macromolecules such as proteins and lipids is vital for the normal growth and development of organisms. However, the elaborate set of biological events ensuring proper glycosylation has not been completely elucidated. *Drosophila melanogaster* embryonic nervous system synthesizes a family of related glycan species that are structurally similar and recognized by antibodies against the plant glycoprotein, Horseradish peroxidase (HRP). The specific presentation of HRP epitopes in the developing insect nervous system provides a platform for examining the genetic and molecular pathways controlling tissue-specific glycan expression. *Drosophila* mutations such as *neurally altered carbohydrate (nac)*, *sugar free frosting (sff)* and *tollo* have been shown to specifically affect the neural expression of HRP epitopes. Transgenic overexpression of a Golgi GDP-fucose transporter or of a *Drosophila* homolog of the vertebrate SAD kinases rescues HRP-epitope expression in nac^{1} and *sff* mutants, respectively. By *in-situ* hybridization, *sff* mRNA is detected at

reduced levels in *tollo* mutants, indicating that *sff* expression is regulated through Tollo signaling. To further characterize the signaling pathways regulating neural specific glycan complexity in *Drosophila* embryos, we undertook differential phosphoproteomic analysis to identify molecular targets of Sff/SAD kinase. Bifocal (Bif), Rasputin (Rin) and Liprin-alpha (Lip-α) were detected among a set of proteins phosphorylated in wild-type but not in *sff* mutant embryos. *Rin and lip-α* genetically interact with *sff* to affect glycoprotein glycosylation. Confocal analysis of Stage 14 embryos reveals an increase in the colocalization of Rin with Golgi compartments in *sff* mutants compared to wild-type. Western analysis detected a decrease in the steady state concentration of Rin protein in *sff* mutants, consistent with the genetic interaction detected between these two genes. In *lip-α/lip-α* mutants, compartmentation of the Golgi apparatus is altered in a manner similar to disruptions previously detected in *sff* mutants, consistent with a role for phosphorylation of this multi-domain protein in modulating glycoprotein glycan processing. Taken together, our results provide mechanistic insights into the signaling pathways through which Sff/SAD kinase influences cellular glycosylation in response to intra and extracellular cues.

INDEX WORDS: Bifocal, Glycosylation, Golgi, Liprin-alpha, Nac/GDP Fucose Transporter, Rasputin and Sff/SAD kinase.

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DEDICATION

To my daddy, I dedicate this work.

Together we gazed and dreamt of making a difference in this world with the power of science. He is no more but to be honest it is a PhD for both of us !! He wanted to understand my doctoral work in its entirety. His illness deprived him of the capacity to breathe, read and think; yet he never failed to share his bit of "scientific" curiosity in the time he has left. No words can describe the man who laid the very foundations of this PhD dream. He taught me to love biology and I owe him every bit of my success, now and in the future. Thank you so much and I wish you could see me at my research station. Just once. To my mother, I want to say I learnt my perseverance from you. The words "Thank you" cannot justify the role you have taken for us. You have always been the silent pillar of support. You let me follow my passion and travel so far even when you so needed me at your side to fight dad's illness. This PhD is dedicated to all your patience. To my younger sister Harika, thank you for taking care of me like a child all these years and being the older sister to me. Thank you for holding my hand and walking every step with me. This journey would be incomplete without you. For my husband Lokesh, I want to say: I have no idea what I would I have done without you. Every Friday, when a blot didn't work, I could call and cry to you. Thank you for standing by me in spite of being 1100 miles away from me. You made sure that I did not falter in my pursuit, ever. This is OUR achievement.

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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

Introduction

Studies in carbohydrate biology underscore the requirement of glycans for normal health and development. However, clear mechanistic insights into the regulation of cell and tissue specific glycosylation events have not been completely elucidated. Insects such as *Drosophila* and Grasshopper present a unique example of tissue specific glycosylation in the form of a family of structurally related N-linked oligosaccharides that are recognized by antibodies against the plant glycoprotein horseradish peroxidase (HRP) and enriched in the developing embryonic nervous system and non-neural tissues such as garland glands, posterior hind gut and anal pads. While HRP epitopes represent a specific class of glycans, our understanding of glycoprotein glycosylation across cells and tissues is still in its infancy. The scope of this dissertation deals with the genetic and molecular mechanisms regulating glycoprotein glycosylation in the *Drosophila* embryo.

In this opening chapter, general aspects of glycoprotein glycosylation, secretory pathway and Golgi signaling will be covered followed by a discussion on HRP epitopes, *Drosophila* embryonic nervous system development and the

mutations affecting these epitopes during embryonic stages of development. Signaling pathways involving Sff kinase are also discussed for an appreciation of the parallels existing in the vesicular trafficking events at different sites in a cell. An overview of these topics will serve as a platform to better understand the molecular and genetic events coordinating glycoprotein glycosylation in using *Drosophila* embryonic nervous system.

Glycosylation- A Post Translational Modification

Glycosylation or the ornate decoration of proteins or lipids molecules with carbohydrate moieties is an important effector of several cellular functions. While glycoproteins and proteoglycans constitute glycan classes where the oligosaccharide and protein backbone are linked covalently, glycolipids on the other hand have glycans linked to a ceramide molecule. Glycosylphosphatidyl (GPI) anchor glycoproteins are the fourth type of glycan class with sugars such as mannose and N-acetylglucosamine (GlcNAc) serving as a link between phosphatidylinositol moiety and an ethanolamine extending out of a protein backbone (Varki, 2009). Glycoproteins are classified into N-linked, O-linked and C-mannosylation classes depending on whether the glycan moiety is attached to an asparagine (amide linkage), serine/threonine (glycosidic) or tryptophan (carbon-carbon bond) residue of the protein backbone respectively. N-linked glycoprotein synthesis begins in the endoplasmic reticulum (ER) and subsequent post-translational alterations take place in the Golgi apparatus before their final transport to the required cellular site of action (Figure 1.1). The ER is a complex milieu of molecular chaperones, glycosylation enzymes and a host of other

proteins that assist in protein folding and initiate post translational modifications such as glycosylation. Glycoprotein cargo proceeds to the Golgi apparatus from the ER, where further processing of N-glycans can take place to yield high mannose, hybrid or complex types. O-glycan modification of transiting cargo proteins specifically also takes place at the Golgi (Moremen et al., 2012; Varki, 2009).

Glycosylation In Development, Health And Disease

Aberrant glycoprotein, proteoglycan or glycolipid glycosylation has been implicated in a myriad of diseases such as autism, congenital disorders of glycosylation, dystroglycanopathies and mental retardation (Hennet, 2012). The diversity of these disease phenotypes reflects the multitude of signaling pathways that are coordinated by this single post-translational modification. Its importance is elegantly demonstrated through the Notch pathways during organismal development. The Notch receptor and its ligands Delta and Serrate orchestrate a paracrine signaling pathway that allows one cell to adopt a neural fate and inhibits the immediate neighboring cells from acquiring the same neural fate during early brain development. Mutations in this receptor or its ligands result in multiple human disorders such as CADASIL and cancers such as T cell acute lymphoblastic leukemia. Humans have four notch genes with mutations in Notch 1 and 2 resulting in an embryonic lethal phenotype (Takeuchi and Haltiwanger, 2010). The Notch receptor is O-glycosylated on its epidermal growth factor (EGF) domains on its extracellular region through the addition of fucose on specific serine or threonine residues by the O-fucosyltransferase-I

enzyme (known as O-fut1 in flies /POFUT1 in mammals). POFUT1 null mutation is embryonic lethal demonstrating that absence of O-fucosylation on the Notch receptor is as severe as the traditional Notch pathway gene knockouts like suppressor of hairless (SuH) or presenilins, clearly emphasizing the role of glycosylation in vertebrate and invertebrate developmental lifecycles (Haines and Irvine, 2003; Shi and Stanley, 2003)

Biosynthesis Of Glycoproteins

N-Glycan synthesis begins with the nascent polypeptide-ribosome-mRNA complex being recognized by the signal recognition particle (SRP) and leading it to the SEC61 translocon complex. Co-translational entry of the nascent peptide through the translocon complex lands the polypeptide into the lumen of the Endoplasmic Reticulum (ER) (van Vliet et al., 2003). Chaperones like Binding Immunoglobulin Protein (BiP), PDI (Protein Disulphide Isomerase), calreticulin and calnexin participate in the protein folding process. One of the earliest steps in N-glycan synthesis is the generation of Dolichol-P-P-$GlcNAc_2Man_9Glc_3$, a lipid linked oligosaccharide precursor that is initially synthesized on the cytosolic face of ER and subsequently flipped to enable transfer of the fourteen sugar oligosaccharide moiety to the Asparagine residue in the polypeptide sequon Asn-X-S/T. Following this, the core glycan moiety is trimmed down by glucosidases (I and II) to yield glycans of $GlcMan_9GlcNAc_2Glc_1$ composition. The monoglycosylated glycoproteins then proceed to be scrutinized by calnexin and calreticulin for an assessment of folding status. Glycoproteins are then acted upon by glucosidase II to remove the single remaining glucose. Through the

discriminative action of the enzyme UGGT1, properly folded proteins are further trimmed by ER mannosidase I and leave the ER through specialized regions known as exit sites to reach the Golgi apparatus. On the other hand, improperly folded proteins are sent for refolding via the addition of a glucose molecule by UGGT1 or trimmed down to remove the sugars and enable the transfer of polypeptide to the proteosome degradation machinery (Moremen et al., 2012).

The export of properly folded proteins takes through the induction of cytoplasmic coat proteins denoted as COPII. It is followed by the formation of vesicles containing the cargo protein molecules along with a host of other proteins. SEC12, a guanine nucleotide exchange factor (GEF) localized to the ER activates Sar-1-GDP by converting it into Sar-1-GTP. This causes the Sar-1-GTP to localize to the ER membrane. Following this, Sec23p/24p and then Sec13p/31p (these make up the COPII coat) are brought to the site of Sar-1-GTP's site of action. Together, these protein complexes along with the selected cargo make the COPII vesicles that subsequently dissociate from the ER membrane and move in an anterograde fashion to reach the ER-Golgi Intermediate Compartment (ERGIC)(Lee et al., 2004). ERGIC, vesiculo-tubular structure formed by the fusion of COPII vesicles is situated between the ER and cis face of the Golgi apparatus (Figure 1.1). A halt in the traffic of secretory molecules from ER to Golgi occurs when temperature is lowered to 15^{o}C and leads to containment of vesicles in the ERGIC (van Vliet et al., 2003). This observation underscores the importance of ERGIC as the first sorting point exhibiting bidirectional trafficking of vesicles.

The Golgi apparatus serves as the next processing station for the transiting cargo molecules such as glycoproteins. It is roughly divided into cis (in vicinity of the ER), medial and trans (towards the plasma membrane) compartmental regions. In mammalian cells, the Golgi apparatus is arranged in the form of flattened stacks called cisternae that are connected with each other through tubular structures and held in place by the cytoskeletal network (Figure 1.1). The biogenesis of Golgi is not strictly driven by the genetic code. The argument that Golgi could have arisen from the ER is supplemented by data where brefeldin A (BFA) treatment results in a collapse of Golgi into vesicles and rerouting of Golgi glycosylation enzymes to ER (Altan-Bonnet et al., 2004). At mitosis, it disperses into the cytoplasm in the form of individual vesicles that get reconstituted into a functional Golgi when the cell transits to interphase again (Shorter and Warren, 2002).

Transport At The Golgi

The vesicular transport and cisternal maturation model dominate our understanding of protein transport through the Golgi (Figure 1.1)(Reynders et al., 2011) . Both models are thought to co-exist at the Golgi. The vesicular transport model emphasizes on the role of cargo-laden vesicles and their movement from one stationary stack of Golgi to the next in an anterograde manner. In contrast to this, the cisternal maturation model calls for a maturation and subsequent transitioning of stacks as a whole from the cis to the trans end, with the cargo proteins maturing as they travel in the stack. The cisternal maturation model accounts for a variant distribution of glycosylation enzymes through the fusion of

retrograde COPI vesicles carrying glycosylation enzymes and budding from the previous mature stack to the incoming stack. The asymmetric distribution is accounted in the vesicular transport model where outgoing cargo is balanced by incoming COPI vesicles containing escaped resident Golgi enzymes (Ungar et al., 2006).

COPI mediated transport assists in retrograde movement of vesicles in intra-Golgi and Golgi to ER transport. Similar to COPII vesicle formation, this reverse transport process begins with the recruitment of cytosolic ARF1 (a GTPase) in its GTP bound state to the Golgi membrane. ARF-1 GTP brings about the localization of COPI coatomer subunits which in turn recognize proteins containing KKXX motifs and designate them as the retrieval proteins. These vesicles have been shown to contain retrograde cargo like p24 family of proteins and KDEL receptor with ER- resident proteins. Thus, COPI vesicles continuously recycle back ER resident enzymes that might have escaped it. The disintegration of COPI vesicles is brought about GTPase activating protein (GAP) which triggers the GTPase activity of Arf-1 and causes GTP hydrolysis and the disassembly of coat proteins from the vesicles (Altan-Bonnet et al., 2004; Lee et al., 2004; Pfeffer, 2007; Spang, 2008).

Localization Of Golgi Resident Glycan Modifying Enzymes

The ability of Golgi to serve as an oligosaccharide-processing center is intricately dependent on the distribution of glycosyltransferases (GT), glycosidases and nucleotide sugar availability across its cisternae (Figure1.2) (Stanley, 2011). A gradient of glycosylation enzymes, reflecting the order of

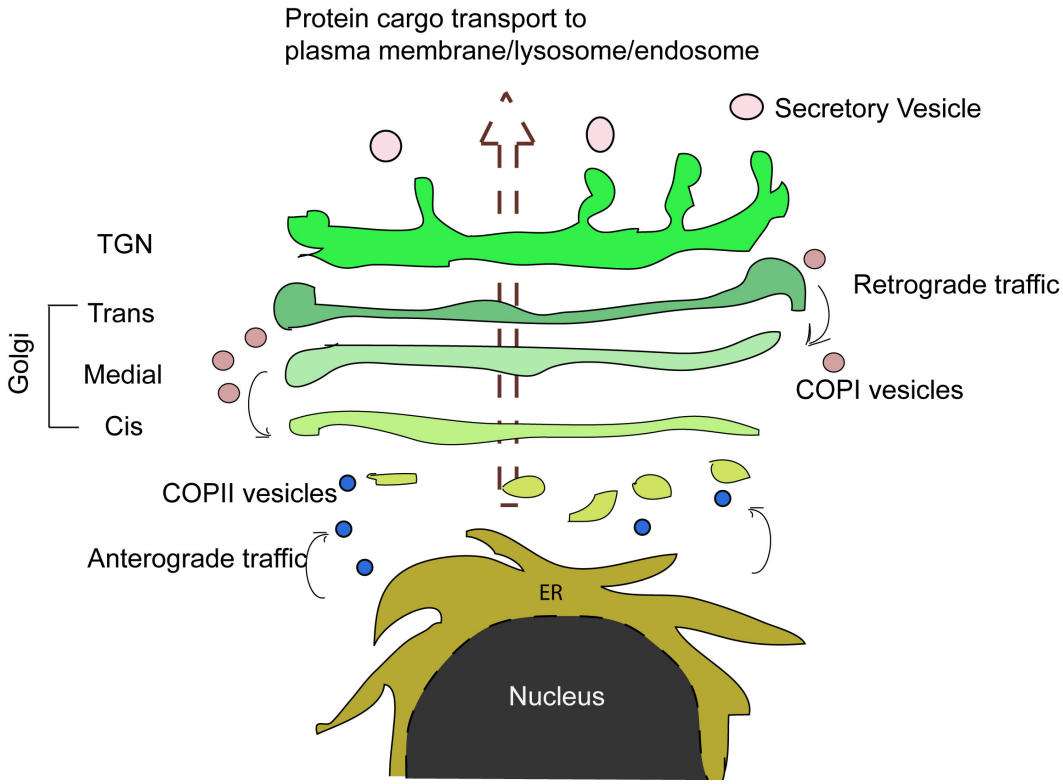

Figure 1.1. A schematic illustration of the secretory pathway. Proteins destined to be secreted to cell surface or various compartments such as lysosomes begin their journey in endoplasmic reticulum (ER), traverse through the Golgi apparatus(cis to trans) and are eventually transported to their final destinations from the trans-Golgi network. Protein cargo and enzyme laden vesicles traverse between different Golgi compartments through COPII and COPI vesicles respectively. Adapted from references(Reynders et al., 2011; van Vliet et al., 2003).

processing exists across the Golgi cisternae. For example, mannosidaseII (ManII) and N-acetylglucosaminyltransferase (GnT-I) are distributed across the cis-medial Golgi with the greatest concentration in medial Golgi. In contrast to this, galactosyltransferase and sialyltransferase enzymes are restricted to the trans side of the Golgi, strongly reflecting the order of processing and addition of individual sugars to the peptide backbone (Pfeffer, 2007; Varki, 2009). Thus, the addition of more complex sugars is seemingly confined to the later compartments of the Golgi apparatus. Based on these observations, it can be predicted that factors leading to a disruption in the restricted distribution of these enzymes can alter the glycomic landscape of many proteins without resulting in a complete loss of glycosylation. Studies on N-glycome profiles of *Drosophila* embryos support this prediction in that the multiple classes of N-glycans are differentially affected due to a mutation in the Sugar Free Frosting Kinase (Baas et al., 2011).

Glycosyltransferases are type II integral membrane proteins. The enzyme is made up of an N-terminus that is short and faces the cytoplasm, a C-terminus consisting of the catalytic domain extending into the Golgi lumen and a transmembrane segment. The short connecting region between the catalytic domain and trans-membrane segment has been demonstrated to be important for the localization of these glycosylation enzymes to Golgi surface (Moremen et al., 2012). Localization of glycosyltransferases is thought to be governed by two mechanisms. The lipid bi-layer sorting model takes into account the lipid differences that exist between ER, Golgi and plasma membrane such that the presence of shorter transmembrane domains in Golgi glycosyltransferases

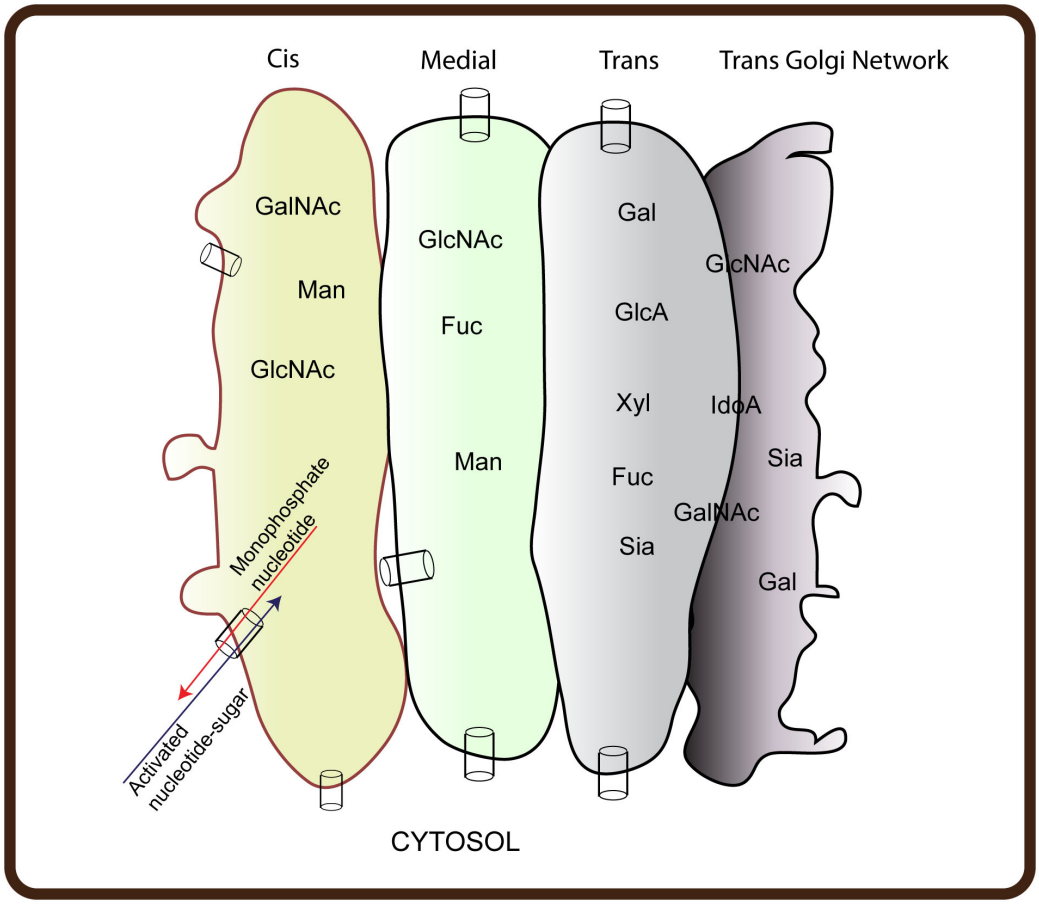

Figure 1.2. Glycan processing in the Golgi. Cis, medial and trans compartments of the Golgi exert biosynthetic control on glycan processing through the availability of glycosyltransferases glycosidases, and nucleotide sugars. These enzymes progressively add or remove sugars at the non-reducing end of the glycan chain to be extended. Activated nucleotide sugars are transported into the Golgi lumen using respective transporter proteins embedded in Golgi membranes across different compartments. Monophosphorylated nucleotide arising from the transfer of the sugar to the glycan chain after a hydrolysis reaction catalyzed by a pyrophosphorylase is transported out of the Golgi lumen. Glycosyltransferases catalyzing the addition of sugars such as sialic acid (Sia), in the later compartments highlights that sialylation is presumably restricted to the later compartments of Golgi. Abbreviations: Fuc, Fucose; Gal, galactose; GalNAc, N-acetylgalactosamine; GlcNAc, N-acetylglucosamine; GlcA, glucuronic acie; IdoA, iduronic acid; Man, mannose; Sia, sialic acid; Xyl, xylose. Adapted from reference (Stanley, 2011).

restricts their confinement in the Golgi membrane in case of vesicular traffic model or contributes to recycling in cisternal maturation model from the relatively more hydrophobic plasma membrane (Pfeffer, 2007). Additionally, proteins such as Vps74p that are peripherally located at the Golgi membranes, enable the sorting of glycosyltransferases by recognizing and binding to a N-terminal motif in these enzymes into COPI vesicles and ensuring their appropriate distribution patterns across the Golgi (Tu et al., 2008). The second model looks at the property of glycosyltransferase enzymes to associate with themselves, promoting the idea of kinship or protein family based association. These proteins have been shown to cluster into oligomers at the medial Golgi by recognizing self type “kin” molecules (Pfeffer, 2007). Similar to vesicular trafficking model and cisternal maturation model of cargo movement, retention of glycosyltranferases and glycosidases is likely governed by both oligomerization and lipid-bilayer sorting models (Moremen et al., 2012).

