REQUIREMENT FOR A CORE 1 GALACTOSYLTRANSFERASE IN THE *DROSOPHILA* NERVOUS SYSTEM

by

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ABSTRACT OF THE DISSERTATION

REQUIREMENT FOR A CORE 1 GALACTOSYLTRANSFERASE IN THE DROSOPHILA NERVOUS SYSTEM

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Glycosylation is important in a lot of fundamental biological processes, including cell recognition, cell adhesion, and cell signaling. Mucin-type *O*-glycosylation involves the synthesis of glycoproteins, expressed in mucous secretions and as transmembrane proteins on the cell surfaces. However, the biological functions of mucin-type *O*-glycans remain incompletely understood. I have pursued genetic and biochemical studies to understand their importance during development in *Drosophila*.

Mucin-type *O*-glycosylation is initiated by the attachment of *N*-acetylgalactosamine (GalNAc) to Ser or Thr residues, and then elongated by additional sugars. To examine the requirements for mucin-type glycosylation in *Drosophila*, I characterized the expression and phenotypes of core 1 galactosyltransferases (core 1 GalTs), which elongate *O*-GalNAc by adding galactose in a β1, 3 linkage. Among *Drosophila* core 1 GalTs, *CG9520* (*C1GalTA*) is expressed in the amnioserosa and central nervous system. A null mutation in *C1GalTA* is lethal. The mutant animals show a

morphogenetic defect in their central nervous system in which the ventral nerve cord is greatly elongated and the brain hemispheres are distorted. Lectin staining and blotting experiments confirmed that *C1GalTA* is required for the synthesis of Gal-ß1,3-GalNAc in vivo. Our observations establish a role for mucin-type *O*-glycosylation during neural development in *Drosophila*.

Overexpression of *C1GalTA* causes a wing blistering phenotype, which occurs when adhesion between the two ventral and dorsal surfaces of the wing blade is lost, and is also commonly seen in integrin mutants. This result implicates mucin-type *O*-glycans in cell adhesion in the *Drosophila* wing blade.

Altogether, these results suggest a role of mucin-type *O*-glycosylation in *Drosophila* development, including the morphogenesis of central nervous system and the formation of the wing blade.

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Chapter I General Introduction

Carbohydrates are essential components of all living organisms. Carbohydrates may be covalently linked to other types of biological molecules to form glycoconjugates, e.g. glycoproteins, glycolipids, and proteoglycans (Varki, 1999; Varki, 2009). Previous studies on Notch glycosylation showed certain sugar modifications on the Notch receptor are essential for regulating the Notch signaling pathway (Haines and Irvine, 2003; Irvine, 2008). In this thesis, I pursued research in *Drosophila* to understand the importance of glycosylation in development.

Glycobiology

Carbohydrates are essential components of all living organisms. Carbohydrates can serve as intermediates in generating energy, signaling molecules, or structural components. The structural roles of carbohydrates are particularly important in multicellular organisms. Since most glycans are on the surface of cellular and secreted macromolecules, they might modulate or mediate cell-cell and cell-matrix interactions that are crucial to the development and function of a complex multicellular organism (Varki, 1999; Haltiwanger and Lowe, 2004; Varki, 2009). They are also in a position to mediate interactions between organisms (e.g., between host and parasite). (Duus et al., 2000; Loukas and Maizels, 2000)

Studies of glycans have lagged far behind those of nucleic acids and proteins. This was mainly due to their structural complexity and their biosynthesis. The structural complexity of glycans comes both from the different sugar components and the multiple different glycosidic linkages (Haltiwanger and Lowe, 2004; Bertozzi and Rabuka, 2009). The biosynthesis of glycans, unlike nucleic acids and proteins, is often branched and not

directed from the DNA template. More recently, combinations of physical and chemical approaches, including mass spectrometery (MS) and NMR, eventually reveal the structures of glycans (Duus et al., 2000; Harvey, 2001). This has made it possible to study the changes in carbohydrate structures and their relation to biological function and disease.