Molecular Tethers Of The Secretory Pathway

The function of Golgi apparatus as a protein-processing center requires a dynamic flux of cargo to and fro from itself. Interactions of the SNARE proteins lead to the ultimate vesicle fusion with the target membranes. However, such fusion events take place only after the vesicles and membranes have been captured and brought together by protein molecules known as “tethers”. Essentially, the process begins with vesicle cargo detaching itself through the budding process, moving along the microtubule cytoskeleton to reach the vicinity of the target membrane. The tethers then latch up the cargo laden vesicle to the

target membrane (Cai et al., 2007). Thus, tethering process involves protein-protein interactions between components present on vesicle surface, cytosol and receiving membrane such as Golgi cisternae. Tethers are important traffic modulators and like any other molecule in a cellular context have to be regulated. The candidate molecules regulating this aspect of golgin function are the small G-proteins belonging to the superfamily of ras proteins known, as Rab GTPases (Bannykh et al., 2005; Barr and Short, 2003; Cai et al., 2007; Goud and Gleeson, 2010). Their operation mode consists of continuous shuttling between their cytosolic GDP (guanine di-nucleotide phosphate) bound and a membrane associated GTP (guanosine tri-phosphate) forms. On a general basis, Rabs can be thought of as “tether receptors “that recruit different effectors onto various regions of membranes (Barr and Short, 2003; Cai et al., 2007; Short et al., 2005). For example, Rab1 recruits p115 and GM130. Rab2 effectors include GRASP 55 and Golgin-45. GM130 has been shown to interact with Rab2 and Rab33b (Barr and Short, 2003; Puthenveedu and Linstedt, 2001). Rab-1 can interact with Golgin-84 found at the cis-Golgi. Rab-6 interacts with Bi-caudal D1 and 2(Goud and Gleeson, 2010). Thus, multiple rabs molecules can interact with the same protein at different or similar stages of a tethering interaction. Studies on Uso1p, yeast homolog of p115 have demonstrated that Rab dependent (Ypt1p) and Rab independent mechanisms can be employed to bind membranes (Sztul and Lupashin, 2006). Additionally, evidence from studies on Golgin-84 and CASP protein suggest that apart from mediating the tethering reactions, Rab GTPases promote interactions between SNARE molecules or orchestrate structural

changes that allow coiled coil proteins such as golgins to better interact with organellar or vesicular membrane(Short et al., 2005; Sztul and Lupashin, 2006).

Integral to the execution and maintenance of protein transport and enzyme localization are tethering molecules like golgins and multimeric complexes such as golgins and COG (conserved oligomeric Golgi). Different types of molecular tether participating in secretory protein trafficking are discussed below.

Golgins

Golgins were first discovered in experiments trying to resolve the cause of specific autoimmune conditions in patients. Subsequently antigens responsible for this condition were found to be associated with Golgi apparatus and hence named as golgins (Barr and Short, 2003). Golgins are associated with the proteinaceous scaffold that makes up the Golgi apparatus (Barr and Short, 2003). Structurally, golgins are characterized by the presence of extensive coiled coil alpha helical domains which provides them with a rod like extension property. Electron micrograph images show the presence of linkages between vesicles and Golgi cisternae and the length of these connections correspond to the length of the observed coiled coil extensions of golgins (Sztul and Lupashin, 2006). p115 golgin was discovered as a participant of the Golgi transport pathway and in 1997 Nakamura et al identified GM-130 on the cis-Golgi membranes as one of its binding partners (Nakamura et al., 1997; Sztul and Lupashin, 2006). It was then proposed that p115 is a peripheral protein recruited from the cytosol on to the COPI vesicles in the intra-Golgi retrograde pathway and/or onto the COPII

vesicles as a part of the ER-Golgi anterograde traffic to mediate tethering events with Golgi membranes by means of a binary or ternary tethering complexes (Nakamura et al., 1997; Sztul and Lupashin, 2006). Resembling myosin chains, p115 is a homodimeric molecule distinguished by globular head domains and four coiled coil domains making up the rod region. In addition to this, it has been suggested that “hinge” regions present in these coiled coil domains are responsible for conferring rotational flexibility, which aids in the tethering process. Localization studies indicates that p115 can be found at ER exit sites, ERGIC and cis-Golgi regions (Altan-Bonnet et al., 2004; Short et al., 2005; Sztul and Lupashin, 2006).

GM130 is a coiled coil domain containing protein and extensively found at the cis-Golgi. Similar to p115, GM130 possesses several coiled coil domains and has been shown to interact strongly with GRASP-65 (discussed later) on the Golgi membranes. Its N-terminus interacts with the C-terminus of p115 and this protein-protein interaction is deemed necessary for the tethering of COPII vesicles from the ER during anterograde transport of cargo (Nakamura et al., 1997). The binding of p115 to GM-130 is dependent on its phosphorylation status with a phosphorylated GM-130 inhibiting the p115-GM130 interaction. This especially happens in the presence of mitotically induced cyclin dependent kinases (Nakamura et al., 1997). Interestingly, it has been observed that GM130 is required in CHO cells for proper functioning and structural maintenance only at high temperatures. Further, anti GM130 antibodies fail to disrupt Golgi structure.

Indicating the presence of mechanisms devoid of GM130 participation in vesicle tethering exist at cis-Golgi membranes (Sztul and Lupashin, 2006).

Giantin is a third member of the golgin family, also found at the cis Golgi. A triangular interaction between p115, GM130 and Giantin has been proposed in the tethering of COPI vesicles onto the cis-Golgi membranes. It is a transmembrane protein stably anchored in the thin Golgi bilayer with the transmembrane region confined to its C-terminus whereas the N-terminus contains its coiled coil regions (Sztul and Lupashin, 2006). Found only in mammalian cells, Giantin is positioned at the edges of medial Golgi and is involved in the tethering of COPI vesicles in the intra-Golgi trafficking pathway. p115 enables the linking up of GM130 on incoming COPI vesicles with Giantin present in the Golgi membranes (Sztul and Lupashin, 2006). Studies supporting this bridging model of tethering demonstrate that in the absence of Giantin, COPI vesicles cannot position themselves at the Golgi and hence are unable to interact with p115. Disruption in any of these proteins results in the accumulation of COPI vesicles that are not able to form tethers and fuse with the membranes. Both GM130 and Giantin bind to the same site on p115, thus competing in the process suggestive of their mutually exclusive roles (Short et al., 2005; Sztul and Lupashin, 2006).

In addition to the above-mentioned cis and cis-medial golgins, a variety of other golgins also exist. Golgin-84 is a transmembrane protein that is responsible for controlling the lateral extension of Golgi cisternae (Sztul and Lupashin, 2006). Golgins such as CASP (CCAAAT displacement protein alternatively splice

product) have been postulated to aid the SNARE fusion process and are type-II membrane proteins present at the ER. Experiments involving mammalian cells demonstrate that the presence of soluble extraneous CASP interferes with the normal wild type CASP and breaks down the retrograde pathway. Interestingly, vesicles bound by one tethering complex seem specific to each other. This has been demonstrated in studies where Golgin-84 –CASP bind a distinctive population of COPI vesicles (Lee et al., 2004).

Amongst those golgins associated at the periphery, GCP-60 is found to be associated with Giantin. Its precise roles in tethering are unknown, however an increased expression of GCP-60 causes a breakdown of transport between ER and Golgi and cripples the Golgi structure. GMAP 210 is a peripheral golgin of the cis-Golgi with a molecular weight of 210 KDa. It is associated with the microtubules and an over expression of GMAP-210 leads to a paralysis in both the anterograde and retrograde trafficking of molecules with vesicles accumulating in the cytoplasm. This suggests a possible tethering role for GMAP-210 (Short et al., 2005; Sztul and Lupashin, 2006). Other golgins like Golgin-45 are predicted to be involved in the tethering of COPII vesicles as experiments interfering with the normal role of this protein result in COPII vesicle accumulation. Two other golgin molecules namely Golgin-245 and Golgin-97 have been shown to have roles at the trans Golgi network. Both Golgin-245 and Golgin-97 participate in regulating vesicle transport from the endocytic pathway into the Golgi but Golgin 245 seems to have additional roles in the movement of

Golgi on the microtubule cytoskeleton leading to the microtubule organizing center (Sztul and Lupashin, 2006).

Golgi reassembly stacking proteins

Belonging to genre of proteins containing coiled coil domains, Golgi reassembly stacking proteins **(**GRASP) family members GRASP 65 and GRASP 55 have been shown to maintain the stacked nature of the Golgi apparatus (Seemann et al., 2000; Sztul and Lupashin, 2006). GRASP 65 is a 65 KDa protein and is localized to cis-Golgi membranes and interacts with membranes through its myristoylated group (Sztul and Lupashin, 2006). GRASP-65 interacts with GM130 serving as an anchor for this golgin. In concert with p115, they are responsible for maintaining the Golgi cisternal architecture (Short et al., 2001). GRASP 55 is the other, 55KDa Golgi stacking protein found on the medial cisternae. Using yeast two hybrid assays, Short and colleagues identified Golgin-45 as the interacting partner of GRASP55 (Short et al., 2001). RNAi studies and protein secretion assays found the GRASP55 –Golgin 45 complex to be essential for Golgi structural maintenance as well as the anterograde secretion of proteins (Short et al., 2001).

COG complex

The COG complex is a bilobed structure found to be peripherally associated with the Golgi stacks. It is characterized by the absence of any transmembrane or lipid binding motifs in its individual subunits which correlated with its localization on the cytoplasmic face of Golgi. The two lobes named A and B each consist of four subunits. Lobe A has Cog1p, 2p, 3p, 4p subunits and lobe

B has Cog5p, 6p, 7p and 8p subunits (Smith and Lupashin, 2008; Ungar et al., 2002). The COG subunits have been shown to interact with the Golgi membranes through protein-protein interactions with golgin molecules such as p115, GM130, v-SNARE and Rab molecules such as Yeast Ypt1.

Mutations in two subunits of the lobe A of COG complex namely Cog1 and Cog2 result in an aberrant profile of N- and O-glycans, glycolipids and defective turnover of the LDL receptor in Chinese hamster ovary cells. Cog5p shows a concomitant reduction in Cog1p and Cog2p mutants while Cog3p is reduced in latter as well indicating that individual subunits in lobe A act in concert with each other for full functional activities (Ungar et al., 2002). Like Cog1p, Cog2p mutants also exhibit a defective glycan profile of the glycoprotein cargo. While the forward trafficking and final localization of these transiting glycoprotein cargo is not compromised, the altered glycan profiles and observed buildup of medial enzymes such as Mannosidase II and GlcNAcT-I in Cog complex dependent vesicles (CCD) vesicles instead of Golgi compartments suggest to a defective enzyme availability to these substrate proteins as they transit through different Golgi stacks (Shestakova et al., 2006; Smith and Lupashin, 2008).

Depletion of COG subunits over an extended period of time has drastic effects on the Golgi. This is particularly evident in depletion of Cog3p and Cog7p subunits from the cells. Short-term effects of Cog3p mirrors the mutational effects in other lobe A subunits including the accumulation of CCD, some breakdown of Golgi and relatively low effects on the glycosylation of glycoproteins. On a long-term basis however, depletion of the Cog3p and Cog7p

subunits manifests into apparent defects in glycoprotein glycosylation. Relatively higher levels of medial Golgi enzymes such as GlcNAcT1 and Man-II have been demonstrated to accrue in CCD vesicles under extended absence of COG3 subunits with a final shunting of these enzymes to the ER (Shestakova et al., 2006).

Studies on fibroblasts derived from patients with a mutation in Cog-7 demonstrate a more detailed role of this subunit in the overall role of the COG complex. Using the redistribution patterns of proteins such as Giantin and βGalT to ER upon BFA treatments, depletion of Cog7 was demonstrated to compromise the retrograde transport rate of Golgi –ER transport traffic (Steet and Kornfeld, 2006). These Cog7 mutant fibroblasts exhibit compromised sialic acid modifications on their N- and O-linked glycoconjugates. Additional defects in these mutants include an increase in the steady state levels of the nucleotide sugars such as uridine diphosphate galactose (UDP-Gal) and cytidine monophosphate-sialic acid (CMP-Sia) and their reduced transport into the Golgi lumen. Tranferase activities of enzymes such as GalT and ST3Gal-I are also compromised in these Cog-7 mutants fibroblasts. Taken together, these results are indicative of aberrant glycosylation changes arising due to compromised function of multiple enzymes in the Golgi cisternae (Wu et al., 2004). In a manner similar to that observed for lobe A subunits, immunofluorescence staining demonstrated that subcellular localization of subunits 5,6 and 8 is also altered in Cog-7 mutant fibroblasts indicating that these lobe B COG subunits require and interact with each other for full functional utility at the Golgi (Steet and Kornfeld,

2006; Wu et al., 2004). In Cog-7 patient fibroblasts, v-SNARE proteins such as GS15 is reduced while GS28 though not reduced, displayed altered subcellular localization patterns. A parallel study which entailed RNAi mediated silencing of Cog-7, enzymes like GlcNAcT1 and GS15 were affected in a manner that reciprocated extended depletion of Cog3 in CHO cells while trans-Golgi resident enzymes like GalNAcT2, were not affected. Further, the glycosylation defects in Cog7 mutants arise before the observed effects in Cog 3 mutants suggestive of its role in transport of proteins between early Golgi and ER compartments. Additionally, Cog-7 depletion results in a concomitant depletion in the levels of t-SNARE protein Syntaxin-5 underscoring the extent of interactions between COG complex and vesicle fusion machinery at the Golgi membranes (Shestakova et al., 2006). In all the above-mentioned observations, the secretion of the glycoprotein cargo is not compromised indicative of a normal anterograde pathway. However, the persistent glycosylation defects along with mislocalization of key glycosylating enzymes to CCD vesicles points to the imperative role of COG complex in maintaining appropriate levels of Golgi resident enzymes and proteins through appropriate recycling events between Golgi stacks and the ER.

Integral proteins resident in Golgi membranes such as GEARs respond to perturbations in the COG complex. These proteins namely, GS28, GS15, CASP, Giantin, Golgin -84, Mannosidase II and glycoprotein GPP130 are reduced in manner that is reflective of the corresponding decrease encountered due to depletion of COG 1 and 2 subunits. Of the seven GEAR proteins, GS28, GS15 and ManII are directly sensitive to the levels of COG complex subunit 1.

Interestingly, the COG complex sub 1 and 2 are interdependent on ε-COP. Mutations in Cog1 and 2 disrupt the equilibrium of ε-COP associated with peri-nuclear region and increase the cytoplasmic pool of ε-COP. On the other hand, depletion of ε-COP at restrictive temperatures of 39.5^0C in yeast cells resulted in a reduction of COG-1 levels. Further, GEAR proteins such as GS28 and GS15 are also affected and reduced upon depletion of ε-COP. Together these results strongly suggest of an highly interdependent relationships between COG and specific COPI subunits to ensure proper localization of GEAR proteins such as GS28 and GS15, which in turn might play a crucial role out in ensuring appropriate recycling of Golgi enzymes and glycoprotein glycosylation (Oka et al., 2004).

Together, the above observations underscore the role of multi-protein complexes in ensuring the steady state distribution of resident proteins and enzymes in the secretory pathway. The glycosylation defects that arise as a result of perturbations in the COG complex subunits stress the importance this multimeric tethering complex in maintaining equilibrium of recycling glycosyltransferases at the Golgi. It has been demonstrated previously that enzymes like GlcNAcT2 traverse through the cis Golgi to the trans Golgi and TGN via the medial Golgi during their lifetime (Shestakova et al., 2006). The COG complex in concert with SNAREs and Rab GTPases helps in this process by tethering the vesicles containing these enzymes during this recycling phase through the Golgi stacks. But in the event of a failure of COG complex to carry out its role, the Golgi glycosyltransferases get mislocalized leading to defective

glycan processing of key proteins (Steet and Kornfeld, 2006; Wu et al., 2004). An interesting question then arises as to what happens to these glycosyltransferases once they are mislocalized. Shestakova et al report that one of immediate effects of Cog7 deficiency is the accumulation of these enzymes in CCD vesicles. Likewise, Cog3 knockdown caused a reduction in the endogenous levels of ManII and degradation of enzymes like GlcNAc-T1. In other COG subunit mutants, for example Cog1, GS28 protein degradation was also observed (Shestakova et al., 2006). While in Cog 7p mutants, nucleotide transport and activity of GalT and ST3Gal-I is compromised. Alteration of localization of these glycosyltransferases and glycosidases reflects in their function and activity. For these enzymes to achieve their maximal optimal activity, the right environment is a necessity. It has been proposed that, these glycosylation enzymes associate with each other to increase the efficiency of the glycosylation process. For example, GlcNAcT1 and ManII have been shown to interact via their stem regions and this interaction contributes to their sequential action on the oligosaccharide moiety (de Graffenried and Bertozzi, 2004). Absence of any of these enzymes due to altered trafficking by a compromised COG complex can reduce or completely nullify the activity of the other glycosylation enzymes. Alternatively, the absence of appropriate microenvironment could channel these enzymes for degradation either through the ERAD or lysosomes (Steet and Kornfeld, 2006). Additionally altered trafficking might lead to their eventual transport to endosomes or lysosomes, but this can be disputed by the observation that the CCD vesicles fail to colocalize with endosomal, ER or

lysosomal markers on cog subunit knockdown (Shestakova et al., 2006; Steet and Kornfeld, 2006). Lack of availability of nucleotide sugars was also suggested to be a reason for altered glycosyltransferase activity (Steet and Kornfeld, 2006). Nearly, all of the COG subunit mutations result in altered glycosylation and in cases where two subunit mutations are present; a much more heightened defect in glycosylation is observed (Shestakova et al., 2006). These and other additional reports attributing COG in retrograde transport mechanisms of Golgi enzymes underscore the importance of this complex in proper functioning of the Golgi and the cell as a whole.

The other proteins that modulate tethering reactions are the GARP, Dsl1, Exocyst and TRAPP complexes. Golgi associated retrograde protein (GARP) complex localizes to the trans-Golgi network. Human GARP has been shown to associate with Rab6 while yeast GARP complex acts in the retrograde pathway between endosome and the trans-Golgi network. TRAPP I and II complexes differ in their localization at the Golgi membranes. Studies on TRAPP I complex demonstrate the existence of strong interactions between COPII and this complex indicating its presence at the cis Golgi (Shestakova et al., 2006; Spang, 2008). On the other hand, TRAPP II is present at the medial Golgi and is responsible for vesicular transport between the Golgi cisternae themselves (Sztul and Lupashin, 2006). In contrast to all other tethering molecules and multiunit complexes mentioned above, the exocyst localizes to the plasma membrane. By doing so, it has been suggested to serve as a landmark for vesicles destined to reach the plasma membrane. Dsl1 is a yeast specific ER associated protein

complex that plays a role in the retrograde trafficking process. All the above protein complexes are evolutionarily conserved which indicates their similar role in the secretory process (Nakamura et al., 1997; Sztul and Lupashin, 2006).

Golgi Glycosylation And Kinase Signaling

Secretory cargo flux is dependent not only on organelles such as ER and the Golgi but also on a multitude of signaling events involving post-translational modifications such as phosphorylation. Protein kinase based signaling has been demonstrated to co-ordinate multiple events in mitotic and interphase Golgi (Nakamura et al., 1997; Preisinger and Barr, 2005). While kinases like Cdk I (cyclin dependant kinase I), Plk (Polo like kinase) I and III, ERK (extracellular-signal-regulated kinase) I/2 and MEK (MAPK/ERK kinase) I participate in the mitotic division of Golgi; Casein Kinase 2, PKA and PKD have been suggested to regulate vesicular traffic at the Golgi (Altan-Bonnet et al., 2004; Nakamura et al., 1997; Preisinger and Barr, 2005). The substrates for these kinases range from Rab GTPases to Golgi matrix proteins like GM130 and Golgi stacking proteins like GRASP65 and GRASP55.

Mitotic fragmentation and orchestrated dispersal of Golgi stacks is a highly regulated process. During mitosis, phosphorylation of proteins such as Rab 1 and GM130 by Cdk1 prevents ER- derived vesicles to fuse with cis-Golgi. Cdk1 phosphorylation of Rab1 has also been proposed to result in fragmentation of Golgi. However, phosphorylation of GRASP65 by the Cdk1 is required for Golgi reassembly suggesting that Cdk1 can play distinct role in Golgi organization during the cell cycle process. Plk1 and 3 are two other kinases that have been

demonstrated to participate in mitotic disassembly of Golgi structure. While Cdk1-cyclin B and Plk1 are absolutely required to unstack individual cisternae, Cdk1-cyclin B essentially drives subsequent fragmentation and dispersal process of the Golgi apparatus (Preisinger and Barr, 2005).

In the interphase part of cell cycle, kinases such as CK 2 and CK 2 like, Src tyrosine kinases, protein kinase A (PKA) and protein kinase D (PKD) have been implicated in controlling trafficking dynamics at the Golgi. While CK2 directed phosphorylation of p115 has been demonstrated to reassemble Golgi stacks, it also leads to increased association of p115 with GM130 and cis-membranes. Ck2 like kinases are involved in vesicular transport between the trans-Golgi network and endosomes (Altan-Bonnet et al., 2004; Preisinger and Barr, 2005). Src tyrosine kinases are known to regulate signal transduction events through tyrosine phosphorylation of proteins such as Ras. Src- activated pools of Ras have been demonstrated to exist at the Golgi and co-ordinate cellular processes such as oncogenic transformation, and differentiation. A depletion of this pool of activated Ras molecules leads to morphological disturbances in Golgi organization and an impaired retrograde trafficking of cargo (Preisinger and Barr, 2005).

The association of multiple different kinases with the Golgi apparatus is also dependent on the polarized nature of different cell types. Pools of protein kinase D are present at the trans-Golgi network and its members PKD1 and PKD2 have been demonstrated to control the vectorial trafficking of secretory cargo from the TGN to the cell membranes on the baso-lateral side of MDCK

cells. While PKD1 co-ordinates vesicle budding through exocyst 6 complex, PKD2 does not seem to utilize this complex (Preisinger and Barr, 2005; Rozengurt et al., 2005). The polarized nature of cells is often perceived to be static, however dynamic processes such as cell migration also confer specific directionality to intracellular organization. Critical to this process is the orientation of the microtubule organizing center (MTOC) and Golgi apparatus in the direction of cellular migration (Mellor, 2004). The association of Ste20 family members YSK1 and MST4 with GM130 has been demonstrated to be crucial for cellular migration as well as polarizing Golgi orientation in this direction. Working under cues from GM130 protein, YSK1 and MST4 work in antagonistic ways to regulate cell migration. YSK1 has been shown to phosphorylate 14-3-3z which in turn regulates several downstream proteins like integrins, Par3/Baz and Raf1 to affect cellular processes such as adhesion, and cell polarity that are integral to migratory behavior (Mellor, 2004; Preisinger and Barr, 2005). AMP activated protein kinases are a recent addition to the family of signaling molecules affecting the Golgi complex. An increase in cellular AMP:ATP ratios results in AMPK phosphorylation of Golgi specific Brefeldin A resistance factor 1 at Thr 1337 and consequent induction of Golgi fragmentation (Miyamoto et al., 2008). Studies on *Drosophila* Sff/SAD kinase, an AMPK family protein, show that in absence of Sff signaling Golgi compartmental dynamics is altered with bidirectional changes in glycoprotein glycosylation and increased overlap of glycoprotein cargo such as Fasciclin II with with trans-Golgi compartments (Baas et al., 2011). These results suggest that perturbations at the level of Golgi organization through these

kinases and their effectors proteins can result in global glycosylation changes in response to various external stimuli.

While kinase signaling can orchestrate changes in the behavior of proteins that regulate Golgi architecture and function, it can also directly influence the activity of glycan processing enzymes. Levels of key enzymes in ganglioside synthesis such as GalNAcT and sialyl transferases I (ST-I) are affected by activation of kinases such as PKA and PKC. In-vitro kinase assays demonstrate that sialyl transferase II (ST-II), III(ST-III) and IV (ST-IV) are phosphorylated by PKA on their cytoplasmic regions. The cytosolic presence of PKA and PKC is aptly positioned for phosphorylating these membrane embedded enzymes on their cytosol exposed amino acid regions. These enzymes can also be phosphorylated on their luminal side by kinases that are resident in the Golgi such as Golgi casein kinase II. Together, these observations suggest that both external stimuli and intracellular cues can mold glycoprotein glycosylation output through phosphorylation-based events.

HRP Epitopes Of The *Drosophila* Embryo

The antigenic determinant responsible for the anti-HRP antibody binding is a fucose moiety in an α-1,3 linkage to the asparagine linked to the inner most GlcNAc of the chitobiose core. These epitopes structurally encompass difucosylated α-1,3 and α1,6 paucimannose, hybrid and complex type N-glycans (Aoki et al., 2007; Baas et al., 2011). HRP epitopes are prevalent in the neural tissues such as the ventral nerve chord (central nervous system), peripheral sensory neurons and non-neural tissues such as the posterior hindgut, garland

glands and anal pads of the *Drosophila* embryo (Jan and Jan, 1982). Interestingly, the expression of these epitopes is not only tissue specific, but also protein specific and developmentally regulated(Katz et al., 1988). HRP epitopes are expressed on all embryonic neurons. Additionally these are also expressed at all stages of development albeit on distinct sets of proteins at different stages of development (Jan and Jan, 1982). Seminal studies on these epitopes in the embryonic CNS of invertebrates like *Drosophila* and Grasshoppers revealed them to be expressed on several neuronal proteins. For instance, Fasciclin I and II on axon fascicles, Neurotactin and Neuroglian involved in adhesion and signal transduction pathways in nervous system, receptor linked tyrosine phosphatases and the more recently discovered Nrv2 protein- Nerve Antigen express the HRP epitopes (Paschinger et al., 2009). The expression of these epitopes is concomitant with the appearance of post mitotic neurons derived from the ganglion mother cells(Jan and Jan, 1982). Thus, the HRP epitopes serve as an excellent marker for tracking the progress of neural development in the *Drosophila* embryo.

Development Of Embryonic Nervous System: *Drosophila* Neurogenesis

Dermal lineages of every cell in an organism can be attributed to one of the three germ layers namely, the ectoderm, mesoderm and endoderm. The developmental process that transits a blastula to an embryo containing these three germ layers is known as gastrulation. It begins with the formation of a ventral furrow and is spatially and temporally coordinated with the assistance of maternal and zygotic gene products. In *Drosophila* embryos prior to gastrulation,

cells destined to become the ectoderm are present dorsally to the cells that will proceed to attain mesodermal fate. On the other hand, cells acquiring endodermal lineage are restricted to the anterior and posterior poles of the pre-gastrulation embryo. With the formation of ventral furrow, the presumptive mesoderm and endoderm invaginate at the anterior part of the embryo followed by the invagination of the presumptive endoderm at the posterior end of the embryo. Eventually this process leads to the movement of endoderm and mesoderm layers into the interior of the developing embryo. Towards the end of gastrulation period, a rearrangement of the germ layers takes place such that the ectoderm is on the outside and endoderm and mesoderm layers are on the inside of the embryo. Gastrulation thus, paves the way for the embryo to initiate the process of forming different organs dedicated towards accomplishing diverse biological functions for the future developmental stages of larvae and adult (Campos-Ortega and Knust, 1990).

Drosophila embryonic nervous system arises from distinct areas of the ectoderm known as neurogenic regions (Urbach and Technau, 2004). The procephalic neurogenic region (PNR) gives rise to the future embryonic brain and the ventral neurogenic region (VNR) gives rise to the ventral nerve chord. Ectodermal cells at the onset of gastrulation are endowed with intrinsic information conveyed through maternal and zygotic gene products and positional/ extrinsic cues dependent on their placement along the dorso-ventral and antero-posterior axis (Leptin, 1999; Perrimon et al., 2012; Stuttem and Campos-Ortega, 1991). The propensity to adopt neurogenic fates is highest in

the ectodermal cells of the PNR region followed by the cells of the VNR. Homotypic and heterotypic transplantation studies of single cell and cluster of cells, have led to postulation that inductive signals in the VNR and inhibitory signals from the Dorsal Epidermal Analage (DEA) contribute towards the specification of neural and epidermal lineages of cells in the gastrulating ectoderm (Luer and Technau, 1992; Stuttem and Campos-Ortega, 1991). Cell culture studies on ectodermal cells isolated at the earliest stages of gastrulation have shown that positional information of cells along the dorso-ventral axis in the ectoderm contributes in the decision of whether a particular ectodermal cell adopts a neural or a epidermal fate (Luer and Technau, 1992). For instance, ectodermal cells isolated form Stage 7 *Drosophila* embryos at 0-5% ventro-dorsal length develop exclusively into neural clones and those isolated from the more dorsal part of the embryo (i.e., 55-100% DV) formed epidermal clones. Interestingly, cell culture studies of isolated ectodermal cells from gastrulating embryos weigh in favor of the intrinsic capacity of cells to adopt a neural vs epidermal fate in comparison to the extrinsic cues provided by either the VNR or DEA (Luer and Technau, 1992). Thus, a complex interplay of intrinsic information and positional cues contributes to the delineation and segregation of neural precursor cells, which then go on to form the embryonic nervous system.

The process of neurogenesis or the formation of the nervous system involves several steps and begins after the delineation of the neural region from non-neural part of the ectoderm (Doe and Goodman, 1985a). Elegant studies carried out on the developing grasshopper embryonic nervous system have

shown that cells from the neuroectodermal lineage eventually can give rise to various differentiated cell types such as neuroblasts and midline precursor cells that will form the future neurons, glial precursors that will develop into glia and several other non-neural cells that will become a part of the developing neuroepithelium and exist to support the developing nervous system. The first step in insect neurogenesis is marked by the swelling of a single neuro-ectodermal cell in a group of equivalent cells to form a stem-cell like neuroblast (NB)(Doe and Goodman, 1985a). This wave of neurogenesis begins in the thoracic segments of the gastrulating embryo and proceeds along the antero-posterior axis. During the time of neuroblast formation and differentiation, the remaining neural ectodermal cells in the vicinity start attaining non-neural characteristics and eventually function to support to the newly formed neuron. The embryo thus, witnesses the transformation of a single layer of neuroectodermal cells into a tissue that will go on to become the future nervous system of the adult insect. Specification of a neuroblast and the associated differentiation of the surrounding neural ectodermal cells into non-neural support cells thus underscores the spatial and temporal precision of signaling cross talk that exists between cells across the entire AP axis during the formation of the nervous system.

Molecular Signals During *Drosophila* Neurogenesis

During gastrulation, the distribution of ubiquitously expressed maternal transcription factor Dorsal along the dorso-ventral axis is critical in specifying ectoderm, neuroectoderm, mesoderm and mesoectoderm regions(Leptin, 1999).

It activates the zygotic gene products of Twist and Snail in the mesoderm progenitor cells that are located ventrally in the gastrulating embryo. Both Twist and Snail are transcriptional factors that carry out relatively opposing functions in the prospective mesoderm cells. While twist acts to promote mesodermal marker gene expression, Snail works to repress the expression of ectodermal genes in the mesodermal cells. Together, Snail, Twist and Huckebein determine the DV and AP borders, of the presumptive mesoderm in the embryo. Dorsal activates Short of gastrulation (Sog) in the ventral part of the embryo which restricts the influence of Decapentaplegic (Dpp) to dorsal part of the ectoderm This division along the DV axis leads to an activation of pre-patterning genes such as *vnd*(*ventral nerve chord defective*)*, ind* (*intermediate neuroblast defective*) and *msh (muscle specific homeobox gene)* such that each of these genes is expressed in a columnar fashion along antero-posterior axes in the ectoderm. Simultaneous action of pair rule and segment polarity genes takes place in a row like manner with their expression pattern stretching from the medial to the lateral part of the ectoderm. Together these events contribute to the activation and expression of proneural genes that will lead to the delamination of neuroblasts from the ventral neuroectoderm (Hartenstein and Wodarz, 2013).

Inside the ectoderm/ neuroectoderm itself, the decision to adopt a neural versus an epidermal fate is decided by the action of two sets of genes: proneural and neurogenic. The proneural genes work to promote neurogenesis in the cells predetermined to adopt neural fate(Campos-Ortega and Knust, 1990). Prior to neuroblast delamination, the positional expression of these genes in the ventral

neuroectoderm generates groups of cells called proneural clusters or equivalence groups. Roughly 10 proneural clusters exist per hemisegment in the embryonic ventral nerve chord and every cell in each of these proneural clusters is capable of attaining a neuroblast fate.

Proneural genes such as Achaete *(ac)*, scute *(sc)* and lethal of scute *(l'sc)*, and asense (*ase*) collectively constitute the AS-Complex and participate in assigning proneural characteristics to the neuroblasts. The *ac, sc, l'sc* genes genes encode for various beta helix loop helix transcription factors and are expressed in NBs prior to delamination. Their expression is down regulated after the neuroblast segregates from the neuroectoderm. Following delamination, *ase* along with *prospero* and *snail*, is turned up in the neuroblasts that have segregated from the proneural clusters in the ectoderm. Together with another transcription factor such as Daughterless, Achaete, Scute and Lethal of scute proteins can dimerize and aid the proneural fate assigning process. In contrast, Extramacrochaete can bind with Ac, Sc and L'sc proteins and prevent their proneural activities. However, the assignment of proneural fate is not completely exclusive to these genes, as mutations in AS-C genes do not result in a complete loss of all the neuroblasts. This observation is suggestive of the presence of additional signals/proteins involved in the proneural fate assignment process. Thus, proneural genes work to promote neurogenesis and lead to the segregation of individual neuroblasts that will gives rise to the future neurons and/or glia (Hartenstein and Wodarz, 2013; Skeath and Thor, 2003).

Neurogenic genes on the other hand, work to suppress the neurogenic potential of neuroectodermal cells such that only 500 out of 2000 cells in the strip of neuroectoderm adopt neural lineage and the rest develop into epidermal cells(Campos-Ortega and Knust, 1990). The action of neurogenic genes thus, causes only one cell from each proneural cluster to attain NB fate and the rest to progress along the epidermal lineage. *Notch, Delta, neuralised, almondex, master mind, big brain* and *Enhancer of Split complex [E(spl)C]* constitute the neurogenic gene group. Transplantation of cells that are mutant for *Notch, delta, neuralised, almondex* and *master mind* genes into wild type ectoderms leads to their development as epidermal cells pointing to their role in the signal sending cell which in this case, would be the cell attaining neural fate. Notch and Delta encode for transmembrane proteins with Delta serving as one of the ligands that interacts with the Notch receptor during neurogenesis. Prior to neuroblast delamination, *delta* RNA is uniformly present in across the neuroectoderm initially but with the delineation of proneural clusters, it expression is restricted to segregating NBs. A feed forward loop ensues to consolidate Delta expression in the neural cells and leads to notch expressing cells adopting epidermal fate. The Enhancer of Split Complex *[E(spl)C]* locus functions in a reciprocal fashion with the Achaete-Scute Complex (AS-C) to regulate neurogenesis. Mutations in this locus result in a phenotype that resembles an enhancement of the *split*, a recessive Notch mutation (Campos-Ortega and Knust, 1990). The *[E(spl)C]* locus gene products are expressed in the neuroectoderm along with the AS-C proteins before the formation of neuroblasts(Hartenstein and Wodarz, 2013).

Gross Anatomy Of The *Drosophila* Ventral Nerve Chord

In *Drosophila*, the central nervous system is made up of the brain and a ventral nerve chord (VNC). The VNC is made up of fourteen neuromeres (or segments) with three in gnanthal (future mouth appendages), eight in abdominal and three in thoracic regions of the embryo (Urbach and Technau, 2004) The precursor cells defining these neuromeres/segments delaminate from the underlying ectoderm at approximately eight hours into embryogenesis (Hartenstein and Wodarz, 2013). In each embryonic hemisegment, roughly 30 neuroblasts form, arranged in a pre-specified manner in the developing nerve chord. The neuroblasts and the resulting lineages of cells are arranged in a laminar manner between the mesoderm on the dorsal and ectoderm on the ventral sides (Ortega and Hartenstein, 1985).

The corresponding neuroblasts across different segments originating at similar times during development follow similar developmental cell lineages. The positional placement of NBs follows a strict pattern of a seven by three grid made up of rows and columns across AP and DV axes, respectively. The 30 NBs in each hemisegment give rise to rise to roughly 350 cells, 80 % of which are interneurons and the rest are motor neurons and glial cells (Urbach and Technau, 2004).

Each neuroblast in a hemisegment has a unique identity that is conferred to it by virtue of its position across the AP and DV axes, timing of birth and the expression profiles of pattering and columnar genes in these axes. It has been postulated that columnar genes also regulate the size of clones that arise from

the initial set of 30 NBs per hemisegment, with the numbers being higher in thoracic versus abdominal segments of the developing embryo speculatively through increased cell division or reduced cell death versus increased cell death in abdominal segments (Skeath and Thor, 2003).

Every cycle of cell division in a neuroblast is unequal and yields a comparatively smaller ganglion mother cell (GMC) and a daughter neuroblast. A given neuroblast can divides a fixed number of times before its disintegration while a ganglion mother cell that divides only once to yield a neuron/glia pair or two neurons. At the molecular level, events are initiated by the transcription factor Prospero, which in complex with Miranda an actin binding protein, recruits the help of apically located Baz/dPar-3/DmPar-6/DaPKC and Gαi/Partner of Inscutebale/Inscuteble complexes to localize to the basal end of the dividing neuroblast. Once the NB undergoes cell division, Prospero selectively gets localized to the newly formed GMC. Each GMC further undergoes a single division event to yield two neurons/glia. Prospero then prevents additional rounds of cell division by suppressing cell cycle genes (Skeath and Thor, 2003). Each GMC that arises has a unique identity assigned to it by the virtue of its time of birth from the respective neuroblast (Doe and Goodman, 1985a, b). The control of cell lineages that arise from specific neuroblasts is regulated through the coordinated temporal expression of five genes (in the order of expression): Hunchback (Hb), Kruppel (Kr), Nubbin and Pdm2 (Pdm), Castor(Cas) and Grainyhead(Gh). These genes encode for various transcription factors and are expressed sequentially during neuroblast divisions. For instance, the generation

of *Drosophila* motor neurons U1-U5 from NB 7-1 begins with the earliest born U1 and U2 neurons requiring Hb expression in the first and second neuroblast division. Following this, the orphan nuclear receptor Seven up Svp, enables a switch from Hb to Kr expression during the second round of division in NB 7-1. The expression of Kr at this stage thus leads to the generation of U3 pair of neurons from the GMC arising after the third division and expressing Kr. This is then followed by the expression of transcription factor Pdm, which facilitates the formation of U4 neurons during the fourth division cycle in NB 7-1. The transcription factor Cas can serve multiple roles depending on the particular neuroblast lineage in consideration. For instance in NB 7-1 and 3-1, Castor acts in concert with Pdm in the specification of U5 and RP5 neuron identity at the end of fifth division. Castor finally acts in the last round of cell division cycles in sculpting interneuron identities in NB7-1 and 3-1 lineages. On the other hand, the temporal expression of Cas in later cell divisions of NB 5-6, allows for its participation with a cocktail of other transcription factors such as Collier (Col), Squeeze (Sqz), Grainyhead (Grh) and Svp, leading to the generation of Apeterous (Ap) neurons from this neuroblast lineage. Controlled temporal expression of these transcription factors thus leads to the generation of unique neural identities in each hemisegment during *Drosophila* neurogenesis(Kohwi and Doe, 2013; Skeath and Thor, 2003).

Mutations Affecting HRP Epitopes' Expression In The Developing Embryo

While HRP epitopes are an excellent tool for studying *Drosophila* embryonic neural development, absence of these epitopes has been shown to

result in a series of wide ranging aberrant phenotypes such as locomotor defects and wing axon directionality defects (Baas et al., 2011; Katz et al., 1988). Thus, the appearance and expression of HRP epitopes is crucial for normal development of *Drosophila melanogaster*. Consequently, an understanding of the HRP epitope expression and regulation will further shed light onto the mechanisms controlling glycoprotein glycosylation in a broader context across animal kingdom. Genes affecting the expression of HRP epitopes can be classified as effectors of their (a) biosynthetic synthesis or (b) cellular signaling events regulating the final expression of these glycans on their respective cell surface proteins (Fig1.3).