Glycosylation is a post-translational modification

Carbohydrates may be covalently attached to other types of biological molecules to form glycoconjugates, e.g. glycoproteins, glycolipids, and proteoglycans. A glycoprotein is a glycoconjugate in which a protein carries one or more oligosaccharide chains covalently attached to a polypeptide backbone.

A glycoprotein is made by glycosylation, which is a post-translational modification that usually occurs in the ER and Golgi. There are mainly two types of glycosylation, *N*- and *O*-glycosylation (Schachter, 2000). *N*-Glycosylation occurs at the amino acid asparagine (Asn). *O*-Glycosylation occurs at serine (Ser) or threonine (Thr) residues.

An *N*-linked glycan is a sugar chain covalently linked to an asparagine residue of a polypeptide chain (Waechter and Lennarz, 1976). *N*-glycans share a common pentasaccharide core region and can be generally divided into three main classes: high-mannose-type, complex-type, and hybrid-type (Figure I-1)(Varki, 1999; Varki, 2009).

An *O*-glycan is linked to the hydroxyl group of a serine or threonine residue on a polypeptide chain (Tabak, 1995; Schachter, 2000). The best-known *O*-glycan is covalently linked to polypeptide chains via *N*-acetylgalactosamine (GalNAc) and can be

extended into a variety of different structural core classes. This is commonly referred to as mucin-type *O*-glycan, because of its association with heavily glycosylated mucin proteins. Other types of "*O*-linked oligosaccharides" do exist (Varki, 1999; Varki, 2009). For example, many intracellular proteins are modified by *O*-linked GlcNAc addition at serine and threonine residues that are also sites of phosphorylation. Vertebrate cells can produce *O*-linked fucose, *O*-linked mannose, and *O*-linked glucose. *O*-linked xylose was produced to initiate proteoglycan formation. In this thesis, I mainly focused on mucin-type *O*-glycans.

Mucin-type *O*-glycans

Mucin-type *O*-glycans are assembled by stepwise addition of single monosaccharides; each sugar is transferred from a nucleotide sugar donor by specific membrane-bound glycosyltransferases (Tabak, 1995; Brockhausen, 1999). The biosynthesis of mucin-type *O*-glycans is initiated by the addition of the monosaccharide GalNAc (from UDP-GalNAc) to serine and threonine residues catalyzed by a polypeptide-*N*-acetyl-galactosaminyltransferase (ppGalNAcT). After this reaction, four common *O*-glycan subtypes could be generated by differential monosaccharide linkage to this unsubstituted GalNAc (GalNAcα-Ser/Thr) (Figure I-2)(Varki, 1999; Varki, 2009).

Most *O*-glycans contain the Core 1 subtype structure formed by the addition of galactose in a β1–3 linkage to the GalNAc, which is catalyzed by the Core 1 β1–3 galactosyltransferase (Core 1 GalT). The branched Core 2-type *O*-glycans are generated by the addition of GlcNAc in a β1–6 linkage to the GalNAc on the Core 1 *O*-glycans.

Core 3 *O*-glycan production is controlled by Core 3 GlcNAcT activity which, like Core 1 GalT, uses the GalNAcα-Ser/Thr substrate (Figure I-2). Thus, Core 3 O-glycan formation may depend on a competition between Core 1 GalT and Core 3 GalT enzymes in some cell types. This Core 3 *O*-glycan can also be a substrate for the formation of branched Core 4 *O*-glycan (Tabak, 1995; Brockhausen, 1999; Brockhausen et al., 2009).

These Core *O*-GalNAc glycans may be further modified by additional sugar linkages. The terminal structures of *O*-GalNAc glycans may contain molecules that block further elongation, e.g. fucose, galactose, *N*-acetylglucosamine, and sialic acid in α-linkages, *N*-acetylgalactosamine in both α- and β-linkages, and sulfate (Brockhausen, 2003).