Glycosyltransferases such as GlcNAcT-I, fucosyltransferase A (FucTA) and glycosidases like Fused Lobes (Fdl), a β *N*-acetylhexosoaminidase, participate at specific steps in the biosynthetic generation of HRP epitopes (Aoki et al., 2007; Katoh and Tiemeyer, 2013). For instance, Fdl enzyme is responsible for the generation of high mannose and paucimannose structures in *Drosophila*. GlcNAc T-1 generates hybrid and complex type of N-glycans. FucTA is the enzyme responsible for the addition of α 1, 3 linked fucose onto the reducing GlcNAc to generate the HRP epitopes. In addition to this, Golgi nucleotide sugar transporters such as GDP fucose transporter (Gfr) are required for the transport of GDP-fucose into the lumen of the Golgi compartments for the relevant downstream enzymatic machinery to act upon it. *Neurally altered carbohydrate* (*nac*) is a mutation in the *Gfr* gene and leads to a progressive reduction in larval, pupal and adult stage HRP epitopes. It causes defects in paths of axon

emanating from the wing sensory neurons (Katz et al., 1988; Whitlock, 1993). Thus, the appearance of HRP epitopes is critically dependent on the functioning of the enzymatic machinery responsible for generation of these epitopes.

Tollo/Toll-8 and sugar free frosting (sff) are genes that fall under the category of effectors that regulate glycosylation through cell signaling events of the secretory pathway. Mutations in these genes affect the expression of HRP epitopes during embryonic stages of *Drosophila* melanogaster life cycle in a very striking manner (Baas et al., 2011; Seppo et al., 2003). In case of *tollo* mutants, HRP epitope expression is completely lost from the embryonic nervous system while it is preserved in non-neural tissues such as garland gland, posterior hindgut and anal pads. *Tollo* encodes for a cell surface receptor that belongs to the toll-like receptor family (TLR); members of this family are homologous to the Toll protein in *Drosophila* (Seppo et al., 2003). Toll and TLRs have been shown to control dorso-ventral patterning and innate immune responses, respectively (Hashimoto et al., 1988; Takeda et al., 2003).

However, Tollo/Toll-8 presents a novel function for the family of TLRs through its participation in the regulation of glycoprotein glycosylation in the *Drosophila* embryo. *In-situ* hybridization studies indicate the presence of *tollo* mRNA in the ectodermal cells surrounding differentiating neurons. This observation coupled with a loss of HRP epitopes in a neural specific fashion has led to the proposition of a signaling mechanism where the Tollo receptor on a closely positioned ectodermal cell surface is involved in the generation of a

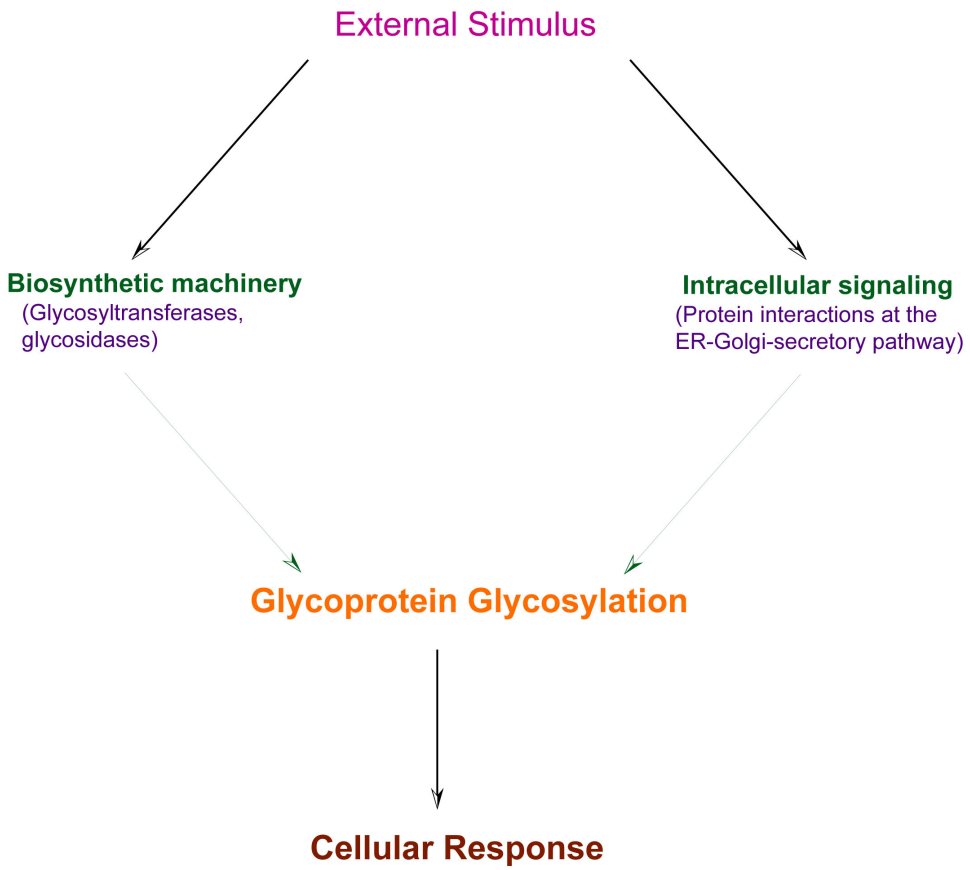

Figure 1.3. Pathways regulating glycoprotein glycosylation. The expression of glycans on cargo molecules transiting the secretory pathway is dependent on inputs from the biosynthetic machinery and cellular signaling events. These arms in turn can be controlled by external stimuli such as developmental, temporal or nutrient cues. The appropriate participation of each regulator feeds into any or both of the two arms controlling glycosylation, which in turn determine the cellular output in response to a particular stimulus.

paracrine signal that directs downstream events in the surrounding and differentiating neurons to express HRP epitopes (Seppo et al., 2003). Glycomic analysis on *tollo* mutant embryos indicates a reduced prevalence of α 1,3 and 1,6 difucosylated glycan structures. However, the trend was not applicable to all fucosylated structures with the prevalence of a major glycan bearing α 1,6 fucose not being altered due to the *tollo* mutation. At the same time, an increase in paucimannose glycan structures predicted that the *tollo* mutation rendered glycomic profiles with similarities to young embryos. However, challenging this idea was the observation where hybrid structures such as $GlcNAcMan_4GlcNAc_2$ (NM4N2) and $GlcNAcMan_5GlcNAc_2$ (NM5N2) were increased (Aoki et al., 2007).

In a manner similar to *tollo* mutants, *sff* also affects multiple pathways during the generation of glycosylated structures in *Drosophila* embryo. A majority of HRP epitope glycans is decreased with atleast a 50% reduction as compared to wild type embryos. However, an increase in prevalence of one complex type HRP epitope glycan namely, N2M3N2F2 has been observed in *sff* embryos. In addition to this complex glycan structures, an increase in NM3N2 and $N3M3N2F^6$ was observed in these mutant embryos. *Sff* as a hypomorphic recessive mutation present on the third chromosome causes a distinct reduction but not complete ablation in HRP epitopes' expression across neural tissues. In the embryonic nervous system, the anti-HRP antibody staining is reduced to the axonal scaffold giving a frosted appearance to the ventral nerve chord. Some amount of reduction in HRP epitope staining is also observed in non-neural tissues such as garland gland, posterior hind-gut and anal pads. The *sff* mutant flies are adult

viable and do not show any gross morphological or anatomical defects. However, these flies exhibit reduced negative geotaxis, a characteristic ability of flies to crawl back against gravity when tapped to the bottom of a culture/growth vial. *In-situ* hybridization and mass spectrometric analysis reveal decreased *sff* mRNA and Sff peptide levels reiterating the hypomorphic nature of the mutation (Baas et al., 2011). Further, embryos transheterozygous for *tollo* and *sff* i.e., with single copies of *tollo* and *sff* mutations, show a composite phenotype with *sff* like frosted staining of the ventral nerve chord and *tollo* like staining in non-neural tissues such as posterior hind gut and garland gland (Baas et al., 2011) in HRP epitope expression suggesting genetic interaction between *tollo* and *sff*. In event of no interaction between the mutant alleles, transheterozygous embryos would have exhibited a wild-type HRP epitope expression pattern. Since Tollo mRNA detected in ectodermal cells surrounding differentiating neurons in the developing embryo, it can be hypothesized that Tollo influences Sff signaling to regulate the expression of HRP epitopes (Seppo et al., 2003).

Sff/SAD Kinase Signaling

Sugar free frosting is the *Drosophila* homologue of *C.elegans* and synapses of amphids defective (SAD) and vertebrate brain specific (BRSK 1 and 2) kinases (Crump et al., 2001; Kishi et al., 2005). Studies in worms, mice and vertebrate cell lines demonstrate the indispensable nature of the SAD kinase in regulating axon-dendrite polarity in neurons, release of neurotransmitter filled vesicles at presynaptic junctions and activation in response to DNA damage and consequent halt in transition from G2(growth) to M(mitosis)phase of Hela cells'

life cycle (Crump et al., 2001; Inoue et al., 2006; Kishi et al., 2005; Lu et al., 2004).

Drosophila Sff kinase modulates the association of atleast one known glycoprotein Fasciclin II with different Golgi compartments, presumably by phosphorylating key substrate proteins that trigger signal transduction events in the secretory pathway leading to altered glycosylation patterns at the cell surface (Baas et al., 2011). This role of Sff kinase is in agreement with its roles at the presynaptic junctions of *C.elegans* and mammals. Studies on the SAD mutants in worms and mice have distinctly shown that this kinase is also responsible for clustering neurotransmitter filled vesicles into restricted domains called actives zones (AZ) at the synapse (Crump et al., 2001; Inoue et al., 2006). The AZ is a dense area with a number of different molecules that contribute in the docking, fusion and release of synaptic vesicles (Schoch and Gundelfinger, 2006). Enriched with proteins from five families namely Munc-13s, RIMs (Rab-3 Interacting molecules), ERCs (ELKS/CAST/Rab-6 Interacting proteins), Liprin-α, Bassoon and Piccolo, the AZ forms a protein scaffold to contain the readily released pool of synaptic vesicles filled with neurotransmitters(Schoch and Gundelfinger, 2006). The RIMs are an evolutionarily conserved set and made up of Zn^{2+} finger domain, PDZ domain in the middle and C2A and C2B domains. Because of these domains, RIM proteins serve as backbone to which the remaining protein families tether on to (Wang et al., 2002). SAD –B is tightly associated with the cytomatrix of the active zone and has been shown to phosphorylate RIM at its C-terminal C2B domain (Inoue et al., 2006). Primarily,

these protein families are restricted to the presynaptic region; but evidence also points to cytoplasmic distribution for some of these proteins such as ERCs, Liprin-alpha, SAD kinase also (Inoue et al., 2006; Wang et al., 2002). Additionally, ERCs have been demonstrated to interact with a Rab-6 GTPase (Wang et al., 2002). Bicaudal D (Bic D), coiled coil domain protein found at the Golgi, has been demonstrated to tether Rab6a coated COPI-independent vesicles to the dynein/dynactin complex at the cell periphery and mediate the transport of COPI-independent retrograde cargo (Girod et al., 1999; Matanis et al., 2002). *In-vitro* studies using PC12 cells demonstrate that SAD-A kinase can phosphorylate cytoskeletal protein Tau. Ablation of SAD isoforms in mouse cortex leads to reduced levels of phosphorylated Tau and increased amounts of dephosphorylated Tau in the dendrite enriched region. A shift in the equilibrium of phospho-Tau to dephospho Tau contributes to aberrant neuronal polarities in neurons emerging from the cortex region (Kishi et al., 2005). Together, these results strongly suggest that high intensity trafficking sites presumably involve similar protein molecules like coiled coil proteins such as Liprin-alpha, golgins, rab molecules such as Rab6 and elements of the cytoskeletal network to carry out vesicular transport.

While much is not known about the downstream effectors of Sff/SAD kinase signaling, various groups have identified key upstream regulators of this serine threonine kinase. Two signaling pathways, one involving a serine/threonine kinase liver kinase B 1 (LKB1) and the other involving Target of Rapamycin 1 (TORC1) can act upon it. Mouse knock-outs of LKB1 and SAD

kinase genes show similar phenotypes such as loss of neuron polarization, an absence of clearly delineated axon and dendrites attributable to a shift in the phospho-Tau and dephospho Tau equilibrium. Additionally, transfecting HeLa cells that endogenously express STRAD-α and MO25 with LKB1 leads to phosphorylation of SAD kinase (Barnes et al., 2007). On the other hand, TORC1 through the GTPase activating protein Tuberous Sclerosis Complex (Tsc)2 and Rheb GTPase to control SAD kinase mRNA levels. In the presence of growth promoting external cues such as brain-derived neurotrophic factor (BDNF) and insulin like growth fact (IGF-1) signaling pathways involving PI-3 kinase and Akt get activated and consequently inactivate Tsc2 (Choi et al., 2008). Inactivation of Tsc2 leads to Rheb GTPase based activation of TORC1which in turn activates post-translational regulators like S6Kinase (Wildonger et al., 2008). An increase in SAD kinase and phosphorylated SAD and Tau levels are also observed in Tsc2 ablated neurons and Tsc1 conditional knock out mice. Loss of Tsc1/Tsc2 also contributed to a multi-axon phenotype, a departure from the presence typical single axons in neurons, suggesting of a negative regulation by Tsc1-Tsc2 complex in specifying neuronal polarity (Choi et al., 2008). Taken together, these observations lay the framework for atleast two different signaling cascades that affect Sff/SAD kinase activity to affect spatio-temporal processes such as neuronal polarization during development.

Summary

Glycoprotein glycosylation is essential for normal development. It is intricately dependent on biosynthetic processing and non-biosynthetic signaling

events in the secretory pathway. Using HRP epitopes as a platform for studying glycoprotein glycosylation, we have addressed two major questions in this dissertation. The second chapter addresses the question of whether overexpression of a *Drosophila* homologs of a Golgi GDP fucose transporter and SAD kinases are able to rescue *nac* and *sff* mutations , respectively . Additional results described in this chapter connect *tollo* and *sff* signaling.

The third chapter delves deeper to understand the mechanisms by which Sff signaling impinges on glycoprotein glycosylation. Previously work on *Drosophila* Sff/SAD identified a novel role for this kinase in affecting the association of a neuronal glycoprotein Fasciclin II with different Golgi compartments. We undertook comprehensive phosphoproteomics and orthogonal validation by genetic and biochemical analyses with the goal of identifying direct substrates of Sff kinase to gain a better understanding of the cellular processes controlling glycoprotein glycosylation in the *Drosophila* embryo. Chapter four further expands on the observations made in chapters two and three and provides mechanistic possibilities into the regulation of glycosylation. These ideas can be extended to other model systems such as mammals and insights into possible mechanisms regulating glycoprotein glycosylation and Golgi trafficking can be gleaned from the data presented here.

CHAPTER 2

VALIDATION OF BIOSYNTHETIC AND GENETIC PATHWAYS REGULATING HRP-EPITOPE GLYCOSYLATION

Purpose Of The Study

Enrichment of HRP epitopes in the *Drosophila* embryonic nervous system presents us with a unique platform to explore the biosynthetic and signaling pathways regulating the glycoprotein glycosylation. Mutants such as *nac*[1], *tollo* and *sff* lead to neural specific reduction or loss of HRP epitopes in *Drosophila* at various developmental stages. Biochemical studies and genomic analysis identified Golgi GDP fucose transporter (Gfr) and CG6114, the *Drosophila* homolog of vertebrate SAD kinases as putative genes, responsible for *nac*[1] and *sff* phenotype. In order to unequivocally demonstrate that molecular lesions in these genes resulted in HRP epitope expression defects, we specifically overexpressed Gfr and CG6114 in *nac*[1] and *sff* mutant embryonic nervous systems, respectively. In this study, our results demonstrate that expression of Gfr and *Drosophila* CG6114/SAD kinase gene product is sufficient to rescue the HRP epitope mutant phenotype in *nac*[1] and *sff* embryos, respectively. These results provide crucial insights into the biosynthetic and signaling mechanisms controlling cellular glycan flux.

Introduction

The expression of HRP epitopes is not only tissue specific but also protein restricted and developmentally controlled (Katz et al., 1988). To this date, only a few molecules such as the neuronal cell surface proteins Fasciclin I and II, Neuroglian, Neurotactin, receptor protein tyrosine phosphatases (DPTP69D, DPTP10D, and DPTP99A) and Nervana have been reported to display HRP epitopes (Paschinger et al., 2009; Snow et al., 1987). In spite of the distinct and enriched display of HRP epitopes in the embryonic nervous system at various stages of development, the specific requirement of these antigens remains elusive.

The *Drosophila nac*^{1} mutation was isolated from a screen looking at genes causing lethality at pupal stages of *Drosophila* development. In *nac*^{1} mutants, neurons derived from imaginal tissues (quiescent cells that will form adult organs during metamorphosis) will not stain with anti-HRP antibodies. The deficiency of HRP epitopes does not compromise basic neuronal functions with adult *nac*^{1} flies being grossly normal at 25^{0}C. However, mutant *nac*^{1} flies display developmental irregularities (female sterility, defective ommatidia, and compromised wing formation) at lowered temperatures of 18^{0}C (Katz et al., 1988; Whitlock, 1993). Studies aimed at uncovering the genetic lesion responsible for this phenotype indicated identified 84F4 to 84F11-12 of Chromosome 3 in the *Drosophila* genome as the region contributing to this phenotype (Katz et al., 1988). Interestingly, one of the genes in this region is the *Drosophila* Golgi GDP fucose transporter (Gfr). Genomic amplification of Gfr locus in *nac*^{1} mutants uncovered a

cytosine to thymidine base pair change at position 86 in the mutant ORF that resulted in a serine to leucine transition. This change compromises the transport of sugar donor, GDP fucose into the Golgi lumen of Sf9 cells (Geisler et al., 2012). Taken together, biochemical and genomic studies strongly suggested that *Drosophila* Golgi GDP fucose transporter gene was the gene affected in *nac*^{1} mutants.

Tollo and *sff* are third chromosome mutations that specifically affect HRP epitope expression in embryonic central nervous system and peripheral nervous system (Baas et al., 2011; Seppo et al., 2003). The effect of these mutations on HRP epitopes' expression in larval, pupae and adult stages of *Drosophila* development is not completely clear (Baas S, Katoh, T. unpublished results). In the embryo however, these mutations lead to a complete absence (in case of *tollo*) or a distinct decrease in neural specific staining of HRP epitopes (in case of *sff*). Previous work through deletion mapping and single embryo PCR methodologies led to the identification of gene affected in *sff* mutants as CG6114. CG6114 is closely related to the vertebrate SAD kinase genes that have been shown to play critical roles in clustering of neurotransmitter vesicles at the active zone of presynaptic junctions (Crump et al., 2001; Inoue et al., 2006). In line with this observation, CG6114 mRNA transcript expression was detected in the embryonic nervous system (CNS and PNS) of *wild-type* embryos. By *in-situ* hybridization, CG6114 mRNA was reduced but not completely ablated in *sff* mutants emphasizing the hypomorphic nature of the *sff* mutation (Baas et al., 2011).

In the present study, two questions have been addressed, (a) does overexpression of the *Drosophila* Golgi GDP fucose transporter rescue the *nac*^{1} phenotype and (b) does overexpression of CG6114 rescues the *sff* mutant phenotype. Our results demonstrate that expression of Gfr and *Drosophila* CG6114/SAD kinase gene product is sufficient to rescue the HRP epitope mutant phenotype in *nac*^{1} and *sff* embryos, respectively. Together, these results demonstrate that both biosynthetic elements such as GDP fucose transporter and signaling proteins such as Sff Kinase participate in coordinating the glycosylation output of a cell.

Materials And Methods

Fly husbandry

OreR, *w*^{1118}, *sff, nac*^{1}, elav-GAL4 and other appropriate balancer fly lines (Bloomington Stock Center, Indiana University, Bloomington, IN) were reared at 25^{o}C.

Immunohistochemistry

Progeny embryos were stained with Rabbit anti-HRP (1:5000) and HRP conjugated Goat anti-rabbit (1:2000) (Jackson ImmunoResearch Laboratories Inc., PA, USA) using to analyze the HRP epitope expression pattern.

Generation of UAS-Gfr and UAS-CG6114 transgenic flies

UAS-Gfr was made by PCR amplifying the full length coding sequence of *Drosophila* GDP fucose transporter using the primers GGAATTCCGAAATGTACAAGAATCTG and GGGGTACCTCAGGCCTTCTGGGTGG. The PCR amplimer was digested with

EcoRI and KpnI to enable sublconing into the pUAST vector (Geisler et al., 2012). A second chromosome UAS-Gfr insertion was homozygosed into the *nac*^{1} mutant background and was crossed with elav-Gal4;+;nac^{1}/nac^{1} female virgin flies. Progeny embryos were stained with anti-HRP antibody to assess for the rescue of HRP epitope phenotype.

To generate the UAS-Sff construct, a vector containing incomplete *CG6114* cDNA (missing 209 bp of coding sequence including the start codon) was obtained from the Berkeley *Drosophila* Genome Project (clone GH13047 in pOT2). To generate the full length coding sequence of CG6114, we PCR amplified the missing region and an additional 97 bp of 5'UTR. With *Eco*RI enabled directionality, both sequences were ligated and cloned into the pBI(KS+) vector (Stratagene) and the integrity of the PCR-amplified region was verified by sequencing. The resulting full-length insert was subcloned into pUAST. Transformant lines were generated by injecting pUAST-*sff* with wcΔ2-3 into *w*^{1118} embryos by standard procedures (Brand et al, 1994). A second chromosome insertion was homozygosed into *sff*^{B22} and crossed to elav-Gal4;+; *sff*^{B22} homozygotes. Progeny embryos were stained with anti-HRP antibody to assess for the rescue of HRP epitope phenotype.

Results

Overexpression of UAS-Gfr rescues HRP epitope defect in *nac*^{1} embyos.

Anti-HRP antibody staining in *nac*^{1} embryos reveals a reduced expression of HRP epitopes in the ventral nerve cord in comparison to wild type embryos (Figure 2.1) (Geisler et al., 2012; Katz et al., 1988). In order to determine that a

nucleotide base pair change at position 86 of *Drosophila* GDP fucose transporter was the cause of *nac*^{1} mutant phenotype, UAS-Gfr was expressed in *nac*^{1} embryos using the pan-neural driver *elav*-Gal4. Elav-gal4 driven UAS-Gfr is sufficient for the neuronal expression of HRP epitopes in *nac*^{1} embryos (Figure 2.1). Anti-HRP antibody could be detected in late stage 10 rescued embryos. HRP epitope expression in wild-type embryos appears only in stages 11- 12. The early stage 10 appearance of HRP epitopes in rescued embryos mirrors *elav* expression, indicating that elav driven *Gfr* expression can autonomyously lead to core α1, 3-fucosylation at such an early stage. However, *elav*-driven expression of *Gfr* resulted in severe embryonic lethality and can be attributed to the overexpression of the UAS-Gfr transgene in all embryonic neurons.

We also generated an additional transgenic line that partially rescued HRP epitope expression in the ventral nerve cord and peripheral nervous system of *nac*^{1} mutant embryos without being crossed to a GAL4 driver line (data not shown). This leaky expression line (UAS-*Gfr*vk2) was homozygous viable and fertile in both mutant and wild-type backgrounds. However, this staining was less than the one obtained by crossing UAS-*Gfr* line to the *elav*-GAL4 driver line. Interestingly, the UAS-*Gfr*vk2 leaky expression line led to survival of more than three-fourths of UAS-*Gfr*vk2/UAS-*Gfr*vk2; *nac*^{1}/*nac*^{1} embryos in comparison to less than one-tenth of *nac*^{1}/*nac*^{1} mutants at 18^{o}C. Thus, a delicate control of Gfr expression is crucial is ensuring optimal HRP epitope expression and survivability into adulthood (Geisler et al., 2012).

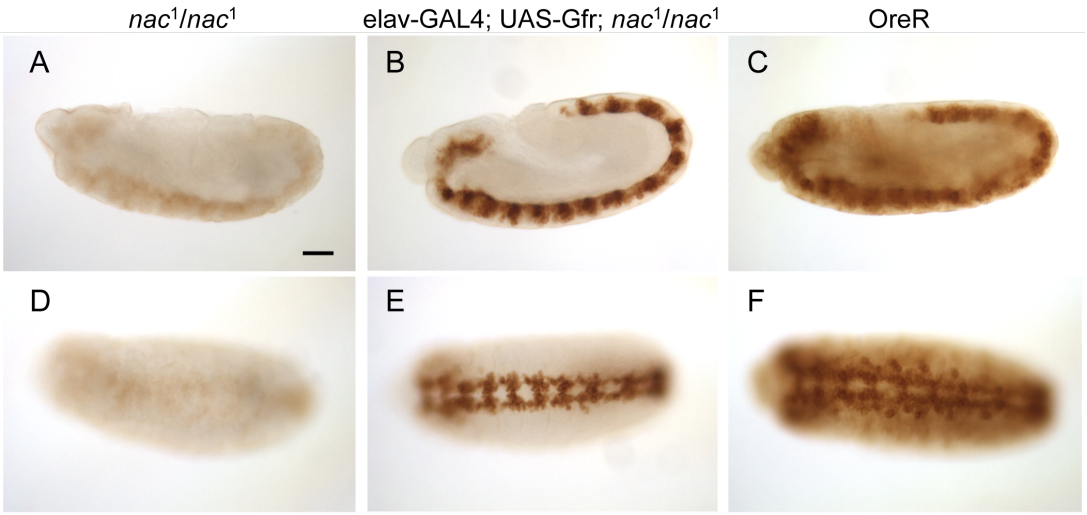

Figure 2.1. **Reduced HRP epitope expression in *nac*^{1} homozygous embryos is rescued by transgenic expression of WT *Gfr*.** *A*, *C*, and *E*, lateral view. *B*, *D*, and *F*, ventral view. All embryos are late stage 12 to early stage 13. In *nac*^{1}/*nac*^{1} embryos (*A* and *B*), HRP epitope expression is reduced in comparison with WT embryos (*E* and *F*). A wilt-type *Gfr* transgene driven by the neuron-specific *elav* promoter rescues neural HRP epitope expression (*C* and *D*). *Scale bar*, 70 µm. Reproduced from reference (Geisler et al., 2012) with permission of American Society for Biochemistry and Molecular Biology. This research was originally published in Journal of Biological Chemistry. Geisler, C., Kotu, V., Sharrow, M., Rendic, D., Poltl, G., Tiemeyer, M., Wilson, I.B., and Jarvis, D.L. (2012). The *Drosophila* neurally altered carbohydrate mutant has a defective Golgi GDP-fucose transporter. The Journal of biological chemistry *287*, 29599-29609. © the American Society for Biochemistry and Molecular Biology.

Overexpression of CG6114 leads to a rescue of *sff* HRP epitope phenotype.

To validate that CG6114 was the gene affected and responsible for the deficient HRP epitope expression in *sff* embryos, a pan-neural driver *elav*-Gal4 was used to drive the expression of UAS-CG6114 in *sff* mutant embryos. As shown in Figure 2.2, transgenic expression of CG6114 was sufficient to rescue HRP epitopes' expression and was evident in early stage 12 *sff* embryos. However, the rescue was not completely penetrant and resulted in high levels of lethality. After several reproductive cycles, the UAS-*sff* was unable to express Sff probably due to transgene silencing, further underscoring the the lethal nature of this insertion.

Discussion

Nac^{1}*, sff* and *tollo* affect HRP epitope expression at different stages of *Drosophila* development. However, *nac/sff* and *nac/tollo* flies are normal for HRP epitope expression (Katz et al., 1988). It therefore suggests of atleast two distinct pathways impinging on HRP epitope expression. *Drosophila nac*^{1} mutation causes a substitution of a conserved serine residue to leucine (S29L), resulting in an impairment of GDP-fucose transport into the Golgi lumen in a manner similar to that of human CDG-IIc. In the data presented here, GDP fucose transporter (Gfr) was validated as the gene affected in *nac*^{1} by rescuing the mutant HRP phenotype through transgenic overexpression of Gfr. Availability of sugar molecules is an absolute requirement for the process of glycosylation and nucleotide sugar transporters are essential for the transport of various activated sugar molecules such as GDP-fucose, UDP-GlcNAc, UDP-Gal,

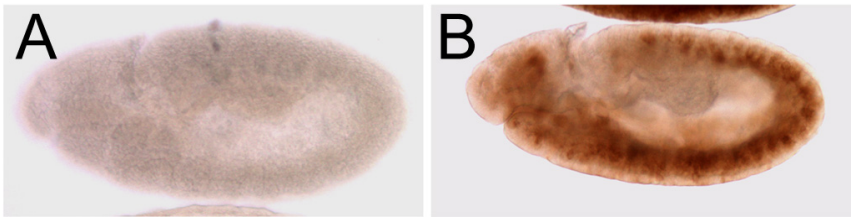

Figure 2.2. The *sff* mutation is rescued by transgenic expression of CG6114. (A) A stage 12 *sff* embryo (anterior to the left) stained with anti-HRP antibody doesnot exhibit epitope distribution characteristic of mid-late stage *sff* mutant embryos. (Baas et al., 2011). Lateral views of stage 12 embryos of genotype elav-Gal4; +/+; *sff*/*sff* (A) and elav-Gal4; UAS-*CG6114*/+; *sff*/*sff* (B), demonstrating rescue of HRP-epitope expression by transgenic Sff. Scale bar: 120 µm. Reproduced with permission from ref. (Baas et al., 2011) and Company of Biologists. The original research was published in the journal Devlopment. Baas, S., Sharrow, M., Kotu, V., Middleton, M., Nguyen, K., Flanagan-Steet, H., Aoki, K., and Tiemeyer, M. (2011). Sugar-free frosting, a homolog of SAD kinase, drives neural-specific glycan expression in the Drosophila embryo. Development *138*, 553-563.

UDP-Glc and UDP-GlcA. Mutations leading to less than ideal concentrations of these sugars (either in the transport, antiport or general luminal availability of these activated sugar molecules) can compromise protein and lipid glycosylation depending on their temporal requirement during this process (Liu et al., 2010). While Gfr as a transporter localized to *Drosophila* Golgi lumen and is known to transport nucleotide sugars like GDP-fucose, *Drosophila* Fringe connection (FRC) is known to transport UDP-GlcNAc, UDP-GlcA and presumably UDP-Glc, UDP-Gal and UDP-Xylose (Liu et al., 2010). Gfr and FRC mutants exhibit notch like phenotype suggesting their requirements in the Notch signaling pathway. However, the presence of reduced but consistent amount of residual HRP epitopes expression in *nac*^{1} argues in favor of an additional fucose transporter at the Golgi (Geisler et al., 2012). *Nac*^{1} flies demonstrate lowered α-1,3 and α-1,6 glycans, an affect that is consistent with GDP fucose transport into the Golgilumen. Our transgenic over expression of GDP fucose transporter clearly demonstrates the requirement of ideal optimal sugar transporter activity for driving glycoprotein glycosylation in the *Drosophila* embryo.

Crux of the Tollo transcellular signaling lies at the generation of an unknown signal from a few ectodermal cells in the vicinity of a subset of differentiating neurons to drive HRP epitope expression on all neurons (Seppo et al., 2003). On the other hand, intracellular Sff signaling coordinates neuron specific glycosylation by influencing Golgi compartmental dynamics in the Drosophila embryo (Baas et al., 2011). In the data presented above, neural specific expression of CG6114 (now termed as Sff Kinase), the *Drosophila*

homolog of vertebrate neuronal SAD kinase rescues the *sff* mutation. Previously, it has been demonstrated Sff interacts with Tollo such that compromised Tollo signaling leads to reduced Sff mRNA expression (Seppo et al., 2003). Therefore it is conceivable that ectodermally expressed Tollo upon stimulation through ligands such Spatzle leads to the production of a paracrine signal that in turn influences Sff signaling in the neighboring neurons to affect glycoprotein glycosylation. Together these results reflect a two level regulation on HRP epitope glycosylation- (a) biosynthetic control exerted by nucleotide sugar transporters and (b) signaling modulation by signaling pathways such as that of Tollo/Sff in the *Drosophila* embryo.

CHAPTER 3

IDENTIFICATION AND CHARACTERIZATION OF PUTATIVE SFF SUBSTRATES THAT CO-REGULATE NEURAL GLYCOSYLATION IN THE *DROSOPHILA* EMBRYO[1]

[1] Varshika Kotu, Peng Zhao, Lance Wells and Michael Tiemeyer. To be submitted.

Abstract

Appropriate glycan expression is dependent on biosynthetic inputs and appropriate co-ordination of intracellular signaling pathways. A complete mechanistic understanding of the pathways regulating glycoprotein glycosylation is lacking. A family of structurally related N-linked glycans known as HRP epitopes are specifically expressed in *Drosophila* neural tissue, providing a platform to understand the regulatory mechanisms controlling tissue-specific glycosylation. Using the *Drosophila* mutant *sugar-free frosting (sff),* we have identified new members of a signaling network that regulate glycoprotein glycosylation in the *Drosophila* embryo. We undertook differential phosphoproteomic analysis of *OreR* and *sff* mutant embryos by LC-MS/MS. Our approach identified Bifocal (Bif), Rasputin (Rin) and Liprin-alpha(Lip-α) as the putative phosphoprotein substrates of Sff kinase. *Bif, rin* and *lip*-α interact genetically with *sff*. In *sff* mutants, the steady state concentration of Rin is decreased. Confocal analysis in stage 14 embryos demonstrates increased colocalization of Rin with Golgi compartments in *sff* mutants. In *lip*-α/lip-α, Golgi compartments and glycoprotein glycosylation are affected in a manner similar to that observed in *sff* mutants. Taken together, our results provide crucial insights into the signaling pathways participating in the regulation of glycoprotein glycosylation.

Introduction

Glycoprotein glycosylation begins in the ER, continues in the Golgi apparatus, and ends with the cargo proteins being sorted to their appropriate

locations. Regulation of this process is achieved through the expression and localization of the enzymes that catalyze the addition or removal of monosaccharide residues and also through the action of the molecular machinery associated with secretory organelle trafficking. These regulatory nodes respond to a variety of intra- and extracellular cues. However, the mechanisms by which specific glycan structures are placed on restricted sets of proteins, either during development or pathological processes, are largely unknown.

In most arthropods, including *Drosophila melanogaster*, the nervous system is significantly enriched in HRP-epitope expression throughout development, presenting opportunities to examine pathways leading to tissue-specific glycosylation. Previously published random mutagenesis screens have identified genes affecting the neural specific expression of HRP-epitopes in the developing embryonic nervous system of *Drosophila melanogaster*. One of these mutations, named *sugar-free frosting* (*sff*), significantly reduces HRP-epitope expression in neural tissue. The *sff* mutation was mapped to the *Drosophila* homolog of a neuronal serine/threonine kinase called SAD-1 Kinase in C.elegans and also Brain specific Kinase 1 and 2 in mammals.

Comprehensive N-linked glycan analysis of *sff* mutant embryos by NSI-MS demonstrated that complex glycans are increased by the mutation, while all but one HRP-epitopes are decreased. The single HRP-epitope showing an increase in *sff* mutant embryos is a complex structure. The observation that the *sff* mutation affects expression of multiple classes of N-linked glycans in opposite

ways indicates that the mutation does not affect a single enzyme or single biosynthetic pathway. One mechanism by which a mutation in a single gene might impact multiple glycan biosynthetic pathways would be by influencing Golgi trafficking. We detected altered colocalization of a protein that bears the HRP-epitope (Fas2) with specific Golgi markers in sff mutant embryos. Fas2 is almost entirely co-localized with a trans-Golgi marker (PNA) in the mutant but only partially co-localized with the same marker in wild-type, suggesting that this cargo protein gains increased access to glycosyltransferases capable of generating complex glycans and that the Sff kinase is responsible for regulating Golgi trafficking.

In *C. elegans*, SAD kinase mutants exhibit altered synaptic vesicle tethering at the neuromuscular junction (NMJ). Analogous to SAD kinase function at the NMJ, we propose that Sff/SAD kinase activity influences Golgi compartmentation by facilitating similar molecular interactions responsible for vesicle tethering at specific Golgi cisternal membranes. Therefore, identification of Sff/SAD substrates is essential for elucidating the molecular mechanisms that underlie tissue-specific glycosylation. To achieve this goal, a differential phosphoproteomic analysis of *OreR* and *sff* mutant embryos was undertaken. By LC-MS/MS, phosphoprotein serine/threonine phosphorylation sites were identified that were utilized in wildtype but not detected in the *sff* mutant. Orthogonal approaches validated the importance of a subset of these phosphorylation sites for HRP-epitope expression.

Materials And Methods

Generation and maintenance of *Drosophila* stocks

All *Drosophila* strains were reared at 25°C unless otherwise indicated. *OreR* refers to wild type strain. P-element insertion lines and genomic deletion stocks were obtained from the Bloomington Stock Center. For analysis of Bifocal interactions, the following stocks were used: y^1 P{SUPor-P}bif ^{KG07899} and bif ^{EP395}/+. For analysis of Liprin-α interactions, the following stocks were used: w^-; lip-$\alpha^{[Fex15]}$/In(2LR)Gla,wg[Gla-1]Bc[1];+/+. In the case of *lip-α*, the parental stock was crossed to *w^-; Kr/Cyo-GFP; +/+* to yield a w^-; *lip-$\alpha^{(Fex15}$)/CyO-GFP* that was used to sort lip-$\alpha^{[Fex15]}$ homozygous embryos for confocal analysis. For quantification of genetic interaction analysis, Rasputin deletion line : *Df(3R)Exel6169,P{Xp-U}Exel6169/TM6B,Tb* and mutant line *Df(3R)rin^2,rin^2/* TM6B, Sb, Tb were used. For light microscopic analysis, *rin*/TM6B, Sb, Tb stock generously provided by Dr. Elizabeth Gavis and Paul Schedl (Princeton University) was used. For confocal analysis, a transgenic stock expressing a Golgi mannosidase II-YFP fusion protein used to mark the medial/trans Golgi compartments in a wild type or *sff* mutant background (Baas et al., 2011), w^-; *lip-$\alpha^{(Fex15}$)/CyO-GFP, w^{1118}* and *sff* stocks were used.

Preparation of protein extracts

For differential phosphoproteomic analysis, staged collections of embryos from *OreR* and *sff* genotypes were obtained. Mating adults were placed in a collection vial for 4 hours and the accumulated embryos were allowed to develop for an additional 3 (*OreR*) or 4 (*sff*) hours. Previous characterization of

embryogenesis in *sff* mutants demonstrated a reproducible delay in reaching developmental milestones such that eight hour old *sff* embryos were morphologically comparable to seven hour old *OreR* embryos (Baas et al., 2011). Collected embryos were dechorionated and stored in heptane at -80^{o}C until further use (Seppo et al., 2003; Snow et al., 1987). Protein extracts were prepared from embryos as previously described (Aoki et al., 2007). Briefly, embryos were delipidated by homogenization in a mixture of chloroform, methanol, and water (4:8:3::C:M:W). The resulting extract was incubated at 4^{o}C with end over end agitation for X hours. Proteins were harvested from the extract by centrifugation, which yielded a protein-rich pellet. The pellet was dried under nitrogen, resulting in a fine white proteinaceous powder that was stored at -20^{0}C until further use.

Protein digestion and peptide fractionation

Protein powder was resuspended in 40 mM NH_4CO_3 and reduced with 10 mM dithiothreitol at 56°C for 1 hour. After cooling to room temperature, free sulfhydryls were alkylated with iodoacetamide (55 mM, dark for 45 min), and the reduced, alkylated proteins were digested overnight at 37°C using sequencing grade modified trypsin (Promega). The tryptic digestion was terminated by addition of 0.1% trifluoroacetic acid, and the resulting peptides were desalted using Vydac C18 Silica spin columns (Nest Group) and dried by vacuum centrifugation (Zhao et al., 2011). The first dimension of peptide fractionation was performed using strong cation exchange (SCX) chromatography on an Agilent 1100 series HPLC system (Agilent Technologies). Dried peptides were

resuspended in solvent A (5 mM KH_2PO_4/30% acetonitrile, pH 2.7) and resolved on a 2.1 x 100 mm Polysulfoethyl A column (PolyLC) at a flow rate of 150 µl/min. Solvent A, solvent B (solvent A with 350 mM KCl), and solvent C (0.1 M Tris/0.5 M KCl, pH 7.0) were used to produce a salt gradient consisting of 5 min at 100% solvent A, 48 min gradient at variable slope to 100% solvent B, 12 min at 100% solvent B, 15 min to 100% solvent C, and 10 min to 100% solvent A. Fractions were collected every 2 minutes for a total run time of ninety minutes. Fractions were combined equally to generate four pooled fractions for each biological sample, which were then desalted and dried.

Phosphoproteome enrichment by immobilized metal ion affinity chromatography (IMAC)

For IMAC enrichment, PHOS-Select Iron Affinity Gel (Sigma) was prepared by washing with load/wash buffer (250 mM acetic acid/30% acetonitrile) and centrifugation at 500 x g according to manufacturer's instructions. Dried peptide pools generated by SCX were resuspended in load/wash buffer and incubated with separate aliquots of resin by end-over-end rotation for 2 hours at room temperature. After incubation, the resin was washed with load/wash buffer and then with water and the resin was collected by centrifugation at 8200 x g. The supernatants containing non-phosphorylated peptides were collected for subsequent analysis. Phosphorylated peptides were eluted by incubating the resin with elution buffer (0.4 M NH_4OH/30% acetonitrile) for 2 min at room temperature and were collected in the supernatant following centrifugation at

8200 x g. Eluted peptides in the supernatant were acidified with 0.1% formic acid and dried by vacuum centrifugation.