Among modifications to the GalNAcα-Ser/Thr structure, the Core 1 to 4 *O*-glycans are most common in mammals. Core 1 and Core 2 *O*-GalNAc glycans are found in both glycoproteins and mucins from a variety of cells and tissues. Core 3 and Core 4 *O*-GalNAc glycans have been found only in secreted mucins of certain mucin-secreting tissues, such as bronchi, colon, and salivary glands (Van Klinken et al., 1995).

Notably, In *Drosophila*, the only mucin-type *O*-glycans identified to date are T antigen and Tn antigen (GalNAc1-Ser/Thr)) (Kramerov et al., 1996). Thus, *Drosophila* may be a suitable model system for investigating the functions of T antigen and the *C1β3GalT* genes during development.

Biological functions of glycans

The importance of glycans varies from different biological contexts. Some glycans might have relatively subtle function, while other glycans might be crucial for the development,

growth, or survival of the organism. The biological roles of glycans can be broadly divided into two groups. One group relies on the structural and modulatory properties of glycans and the other relies on specific recognition of glycan structures by other molecules (generally receptor proteins or lectins)(Montreuil, 1980; Spillmann and Burger, 1996; Varki and Lowe, 2009).

The glycans attached to molecules in extracellular matrix such as collagens and proteoglycans are important for the maintenance of tissue structure and integrity (Iozzo, 1998). The external location of glycans on most glycoproteins can provide a general shield. For instance, mucin-type glycosylation is thought to form a protective barrier and extracellular lubricant.

There are many examples, in which glycosylation can modulate the interaction of proteins with one another. Glycosylation of a polypeptide can also mediate an on-off or switching effect. For example, glycosylation of Notch receptors modulates Notch-ligand interactions and regulates the Notch signaling pathway (Haines and Irvine, 2003; Irvine, 2008).

Specific recognition of glycan structures by other molecules is also crucial in cell-cell recognition and cell-matrix interactions. For example, the mucin-type *O*-glycans expressed on the mouse egg coat (the zona pellucida) have been reported to function in sperm binding to the egg (Bleil and Wassarman, 1988; Kitazume-Kawaguchi et al., 1997). The hematopoietic system of vertebrates also involves glycans during its development and function. Mucin-type Core 2 *O*-glycans were reported to function as ligands for neutrophil recruitment in peritoneal inflammation (Ellies et al., 1998).

Glycosylation changes in cancer

Glycosylation changes have been associated with several diseases including cancer (Fabienne et al., 1994; Kim and Varki, 1997; Yang et al., 2005), autoimmune diseases such as rheumatoid arthritis (Backlund et al., 2002; Balik et al., 2005), and cystic fibrosis (Xia et al., 2005).

Altered glycosylation is often found on the surface of cancer cells. *O*-glycosylation in cancers is often reduced which leads to the accumulation of simpler core 1 *O*-glycans, including the disaccharide Galβ1–3GalNAcα-Ser/Thr (T antigen, TF antigen, or Thomsen–Friedenreich antigen), and the monosaccharide GalNAcα-Ser/Thr (Tn antigen). Aberrant mucin-type glycosylation is often associated with tumor metastasis (Brooks et al., 2008). Interestingly, certain tumor-associated sugar epitopes were shown to be actively involved in metastasis by facilitating intravasation of cancer cells, binding of the circulating cancer cells to the endothelium of distant tissues, extravasation and colonization at distant sites (Kim et al., 1998; Borsig et al., 2001).

Glycosylation in Development

The complex carbohydrates on cell surfaces have been predicted to play important roles in developmental processes because of the observation that specific carbohydrate structures appear in specific spatial and temporal patterns throughout development. Studies on several model organisms (e.g., mice, *Drosophila*, and *C. elegans*) have demonstrated that a number of glycoconjugates would function in cellular communication and involve crucial processes during development (Haltiwanger and Lowe, 2004).