Nanospray ionization liquid chromatography mass spectrometry

Peptide and phosphopeptide fractions were resuspended in 0.5 µl of solvent B (0.1% formic acid/80% acetonenitrile) and 19.5 µl of solvent A (0.1% formic acid) and loaded onto a 75 µm i.d. x 105 mm C18 reverse phase column (packed in house, YMC GEL ODS-AQ120ÅS-5, Waters) by nitrogen bomb. Peptides were eluted directly into the nanospray source of an LTQ Orbitrap Discovery™ (Thermo Fisher Scientific) with a 160-min linear gradient consisting of 5-100% solvent B over 100 min at a flow rate of approximately 250 nl/min. The spray voltage was set to 3.0 kV and the temperature of the heated capillary was set to 210 °C. Full MS scans averaged over 3 microscans were acquired from m/z 300 to 2000 at a resolution of 30000 (full width at half maximum (FWHM) at m/z 400) in the Orbitrap, with a maximum ion injection time of 1000 ms, and an automatic gain control (AGC) setting of 1000000 ions. Collision induced dissociation (CID) MS^n scans were acquired in the ion trap with the AGC target of 300000, isolation window of 2.2 amu, and 35% normalized collision energy, using 1 microscan with a maximum injection time of 100 ms. A dynamic exclusion window was applied which prevented the same m/z value from being selected for 6 seconds after its initial acquisition. Data acquisition utilized Xcalibur® (ver. 2.0.7, Thermo Fisher Scientific) and employed a workflow consisting of a full MS scan followed by data-dependent MS/MS scans of the 6 most abundant ions and subsequent MS^3 scans triggered by the detection of neutral losses of phosphoric

acid (98 Da) between precursor ions in full MS and the 3 most intense product ions in MS/MS.

Analysis of MS data

Raw MS spectra were extracted and searched against the *D. melanogaster* protein database (UniProt, updated at Nov. 18, 2010) using SEQUEST as integrated into Proteome Discoverer (ver. 1.2, Thermo Fisher Scientific). The database search output was filtered to obtain 1% false discovery rate on the protein level using the ProValT algorithm as deployed in PROTEOIQ. After data consolidation, the phosphorylation sites assigned by SEQUEST were further validated by manually examining the raw spectra (Weatherly et al., 2005).

GO-ID classification and AMPK consensus site prediction

The Cytoscape Plugin BiNGO was employed to identify phosphoprotein functional categories (GO: Biological Process annotation) detected in the proteomic samples prepared from the unique developmental stages analyzed above (Maere et al., 2005) . The presence of AMPK consensus Serine/Threonine phosphorylation site(s) in the recovered phosphopeptides was analyzed using Scansite and Group Based Prediction System, GPS 2.0 set at low, medium and high cut off thresholds (Obenauer et al., 2003; Xue et al., 2008).

Immunohistochemistry and immunofluorescence

Primary antibodies used were: Rabbit anti-Horse Radish Peroxidase (1:5000, Jackson Laboratories) and anti-Rasputin (1:1500, a gift from Dr. Elizabeth Gavis, Princeton University, New Jersey). Secondary antibodies used were: HRP conjugated Goat anti-rabbit (1:2000, Jackson Laboratories) and Alexa

Fluor 488 Goat anti-Rabbit was used at 1:1500. For immunohistochemistry and antibody staining of whole mount embryos, dechorionated embryos were fixed, divitellinized and stained using primary and secondary antibodies as previously described (Patel et al,1994; Seppo et al,2003; Baas et al, 2011). Zeiss Axioskop microscope fitted with a Retiga 2000R CCD camera was used for acquiring light micrographs at 10X magnification (Q Imaging, Surrey, Canada).

Confocal microscopy

Embryos were dechorionated, fixed and devitellinized as previously described. In triple staining experiments, embryos were blocked in appropriate blocking buffers and stained with sequentially with each primary and secondary antibody pair. The following antibodies were used to delineate Golgi compartments and target proteins: Goat anti-TGN245 (marks trans-Golgi network compartment, 1:2000, a generous gift from Dr. Sean Munro, MRC Laboratory for Molecular Biology, Cambridge, UK), Biotin Conjugated Peanut Agglutinin (1:2000, Vector Laboratories), Rabbit anti-GM130 (1:1000, Abcam), anti- Rasputin (1:1500, a gift from Dr. Elizabeth Gavis, Princeton University, New Jersey), monoclonal mouse anti-GFP (cross reactive with YFP, 1:1500, Life-Technologies). In addition to Streptavidin-568, the following Alexa Fluor secondary probes (Invitrogen) were used: Donkey anti Goat-488, Goat anti Rabbit-647, Goat anti mouse-568. All secondary antibodies were used at a 1:500 dilution. Blocking buffers used are: TGN Buffer for TGN245 primary and secondary antibodies (Normal Donkey serum in 1X PBS and 0.3% Triton X-100), PNA Buffer for Biotin-PNA and SA-568 antibodies (0.1% BSA, 1X PBS, 0.3%

Triton-X 100) and Goat Serum Blocking Buffer for Rasputin and GM130 primary and secondary antibodies (0.1% Normal Goat Serum, 0.1% BSA, 1X PBS and 0.3% Triton-X 100).

Triple stained embryos were dissected for ventral nerve chord imaging and mounted in ProLong Gold Anti-fade (Invitrogen) before image analysis. Confocal images were acquired on an Olympus FV1000 laser scanning microscope (generous usage provided by Dr. Heather Flanagan-Steet at the Complex Carbohydrate Research Center, University of Georgia, Athens, GA) with 60X oil objective, a numerical aperture of 1.42 and a z-plane thickness of 0.45um as previously described (Baas et al). Instrument related parameters were identical across all embryos. Image analysis was performed with Slidebook software package (Intelligent Imaging Innovations, Denver, CO, USA) on non-compressed Z-stacks. Each region of interest (ROI) analyzed for co-localization spanned a single segment of the ventral nerve chord along the dorso-ventral and antero-posterior axes. For each genotype, we imaged $n \geq 14$ segments derived from three different embryos at stage 14 of embryonic development. Confocal stacks for each channel were manually adjusted for background using the software supplier's recommended algorithm. Total fluorescence intensity and colocalized fluorescence intensity for each marker was quantified within selected ROIs. Colocalized fluorescence intensities were normalized to the total fluorescence intensity of the marker of interest to calculate the percent colocalization for each marker.

Western blotting

OreR and *sff* embryos were homogenized in lysis buffer (HEPES, KCl, EDTA, EGTA, Glycerol, DTT, Triton-X, Phosphatase Inhibitor and Protease Inhibitors) using a handheld electric homogenizer fitted with a plastic pestle designed for 1.5 ml Eppendorf tubes. The lysates were centrifuged at 14,000 rpm for 30 mins at 4^{o}C. Equal amounts of supernatant and Laemelli buffer were mixed and boiled for 5 minutes. Protein samples were resolved on 4-20% Mini-PROTEAN TGX gels (Bio-Rad) and transferred to a PVDF membrane. The membrane blots were blocked in 1X TBS, 0.1% Tween-20 and 2% BSA overnight. The blots were probed with Rabbit anti-Rasputin (1:3000, a gift from Dr. Elizabeth Gavis, Princeton University, New Jersey), and mouse anti-Actin (1:5000, MP Biomedicals LLC, OH). The membranes were washed in 1X TBS, 0.1% Tween-20 and probed with HRP-conjugated secondary antibodies Donkey anti-rabbit (1:1000, Jackson Immuno Research Laboratories Inc., West Grove, PA) and Goat anti-mouse (1:5000,Jackson Immuno Research Laboratories Inc., West Grove, PA). Enhanced chemiluminescence was used to detect antibody binding (Thermo Scientific, IL). Blots were stripped to remove Rasputin primary and secondary antibodies using stripping buffer (100mM β-mercaptoethanol, 2% SDS and 62.5 mM Tris-Cl pH 6.8) and reprobed with actin primary and secondary antibodies. Protein quantification was done using NIH Image J software.

Results

The phosphoproteome of stage 10-11 embryos is enriched in proteins affecting cytoskeletal organization, axon guidance, and catabolism

Protein phosphorylation has been previously well characterized in adult stages, but the portfolio of proteins that are phosphorylated during a restricted window of development that is characterized by extremely dynamic changes in cellular fate and embryonic morphology has not been carefully investigated (Zhai et al., 2008). Approximately five to six hours (Stage 10-11) into *Drosophila* embryogenesis, neuroblast divisions lead to the appearance of ganglion mother cells in the presumptive ventral nerve chord (Ortega and Hartenstein, 1985). This stage is also marked by the appearance of faint anti-HRP epitope staining, which stains cell bodies of ganglion mother cells, all neurons, and their pioneering axon extensions (Baas et al., 2011; Jan and Jan, 1982; Katz et al., 1988; Zhai et al., 2008). Wild type and *sff* mutant embryos were collected during this stage of development and subjected to phosphoproteomic analysis by affinity enrichment and LC-MS/MS (Figure 3.1).

Wild-type phosphoproteome of this restricted stage of development was characterized by enrichment in protein categories contributing to catabolism, cytoskeletal organization and axonal guidance relative to *Drosophila* genome (Figure 3.2). This is expected since embryos are at a stage when newly born neurons are ready to charter the course set by pioneer neurons and find appropriate target for initiating the process of synaptogenesis soon after.

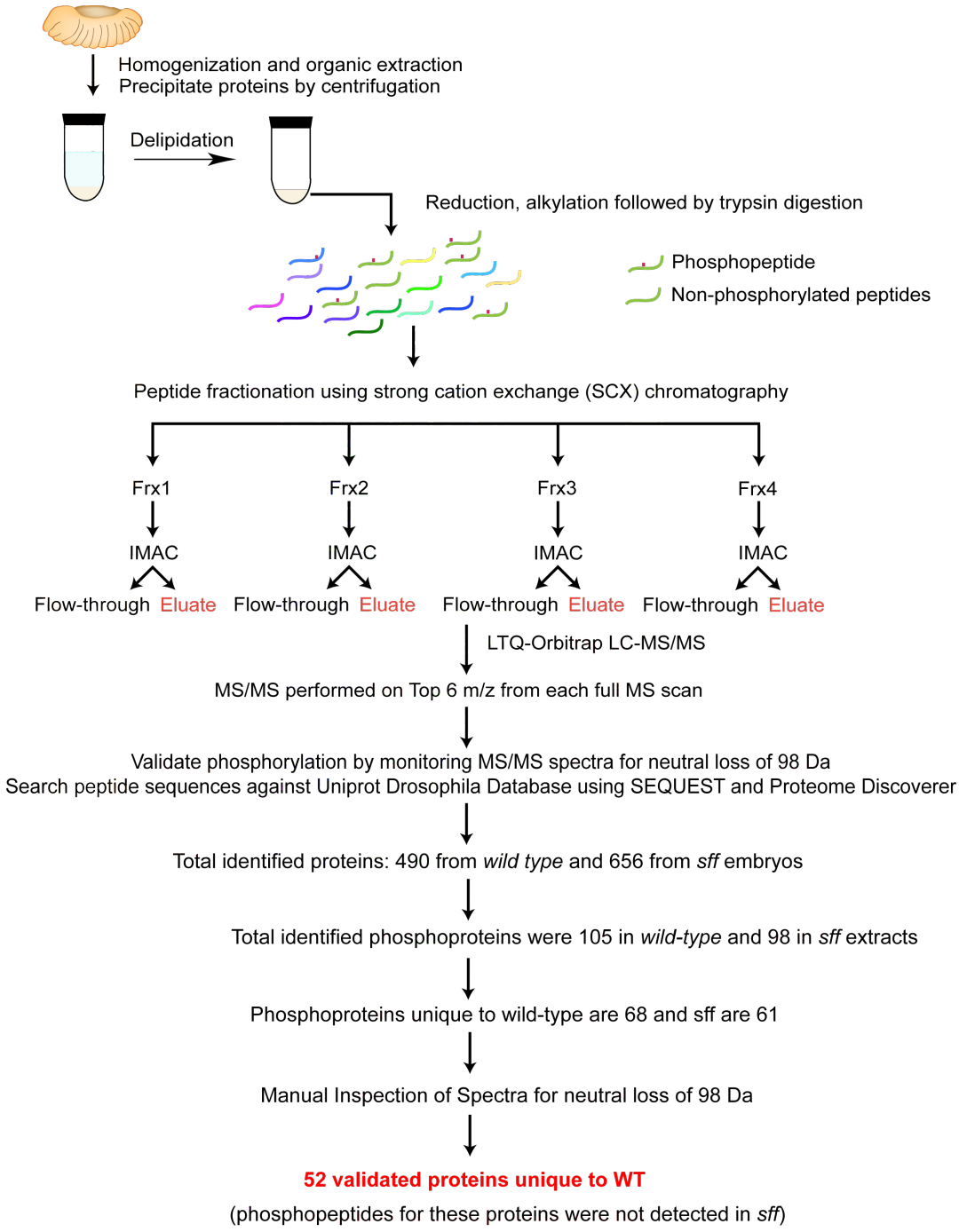

Figure 3.1. Schematic illustration of the workflow involved in phosphoproteomic analysis of *Drosophila* embryos.

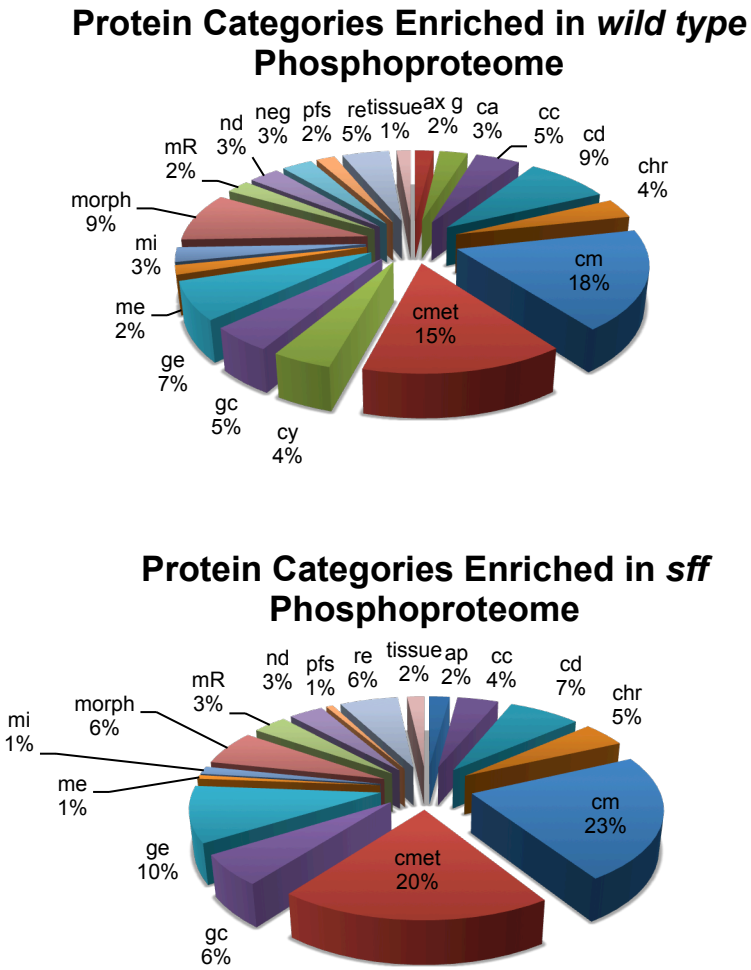

Figure 3.2. Analysis of protein categories enriched in wild-type and *sff* phosphoproteome. Abbreviations are as follows: Ap stands for apoptosis, ax g stands for axon guidance, ca for catabolism, cd for cell development and differentiation, chr for chromosome segregation, cm for cell morphology, cmet for cell metabolism, cy for cytoskeletal organization, gc for germ cell process, ge for gene expression, me for meiosis, mi for mitosis, morph for morphogenetic development, mR for mRNA processing, stability and localization, nd for neural development, pfs for protein folding and stability, re for reproduction and tissue for tissue development and maintenance.

In mutants however, these protein categories either disappear or are reduced to very low levels. As a category, apoptosis related proteins are enriched in *sff* mutants, suggesting that loss of Sff protein activity may predispose cells for destruction.

Phosphoproteomic analysis identifies proteins that are differentially phosphorylated in wild-type (*OreR*) and *sff* embryos.

Sff expression is temporally co-incident with the appearance of the HRP epitope, which begins at stage 11 of embryonic development, well before synaptogenesis begins in the embryo. To identify substrates of Sff kinase that might potentially affect glycoprotein glycosylation, we focused on a time window of stages 8 and 12 of *Drosophila* embryonic development (see materials and methods). LC-MS/MS analysis identified 2343 peptides defining 490 candidate phosphoproteins in wild-type (OreR) extracts and 2802 peptides defining 656 candidate phosphoproteins in *sff* mutant extracts. We identified 52 phosphoproteins defined by 108 phosphopeptides that were unique to wild-type but absent in *sff* embryos. This group of phosphoproteins defines a set of putative substrates/functional interactors of Sff/SAD kinase. We also identified 46 phosphoproteins, defined by 119 phosphopeptides that were detected in *sff* embryos but not in wild-type. This group of phosphoproteins defines a set of proteins whose phosphorylation status may be indirectly or not affected by Sff/SAD kinase activity. Sff/SAD kinase is most closely related to the AMPK/CaMK2 family of kinases (Crump et al., 2001).

AMP family kinases preferentially phosphorylate serine (S) or threonine (T) residues within a consensus that includes a hydrophobic-basic-hydrophobic amino acid motif preceding the target S/T residue (Hardie, 2011). The phosphoproteins identified by LC-MS/MS were queried to identify S/T sites consistent with the AMP family consensus using Scansite and GPS 2.0 (Obenauer et al., 2003; Xue et al., 2008). Phosphopeptides identifying six proteins unique to wild-type embryos and absent in *sff* mutants that met the consensus for phosphorylation by AMPK family were detected (Table 1). This set of differentially phosphorylated proteins unique to wild-type embryos provided a high priority set of candidate protein substrates for Sff kinase.

Genetic validation of putative phosphoprotein interactions with *sff*.

To validate that the identified phosphoproteins were relevant for neural specific glycosylation, existing mutations in putative substrates were crossed to *sff* in order to assess whether transheterozygotes presented deficient HRP epitope expression. In *sff* mutant embryos, HRP epitope staining is reduced to the axonal scaffold and shows up only as a slight smattering at the nerve chord. Wild type embryos or embryos with only one copy of *sff* mutant allele have normal HRP epitope expression (Baas et al., 2011). Based on this consensus as well as the availability of reagents, mutants, and additional functional knowledge, the interactions of *sff* with lip-α, *Rasputin* (rin), and *Bifocal* (bif) were further characterized. Transheterozygotes of the genotypes *lip-*α^{Fex15}*/+; sff/+, rin/sff* and *bif/+; + ; sff/+* are fertile and viable.

Table 1: Classification of six phosphoproteins unique to WT and absent in *sff*, on the basis of AMPK consensus S/T site. Phosphoproteins unique to wild type and absent in *sff* were subjected to genetic and bioinformatics analysis. Out of the six identified genetic interactors, recovered phosphopeptides for only three phosphoproteins exhibit the presence of putative AMPK S/T sites.

Phosphoprotein unique to WT	HRP+/HRP- When in combination with Sff	AMPK (S/T identified) /non-AMPK
Vulcan (Guanylate Kinase Associated Protein	HRP-	Non-AMPK
Ptp61f	HRP-	Non-AMPK
Msp-300	HRP-	Non-AMPK
Bifocal	HRP-	AMPK, S480
Rasputin	HRP-	AMPK, S470
Liprin-alpha	HRP-	AMPK, S542

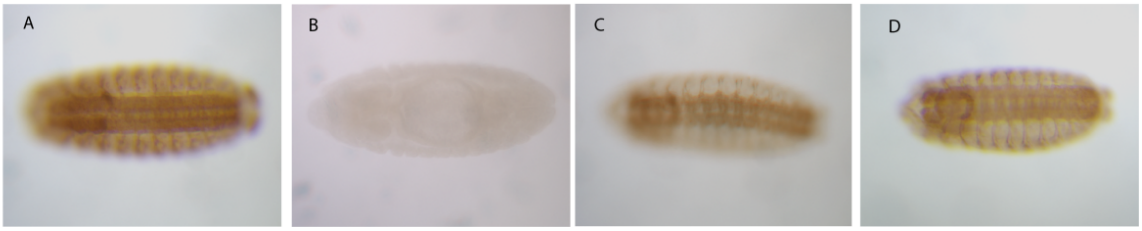

Figure 3.3. *Lip-α* genetically interacts with *sff*. In comparison to HRP epitope expression patterns, that are normal in wild-type (A) embryos and completely penetrant in *sff*/*sff* (B), transheterozygous *lip-α/+; sff/+* (C) and *lip-α*/*lip-α*; +/+ (D) homozygous mutant embryos exhibit slightly reduced HRP epitope staining. A-D are ventral views of embryos stained with anti-HRP epitope antibody. Magnification 10X.

Figure 3.4. *Rin* genetically interacts with *sff*. In comparison to wild type (A) embryos, *sff*/*sff* (B) and embryos transheterozygous for *rin* and *sff, rin*/*sff* (C) exhibit reduced HRP epitope staining. The expression of *rin/sff* transheterozygotes is intermediate between wild type (A) and highly penetrant *sff/sff* (B) patterns at this stage of embryonic development (Stage 11). A-C are lateral views of embryos stained with anti-HRP epitope antibody. Magnification 10X.

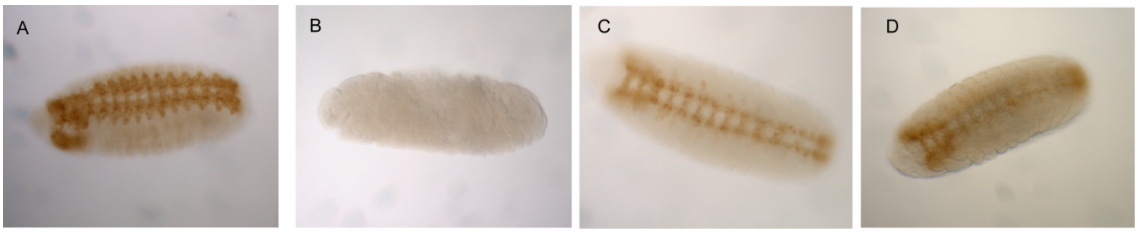

Figure 3.5. *Bif* genetically interacts with *sff*. In comparison to wild-type (A) embryos, *sff*/*sff* (B), *bif* /+;+;*sff*/+ (C) and *bif*/*bif* (D) exhibit reduced HRP epitope expression. C,D show intermediate HRP epitope staining pattern ranging between wild-type and *sff* mutant patterns. A-D. Ventral views of stage 13 embryos stained with anti-HRP epitope antibody. Magnification 10X.

Embryos with one copy of a single mutant allele in any of these genes over a wild type chromosome show normal HRP epitope expression. However, transheterozygous embryos that carry one copy of a *lip-α*, *rin* or *bif* mutant allele in combination with one copy of a sff mutant exhibit affected HRP epitope staining pattern with varying degrees of penetrance (Figure 3.3, 3.4, 3.5). The penetrance of the interactions between *sff* and *lip-α, rin* and *bif* were further quantified by scoring HRP epitope expression in staged embryos obtained by mating transheterozygous adults. In comparison to progeny harvested from control genotypes (*w^-; +/CyO; sff/+ and w^+; +/+; sff/+*), where the observed distribution of HRP+ to HRP- embryos is close to the expected Mendelian ratio of 3:1, the crosses between Rasputin and Sff and Bifocal and Sff exhibited substantial shifts towards greater percentages of embryos with reduced HRP epitope expression (Table 2).

Association of TGN Golgin 245 and cis Golgin GM130 is altered in the CNS of *lip-α/lip-α* and *sff* embryos.

Previous analysis demonstrated that *sff* mutants exhibit altered Golgi organization(Baas et al., 2011). Therefore, the distributions of Golgi markers and PNA, a plant lectin, was assessed in putative Sff substrate mutants and in *sff/sff* double mutants. Staining with the PNA, which recognizes an O-linked glycan structure (Core 1, Galβ1,3GalNAc linked to Ser/Thr residues), revealed a significant change in O-linked glycosylation in *lip-a^{Fex15}/lip-a^{Fex15}* and *sff/sff* mutant embryos.

Table 2. Population analysis of HRP epitope expression patterns in embryos derived from matings of siblings transheterozygous for *sff* and *bif* or *lip-α* or *rin.* Progeny embryos were stained with anti-HRP antibody and scored for wild type, or affected HRP epitope expression. The number of progeny embryos scored for each cross is indicated in brackets (n>500). w^-; +/*CyO*; *sff*/+ and w^+ ; +/+ ; *sff*/+ are control genotypes. The expected distribution of HRP+: HRP- progeny is 3:1 if no genetic interaction occurs between *sff* and the indicated genes.

Genotypes used for sibling matings	n, Number of embryos	% of HRP+ embryos	% of HRP- embryos	Ratio of HRP+: HRP-
+/+ ; *sff*/+	672	74.2	25.8	2.9
+/*CyO*; *sff*/+	1027	75.8	24.2	3.1
rin^{2}/*sff*	540	62.6	37.4	1.7
rin^{6169}/*sff*	702	66.0	34.0	1.9
liprin-α^{R60}/*CyO*; *sff*/+	1330	68.6	31.4	2.2
liprin-α^{F3ex15}/*CyO*; *sff*/+	523	68.3	31.7	2.2
bif ^{EP395}/+;*sff*/+	936	65.1	34.9	1.9
bif ^{KG07899}/+; *sff*/+	618	63.8	36.2	1.8

Both, *lip-a*^{Fex15}*/lip-a*^{Fex15} and *sff/sff* mutants exhibit a decrease in Core 1 O-glycans as demonstrated by PNA staining, indicating that the changes in Golgi organization impact both N- and O-linked glycoprotein glycosylation (Figure 3.6). In *lip-a*$^{[Fex15]}$*/ lip-a*$^{[Fex15]}$ and *sff/sff* mutants, the mean object volume for a trans-Golgi network marker (TGN golgin 245) is significantly increased in comparison to wild-type embryos (Figure 3.7). These genotypes also demonstrated a significant increase in the colocalization of a cis-Golgi marker (GM130) with the trans Golgi network (Figure 3.8).

Rasputin colocalizes with Golgi compartments and its association with TGN Golgin-245 is increased in ventral nerve chord of *sff* embryos.

Altered HRP epitope staining in *rin/sff* transheterozygous embryos suggested that Rin and Sff kinase might interact to drive neural specific glycosylation in the embryonic nervous system. In order to dissect this proposed role of Rasputin, its expression and distribution pattern in Stage 14 embryos was examined by confocal microscopy. Prior to stage 13, neural precursor cells are intermingled with other ectodermal cells within the ventral part of the embryo, making it difficult to distinguish between neural progenitors, differentiating neural cells, and other ectodermal lineages. By mid-stage 13, the ventral nerve cord has consolidated into a distinct structure and becomes well separated from the underlying ectoderm as development proceeds. However, upon dissection for confocal analysis, the ventral nerve cord collapses onto the underlying ectoderm, making it difficult to clearly resolve the boundaries of each tissue.

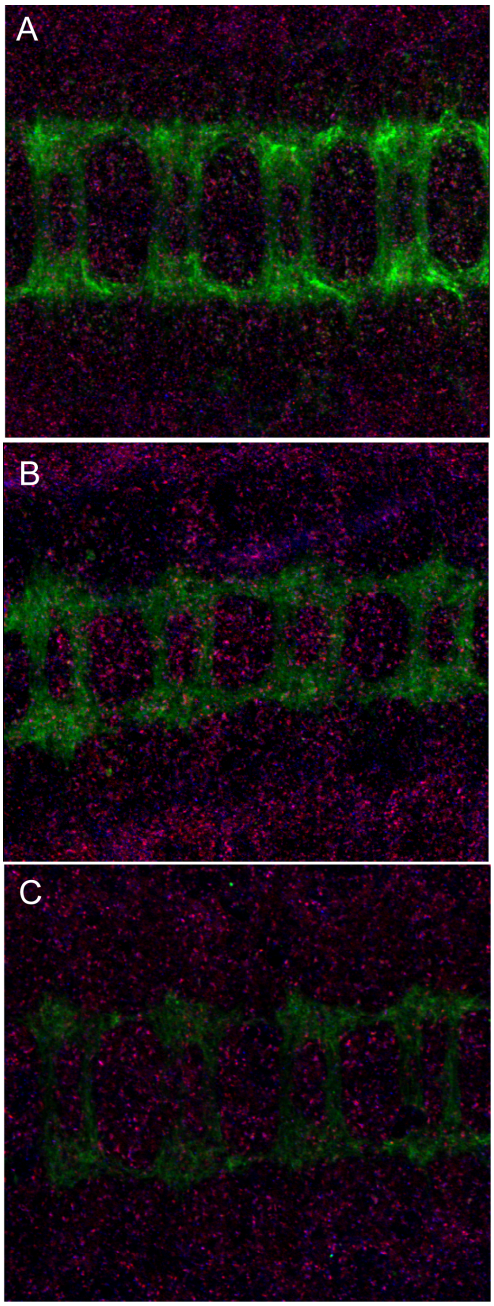

Figure 3.6. Core 1 O-glycans are reduced in *lip*-α and *sff* mutants. *Drosophila* embryos (Stage 14) stained with PNA lectin recognize Core 1 O-glycans (green) and antibodies against cis Golgin GM130 (blue) and trans-Golgin network golgin 245 (red). A-C are ventral views of nerve chord imaged at 63X magnification. A. w^{1118}(wildtype) . B. *sff* .C.*lip*-$\alpha^{[Fex15]}$ / *lip*-α $^{[Fex15]}$.

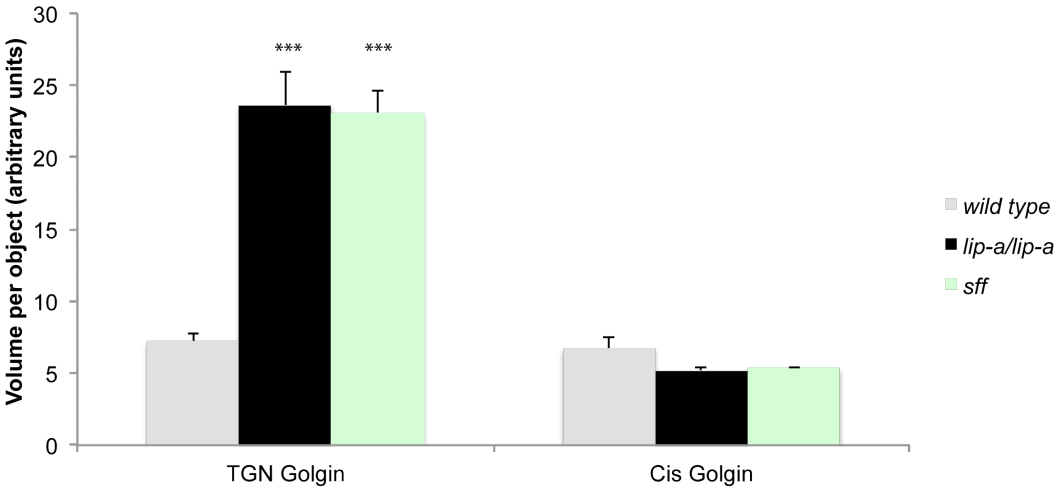

Figure 3.7. TGN expansion is phenotypically similar in *lip-α* and *sff* mutants. Quantification of volume per object (total volume/total number of punctae or objects) in each genotype was calculated for trans Golgi network golgin TGN245 and cis Golgi golgin GM130. In case of TGN245, the mean volume of individual objects is significantly increased in *lip−α* and *sff* mutants while mean volume of GM130 is not altered. ***p<0.00001 and n= 14-18 nerve chord segments from three age matched embryos at stage 14 of development were used for quantification.

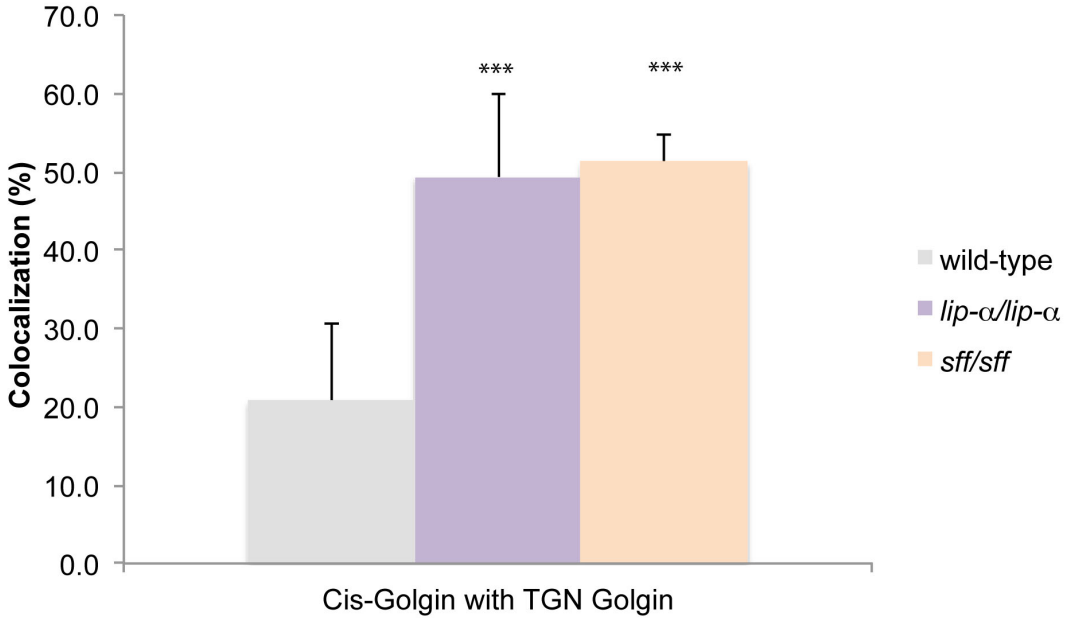

Figure 3.8. cis-Golgi colocalization with TGN is increased in *lip-α* and *sff* mutants. The percentage overlap between cis golgin marker GM130 and trans golgi network marker TGN245 was quantified in age matched *lip-α* and *sff* mutant embryos. In comparison to wild-type, *lip-α* and *sff* homozygous mutants show a significant increase in co-localization of cis-Golgi compartments with the trans Golgi network. ***p<0.00001.

Nonetheless, it is possible to image and quantify fluorescence intensities within regions enriched for ectoderm or nerve cord. By confocal analysis, the distribution of Rin in the nerve cord differs in intensity and organization in comparison to the ventral ectoderm. While both tissues exhibited punctate staining reminiscent of Golgi-like objects, cells of the ventral ectoderm also possessed a diffuse cytoplasmic pool of Rin. By contrast, Rin within the nerve cord is completely consolidated into punctate objects (Figure 3.9 A, B). In comparison to wild type, *sff* embryos show decreased abundance of Rasputin positive objects in the nerve cord (Figure 3.9 A, B). To investigate whether the Rin punctae visualized in the nerve cord were associated with an identifiable Golgi compartment, colocalization with a medial/trans-Golgi marker (YFP-Mannosidase-II) and a trans-Golgi network (TGN) marker (TGN golgin 245) was quantified in the nerve cord of triple-stained embryos (Figure 3.9 C, D). In comparison to wild-type embryos, 131% of more Rin colocalized with the medial/trans Golgi marker and 85% of more Rin colocalized with the TGN marker in *sff* mutants (Figure 3.9 E). Further, western analysis of whole embryo extracts demonstrated a 60% decrease in Rin protein in the *sff* mutants (Figure 3.10).

Discussion

Sff/SAD kinase expression slightly precedes the appearance of HRP epitopes during embryonic development. It has also been shown to regulate neural specific glycosylation in *Drosophila* embryos by affecting Golgi compartmentation (Baas et al., 2011). However, its substrates and effectors that might interface with the secretory apparatus have not been identified. In this study, we report the

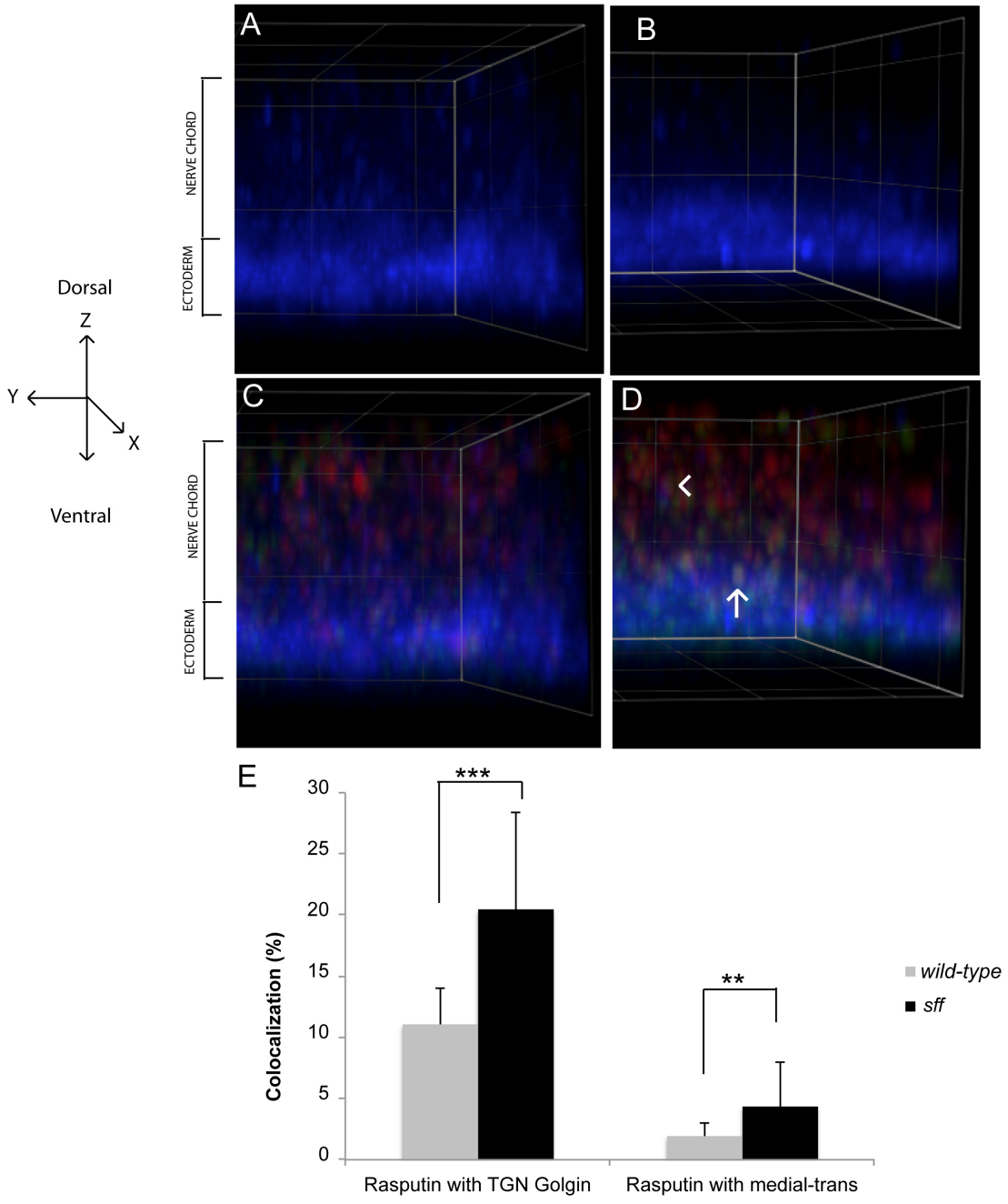

Figure 3.9. Rasputin localizes to Golgi and its association with medial-trans Golgi and trans Golgi network is altered in *sff* mutants. Embryos were imaged across all sections beginning at the ectoderm and ending dorsally for wild-type (A,C) and *sff* (B,D). Representative section from a single segment along the ventral nerve chord in all z-planes was selected to create a three

dimensional projection. (A, B) Diffused expression pattern of Rasputin (blue) is observed in the ectoderm while a distinct punctate presence is seen in the nerve chord. (C, D) Rin localizes to medial-trans Golgi (red) compartments and TGN (green). E. Quantification of the percentage overlap of Rin with TGN and medial trans marker (red) was carried out in the neuronal region away from the ectodermal region. Rin co-localization with medial-trans compartments and TGN is significantly increased in sff/sff mutants. The arrowheads point to overlapping punctae between Rasputin and TGN. The arrows point to the overlapping punctae between medial trans and Rasputin markers. ***$p<0.001$ and **$p<0.05$.

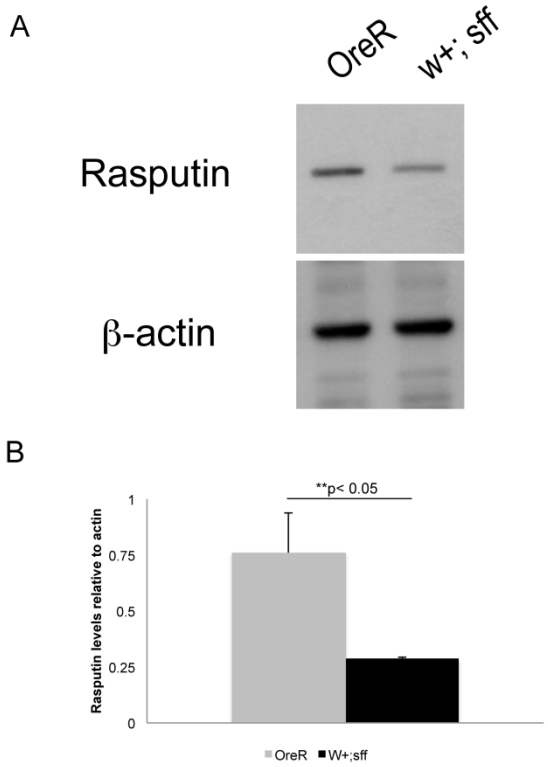

Figure 3.10. Rasputin expression is reduced in *sff* mutant embryos. A. Western blot analysis of wild-type and *sff* embryo extracts (n=3, protein amount loaded was normalized normalized to β-actin) measuring steady state protein concentration of Rasputin(~ 80KDa). B. Quantification of Rasputin levels relative to actin from three different protein extracts for each genotype. Rin expression levels are significantly decreased in *sff* mutants. **p<0.05.

identification of Liprin-alpha, Rasputin, and Bifocal as candidate effectors of Sff kinase signaling in the regulation of glycoprotein glycosylation through the modulation of Golgi organization.