Prior studies in our laboratory have shown that the Notch signaling pathway, which plays important roles in animal development and physiology, is modulated by Fringe-dependent glycosylation (Irvine, 1999; Moloney et al., 2000a; Haines and Irvine, 2003; Irvine, 2008). The *fringe* gene was first identified as a gene involved in signaling interactions between dorsal and ventral cells that are essential for the growth and patterning of the Drosophila wing imaginal disc. Fringe inhibits the activation of Notch by one ligand, Serrate, and potentiates the activation of Notch by another ligand, Delta. The Fringe-dependent Notch glycosylation influences the binding of Notch ligands to Notch receptors, which in turn restricts Notch activation to the dorsal-ventral border of the developing Drosophila wing. Studies on fringe-related genes in mammals also demonstrated that glycosylation regulates Notch signaling and plays essential roles in the development of many other tissues.

Glycosyltransferases

Glycosyltransferases are enzymes responsible for glycosylation. Glycosyltransferases recognize specific sugars, sugar sequences and often peptide moieties of substrates, and catalyze the transfer of sugar moieties from activated donor molecules to specific acceptor molecules, forming glycosidic bonds. Each distinct linkage is usually synthesized by one or more members of a glycosyltransferase family. (Spiro, 2002; Ten Hagen et al., 2003a; Narimatsu, 2006)

Biochemical characterization has shown that Drosophila and mammalian *fringe* homologs encode UDP-*N*-acetylglucosamine:fucose-*O*-Ser ß1,3-N-acetylglucosaminyltransferases. Fringe catalyzes the second step in the formation of an

O-linked tetrasaccharide, adding *N*-acetylglucosamine in a β1,3 linkage onto *O*-fucose on Notch receptor and its ligands (Moloney et al., 2000a; Okajima and Irvine, 2002; Haines and Irvine, 2003; Irvine, 2008).

The *Drosophila* genes *fringe* and *brainiac* are both involved in the regulation of Notch activity in *Drosophila* (Moloney et al., 2000b; Muller et al., 2002a). Based on sequence similarity, Fringe, brainiac and 19 other putative glycosyltransferases are categorized into a large family of β1, 3-glycosyltransferases in *Drosophila* (Correia et al., 2003). These β1, 3-glycosyltransferases are related to mammalian β1, 3-glycosyltransferases. Eleven distinct mammalian homologs have been demonstrated to encode functional enzymes forming ß1-3 glycosidic linkages with different UDP donor sugars and acceptor sugars (Muller et al., 2002b; Togayachi et al., 2008).

I am interested in understanding the importance of glycosylation in development. According to the striking phenotypes of *fringe* and *brainiac* mutants, we hypothesized that there might be some genes in this β1, 3-glycosyltransferase family mediating important biological processes in *Drosophila*.

In this thesis, I am reporting genetic and biochemical studies on two genes in this family, *C1GalTA and CG8668*. In the first part of my thesis, genetic and biochemical studies on C1GalTA demonstrate that *C1GalTA* is required for the morphogenesis of central nervous system. Our results identify a role for mucin-type *O*-glycosylation during neural development in *Drosophila* (Chapter II and III).

The second part of this thesis presents experiments that have been performed to characterize CG8668. Our results suggest *CG8668* is not essential for *Drosophila* development (Appendix).

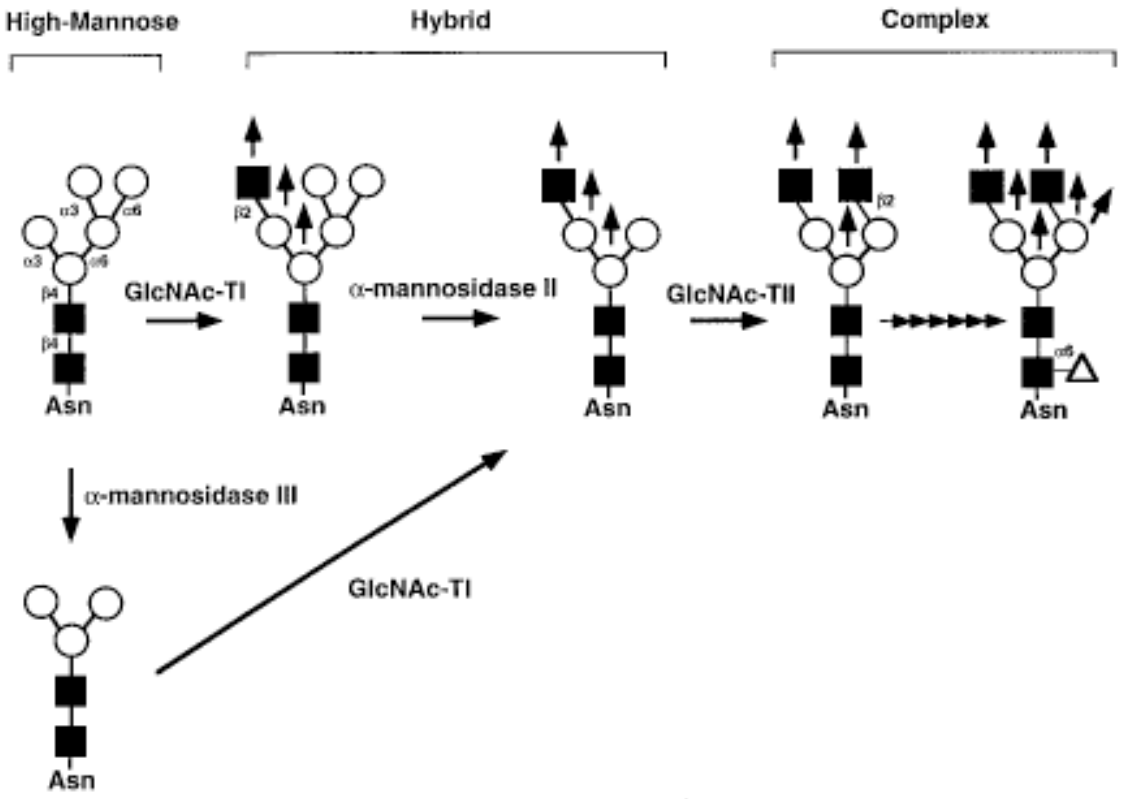

Figure I-1 Vertebrate N-glycan diversification

Vertebrate N-glycan diversification in the Golgi as shown generates three N-glycan subtypes: high-mannose, hybrid, and complex. The vertical arrows depict locations of branch formation in N-glycan diversification, not all of which occur on any single N-glycan.(Varki, 2009)