The Golgi apparatus is a complex, multi dimensional organelle with distinct compartment subunits that can be distinguished based on the expression of molecular markers. In vertebrates, this organelle exhibits organization at two levels. Firstly, compartments roughly marking cis, medial, trans /TGN are arranged relative to each other in a stack like manner to yield “mini-stacks”. At the next level, these mini-stacks are arranged in close proximity with compartments of the same type aligned with each other to form a ribbon like arrangement (Jarvela and Linstedt, 2014). However, in invertebrates such as *Drosophila*, the Golgi apparatus does not have the characteristic mammalian ribbon like appearance of these stacks and exhibits the mini stack arrangement exclusively (Kondylis and Rabouille, 2009; Yano et al., 2005).

Linking of similar compartments across different mini-stacks in mammalian cells has been shown to enable a uniform distribution of Golgi resident enzymes. Disruptions in these linkages and resultant defects in glycoprotein glycosylation such as sialylation have been attributed to altered enzymatic processing on cargo molecules(Puthenveedu et al., 2006). Analogously, an increase in membrane bound golgin molecules may alter vesicle flux through the TGN. These events might reflect in increases in the size of particular Golgi compartments, greater overlap between different compartments, a concomitant disruption in the normal distribution of enzymatic machinery and robust

processing of glycoprotein cargo. This situation predicts that enzymes residing in TGN/trans-Golgi compartments might have greater temporal and spatial access to glycoprotein cargo and consequently affect the addition of terminal sugars such as N-acetyl glucosamine by N-acetyl glucosamine transferases –II and IV. It can also be envisaged that glycosylation related events through cis and medial Golgi compartments might also be affected due to greater overlap of cis and trans compartments. This latter observation is supported by comprehensive N-linked glycomic analysis of wild-type and *sff* embryos, in which increased prevalence of N-acetylglucosaminyl transferase –II and IV products was detected. The cell surface expression of N-linked glycoproteins is not affected in spite of defective glycosylation suggesting that vesicular transport from Golgi apparatus to the plasma membrane is not affected (Baas et al). However, whether increased size of trans Golgi/TGN as marked by golgin 245 leads to greater membrane bound golgin molecules remains to be determined.

While distinct classes of coiled coil domain containing proteins such as golgins localize to different Golgi membranes, the roles of non-golgin coiled coil proteins in secretory protein trafficking is only now gaining momentum. Liprin-alpha, an integral component of the synaptic active zone, contains an N-terminus coiled coil domain that has been shown to associate with other coiled coil proteins, such as ERC, and PDZ domain proteins, such as RIM (Serra-Pages et al., 1995; Serra-Pages et al., 1998; Stryker and Johnson, 2007; Zurner et al., 2011). *Drosophila* Liprin-alpha has been shown to interact with Kinesin-3 and

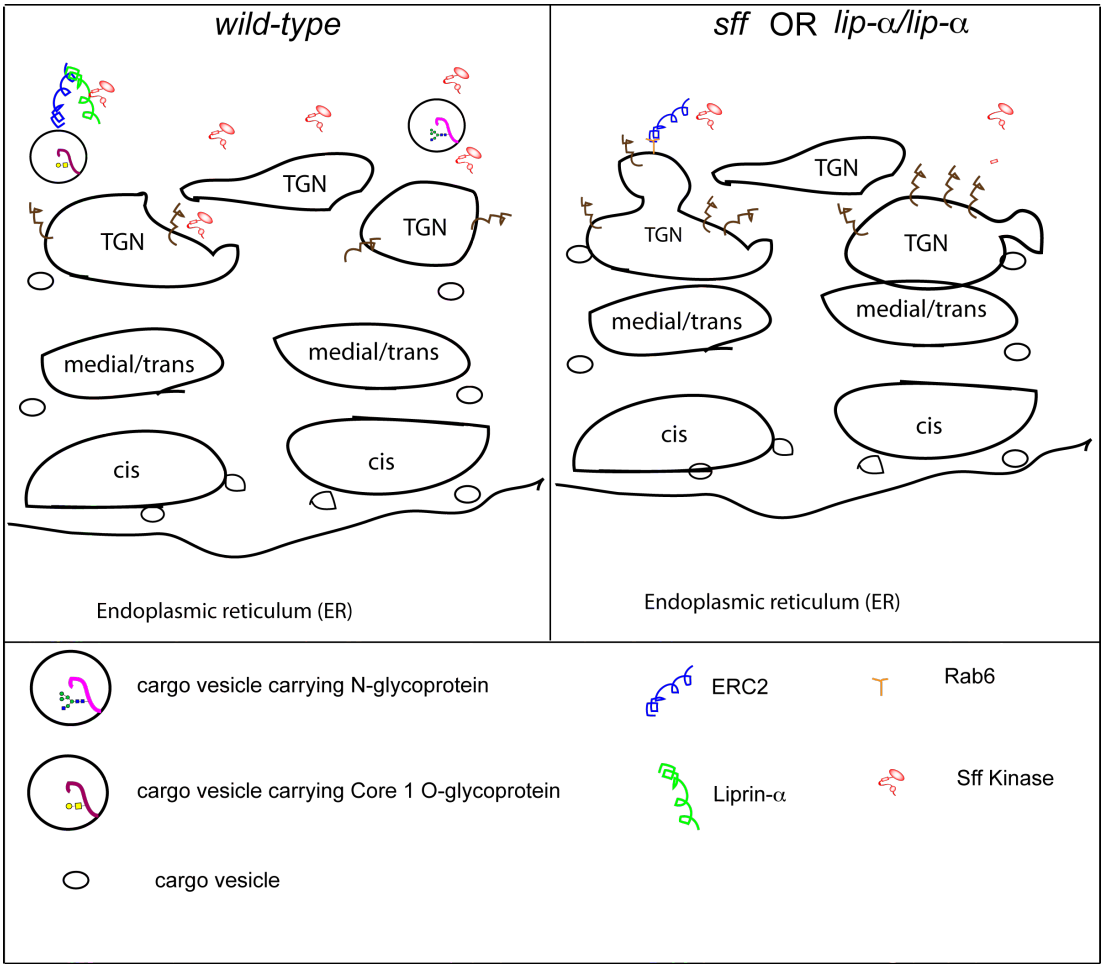

Figure 3.11. A model illustrating the roles of Sff kinase and Liprin-α at Golgi membranes. ERC2 (and Liprin-α) localize to the TGN membrane. While ERC2 gets recruited to TGN with the help of Rab6, Liprin can associate with TGN directly or indirectly through its interaction with ERC2. Liprin-alpha and ERC2 might act on specific domains on TGN such that particular classes of cargo such as O-linked glycoproteins are under their regulation. In *liprin-α* mutants, loss of Liprin-alpha leads to a defective budding of cargo vesicles from the TGN leading to an accumulation and consequent enlargement of the TGN. In *sff* mutants, similar mechanisms might exist where loss of downstream signals via Liprin-alpha contribute to an accumulation of vesicles that are not able to leave the TGN. Documented functions of Liprin-alpha include trafficking of AMPA receptor to post-synaptic sites and regulation of active zone morphology(Kaufmann et al., 2002; Miller et al., 2005; Spangler and Hoogenraad, 2007).

mediate bidirectional transport of synaptic vesicles. Liprin-alpha mutants exhibit decreased forward transport of synaptic vesicles and increased reverse transport from the plasma membrane (Miller et al., 2005). Our observations pertaining to decreased expression of epitopes recognized by peanut agglutinin (PNA) suggest that O-linked (galactose β1-4 N-acetylgalactosamine) glycoproteins are markedly affected in *liprin-α* double mutants. Interestingly, coiled coil proteins such as Bassoon, Piccolo, and ELKS, that are also constituents of presynaptic active zone matrix show association with membranes of the trans Golgi network while Munc 13-1 shows localization with cis-Golgi compartments. On the other hand, RIM1a does not co-localize with either trans Golgi network or cis-Golgi compartments and is believed to attach to membranes of post-Golgi vesicles. It was also observed that ELKS2 recruitment to TGN membranes did not require Bassoon and Piccolo but at least one of them was required for ELKS2 transport on Golgi derived vesicles to the neuronal synapses (Maas et al., 2012). Additionally, both ELKS2/ERC2 and Liprin-alpha have been shown to co-localize with each other in cultured hippocampal neurons and COS-7 cells while the latter protein has been shown to be present in light membrane fractions also (Ko et al., 2003a; Ko et al., 2003b; Shin et al., 2003). Based on these observations, it is reasonable to propose that Liprin-alpha may function at Golgi membranes, more specifically at the trans Golgi network. In this context, Liprin-alpha and ERC2 might interact with each other in an Sff-dependent manner at TGN membranes and control the transport of Golgi derived synaptic vesicles (Inoue et al., 2006).

Golgi structure is intricately linked to golgin levels in various compartments. A particularly striking feature of TGN golgin over-expression is the specific increase in a subset of TGN membranes emphasizing that these golgins are devoted to specific regions of TGN and hence control specific cargo trafficking at these points (Goud and Gleeson, 2010; Lieu and Gleeson, 2010). The increase in average volume of TGN Golgin 245 in *liprin-*α double mutants is phenotypically similar to that of *sff* mutants and to the observed O-linked and N-linked glycan changes detected respectively in *sff* mutants. This raises the possibility that signaling events mediated through Liprin-alpha/ERC family could have functional similarities with events downstream of Sff kinase at the TGN. Perhaps, localization of proteins such as ERC2, Sff/SAD and Liprin-alpha to TGN membranes, Munc-13 to cis-Golgi, and RIM-1 to post Golgi compartments is a mechanism of differentiating between different glycoprotein cargo that are required to be transported to the cell surface. While Liprin might be affecting trafficking dynamics in addition to altering TGN morphology, Sff kinase probably functions specifically in the structural maintenance of a subset of TGN compartments in the *Drosophila* embryo. This scenario would place Sff kinase upstream of Liprin-alpha with the possibility of it being redundantly phosphorylated by other members of AMPK family and would thus explain the variable penetrance of HRP epitope expression that we observe in *lip-a/+;sff/+* embryos. The phenotypic similarities between these mutants in terms of altered TGN units marked by Golgin 245 suggest that in both of these mutants coiled coil proteins play an essential role in membrane maintenance and trafficking. It is

possible that Liprin-alpha, like ERC2, serves as an anchor through its coiled coil domain to facilitate the budding or fusion of cargo containing vesicles from the TGN. However, whether it executes this function directly by associating with specific TGN membranes or does so with the help of golgins is not clear.

Golgin function is mediated through their capacity to serve as tethering molecules and also through interactions with small GTPases such as Rabs, ADP ribosylation factors (ARF) and ARL (ARF like) proteins (Barr and Short, 2003; Behnia and Munro, 2005; Lord et al., 2013; Munro, 2011). Each golgin molecule can associate with more than one small G protein and vice versa to accomplish targeting to Golgi membranes or bringing vesicles together to these membranes to form a scaffold like environment. For instance on the TGN, ARL-1 works to localize GRIP domain containing golgins such as TGN245. However, golgin-245 has also been shown to bind Rab 2 through its coiled coil domain. On the other hand, Rab6 has been shown to be enriched at the TGN and aid in the association of coiled coil containing proteins ELKS, to these membranes (Behnia and Munro, 2005; Goud and Gleeson, 2010; Munro, 2011). Thus, interplay between golgin molecules and small GTPase proteins serve to bring specificity of recognition and recruitment to individual membranes of the secretory pathway.

Rasputin, one of the phosphoproteins identified as differentially phosphorylated in our proteomic analysis of *wild type* and *sff* mutants, is a positive effector of Ras signaling through its interaction with the SH3 domain of RAS GTPase activating protein (RAS-GAP) protein in vertebrates (Duchesne et al., 1993; Pazman et al., 2000; Schweighoffer et al., 1992). In addition to this,

Rasputin has been shown to aid in the deubiquitination of Bre5p, the yeast homolog of Sec23, which serves as the GAP for Sar1 in COPII mediated anterograde transport. In a related context, action of GTPase families such as Rabs, ARFs and ARL is intricately connected to their active GTP bound state that is initiated by gunanine nucleotide exchange factors (GEFs). On the other hand, GTPase activating proteins (GAPs) function as signal terminators, thereby attenuating the signaling activities of these small G-proteins. Constitutive activation of ARL-1 results in Golgi expansion and the formation of massive vacuolar and tubulo-vesicular structures reminiscent of TGN (Bhave et al., 2014; Lu et al., 2001). On the other hand, depletion of golgins has been shown to result in fragmentation of Golgi (Goud and Gleeson, 2010). Therefore, Golgi architecture is under the complex, balanced control of both golgin and small G-proteins such as Rabs, ARLs and ARFs. We observe an expansion in the Golgi compartment defined by golgin 245 as well as altered Rasputin localization to medial/trans Golgi and TGN in *sff* mutants, suggesting that Rasputin phosphorylation (by Sff kinase) negatively regulates effector of GTPase signaling such that its loss leaves the ARFs, Rabs or ARL molecules in a constantly active GTP bound state that in turn continuously promotes the recruitment of vesicular membranes onto the TGN compartment (Cohen et al., 2003; Irvine et al., 2004). While Rasputin has been shown to associate with the SH3 domain of RAS GAP protein, it remains to be seen if its association with this domain is preserved in other G-proteins at the Golgi (Costa et al., 2013; Duchesne et al., 1993; Parker et al., 1996; Pazman et al., 2000; Schweighoffer et al., 1992). Alternatively, one

cannot exclude the possibility that Rasputin, like its modulation of c-Myc mRNA levels through it 3'endoribonuclease activity, can also regulate the expression of proteins responsible for Golgi organization such as Golgins, GRASPs and even enzymes such as glycosyltransferases and glycosidases (Gallouzi et al., 1998).

Cytoskeletal proteins contribute to Golgi structural dynamics. Bifocal regulates actin organization through its interaction with F-actin (Bahri et al., 1997). It genetically interacts with and is phosphorylated by the serine/threonine kinase Misshapen (Msn). Bifocal's interaction with F-actin is Msn-dependent, such that in the absence of Msn Bifocal triggers significant changes in F-actin organization (Babu et al., 2005). Bifocal expression in *Drosophila* embryos is restricted to a subset of neurons in the ventral nerve chord. Its primary expression pattern has been observed to begin at stage 13 and become restricted to the commissures of the embryonic CNS (Bahri et al., 1997). The observation that Sff kinase begins only a stage earlier than this indicates that Sff kinase might be interacting with Bifocal to phosphorylate it in the *Drosophila* embryo. Arguing in favor of this hypothesis is the identification of a S/T site within Bifocal, which has a high propensity to be phosphorylated by an AMPK family kinase. In light of these observations, we propose that the F-actin polymerization modulated by the phosphorylation state of Bifocal influences Golgi dynamics. The integrity of the cytoskeletal system is crucial to glycoprotein glycosylation and the exact mechanisms by which Bifocal might be playing a role in Sff mediated regulation of glycosylation remains to be answered.

Taken together, our phosphoproteomics approach had led to the identification of three novel interaction partners of Sff kinase namely, Liprin-alpha, Rasputin and Bifocal in the regulation of glycoprotein glycosylation. Signaling mechanisms orchestrated by each of these proteins have the capacity to receive stimuli from upstream regulators such as Sff kinase and react to alter glycoprotein glycosylation as cellular, spatial and developmental cues.

CHAPTER 4

CONCLUSIONS AND FUTURE DIRECTIONS

Glycoprotein glycosylation is a highly complex post-translational modification that is regulated at multiple levels (Moremen et al., 2012). While one level of control can be achieved through the expression of enzymes such as glycosyltransferases and glycosidases, glycan diversity and specificity at the individual protein, cell or tissue level cannot be completely explained by this single variable alone. In this study, we propose that appropriate glycoprotein glycosylation output is dependent on proper Golgi organization and structure. While, *Drosophila* Golgi GDP fucose transporter affects the synthesis of fucosylated glycans by regulating the amount of GDP-fucose available, Sff kinase (and indirectly Tollo) have been shown to regulate neural specific glycosylation output through signaling at the Golgi (Baas et al., 2011; Geisler et al., 2012; Seppo et al., 2003). In spite of these crucial insights, our understanding of glycoprotein glycosylation across cells and tissues in intact organisms is still rudimentary. Using differential phosphoproteomics and orthogonal genetic and molecular analysis, we have identified and characterized Liprin-alpha, Rasputin and Bifocal as putative substrates of Sff kinase, that co-regulate neural specific glycosylation in the *Drosophila* Embryo. The results presented in chapters 2 and

3 of this study are thus aimed to further delineate the mechanisms by which signaling molecules such as Sff kinase function to ensure the required glycan output as per the cellular demand.

Role Of Liprin-alpha As A Modulator Of N And O-Linked Glycosylation

Dliprin-α together with *Drosophila* LAR receptor tyrosine phosphatase (RPTP) has been shown to be necessary for proper pre and post synaptic development across various species (Kaufmann et al., 2002; Serra-Pages et al., 1995). Additionally, *lip-α* mutants have been shown to exhibit compromised axon transport dynamics similar to that of mutants in axonal motor proteins such as kinesin-3 (Kaufmann et al., 2002). Protein-proteins interactions involving Liprin-α are executed through its N-terminal coiled-coil and C-terminal SAM domains. Based on its capacity to interact with multiple proteins, Liprin-α is uniquely positioned to manipulate multiple signaling pathways involving intra and extra cellular environment.

Our analysis on Liprin-α mutants indicates that an increase in a subset of TGN compartments causes a shift in the compartmental association of TGN with cis Golgi. While our immunofluorescence analysis has uncovered Core 1 O-glycans as being markedly reduced in *Liprin-α* and *sff* mutants, it remains be seen if other O- and N-linked glycans are also affected due to these mutations separately or in combination with each other. Comprehensive glycomic profiling for *lip-α/lip-α and lip-α/+;sff/+* embryos will thus be able to reveal any such changes. An interesting aspect that might arise is the preferential affects on glycan classes such that less of Core 1 O-glycan structures are a result of

increased glucuronylation of Gal β1-4 GalNAc structures which leads to them not being recognized by PNA lectin. Our phosphoproteomics uncovered a single S/T site that has a high propensity to be phosphorylated by AMPK family of kinases such as Sff. A GPS 2.0 based bio-informatics analysis yields eight other high potential APMK sites out which seven are in the N-terminal domain of Liprin alpha. This suggests that if Liprin-alpha is direct substrate, then these might be the residues around which Sff kinase phosphorylation might be targeted. Nevertheless, the question of whether or not Liprin- α is a direct substrate of Sff kinase can be answered by using *in-vitro* cell culture system and the regions/sites being phosphorylated can be uncovered by making appropriate constructs, expressing them with Sff kinase and carrying out phosphosite identification by proteomics. Alternatively, *in-vivo* studies can be carried out UAS-Liprin transgenic flies for an assessment on phosphorylation of these sites in a wild type and sff background. Additionally, the availability of a UAS-Dliprin-YFP can also be potentially used to identify interaction partners of Liprin-alpha with potential glycosylation relevant roles.

Role Of Rasputin In Sff Signaling

Rasputin (Rin) and its vertebrate homolog G3BP are ubiquitously present in the cellular cytosolic milieu. The observation that all of the S/T present in Rasputin's proline rich acidic domain can be potentially phosphorylated by members of the AMPK family suggests that Ser/Thr phosphorylation could be an important effector of its downstream functions in various signaling pathways. Consequently, an analysis of these phosphosites through antibody pull down

analysis followed by phosphopeptide proteomics in wild-type and *sff* mutant embryos would be informative with respective to its role in glycoprotein glycosylation. Alternatively, one can address this question by making expression constructs with different parts of the Rin protein to see which region is being or not being phosphorylated by Sff kinase. This serves the dual purpose of identifying if Rin is a direct or indirect substrate of Sff kinase with a second serine/threonine kinase in between Rin and Sff. A third approach to further delineate Rasputin's role in neural specific glycosylation would be to unravel the its interaction partners from *Drosophila* cDNA library by using its proline rich domain as a bait in yeast two hybrid methodologies.

Gauging Bifocal's Role In Glycoprotein Glycosylation

The enrichment of proteins affecting cytoskeletal organization in the wild-type phosphoproteome of Stage 10-11 embryos as compared to sff mutants underscores the importance of cytoskeletal machinery in the movement and shuttling of proteins from their site of synthesis to their site of action. Recovering Bifocal as one of the proteins whose phosphorylation is differentially affected in wild-type and *sff* embryos brings forth the possible role of cytoskeletal regulation in glycoprotein glycosylation. To understand the role of cytoskeletal scaffold in glycoprotein glycosylation, it is imperative to dissect the mechanisms by which Bifocal could be modulating Sff kinase mediated regulation of glycosylation. As a step in this direction, analyzing Golgi compartmental associations and glycomic changes in *bif* double mutants by themselves and in combination with *sff* will yield crucial insights into the role of this protein in glycoprotein glycosylation.

Glycan Flux Modulation In The Secretory Pathway

Taken together, our observations present signaling paradigms contributing to the regulation and fine-tuning of glycoprotein glycosylation. While Liprin alpha and Rasputin control secretory glycan output by affecting Golgi architecture and vesicle dynamics, Bifocal extends regulation through by involving the cytoskeletal network. While these proteins can have many different functions in the cellular environment, their unique signaling roles in sculpting glycan profiles, situates them to carve appropriate cellular output in response to multiple external stimuli.

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