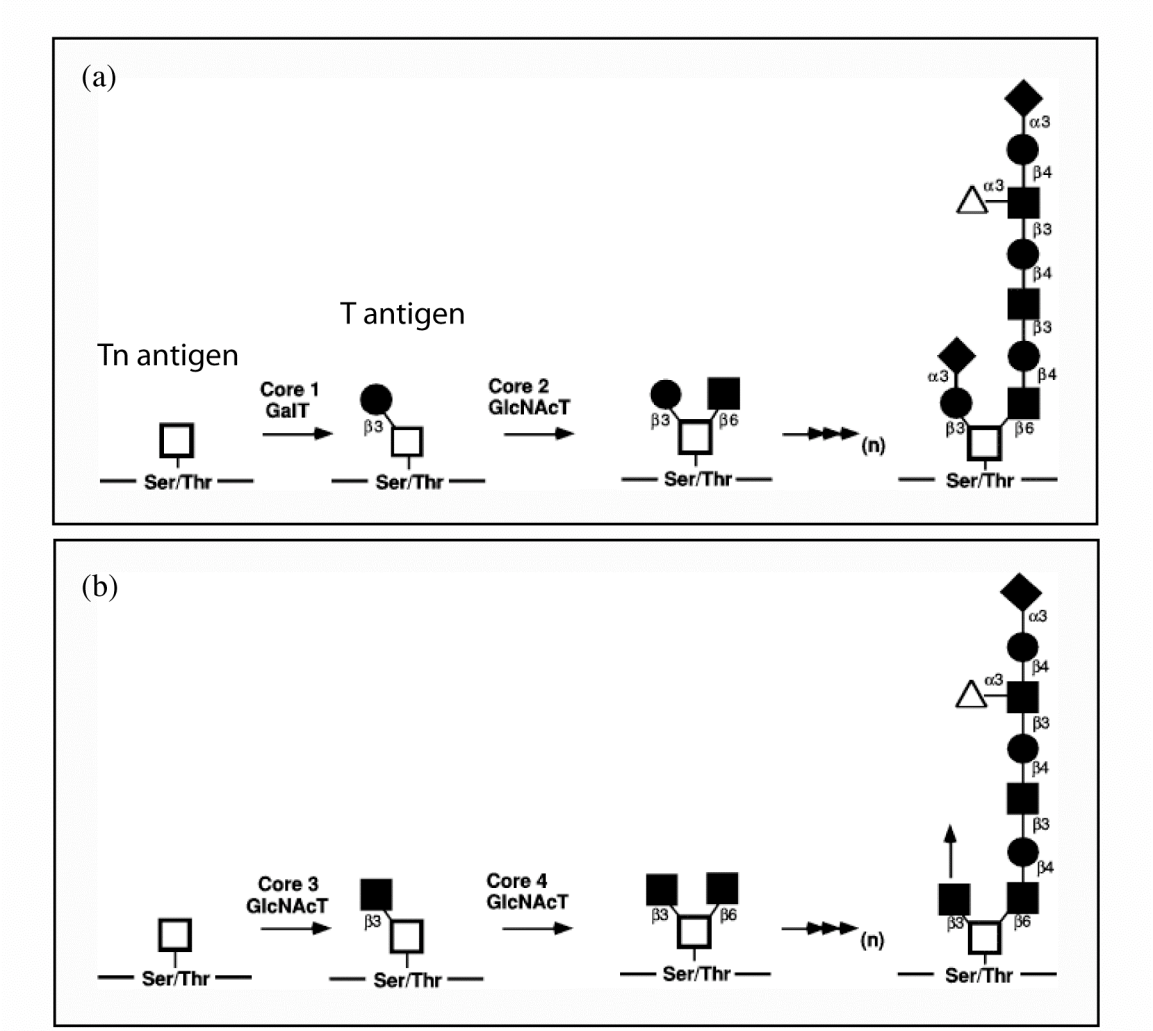

Figure I-2 Vertebrate *O*-glycans.

(a). Core 1 and Core 2 *O*-glycan subtype formation is controlled by the activity of Core 1 GalT and Core 2 GlcNAc-T enzymes. **(b).** Core 3 and Core 4 *O*-glycan subtype formation is controlled by the activity of the Core 3 GlcNAcT and Core 4 GlcNAcT enzymes. Additional biosynthesis can yield *O*-glycans bearing fucosylated and sialylated lactosamines. Further linkages may subsequently occur (*vertical arrow*).(Varki, 2009)

Notably, in *Drosophila*, the only mucin-type *O*-glycans identified to date are T antigen and Tn antigen (GalNAc1-Ser/Thr)(Kramerov et al., 1996).

Chapter II Requirement for a core 1 galactosyltransferase in the *Drosophila* nervous system

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Yuh-ru Lin contributed Figures 2, 3, 4B-D, 5B-I, 6, 7A-B and Tables 1 and 2

ABSTRACT

Mucin type *O*-glycosylation is a widespread modification of eukaryotic proteins, but its functional requirements remain incompletely understood. It is initiated by the attachment of N-acetylgalactosamine (GalNAc) to Ser or Thr residues, and then elongated by additional sugars. We have examined requirements for mucin-type glycosylation in *Drosophila* by characterizing the expression and phenotypes of core 1 galactosyltransferases (core 1 GalTs), which elongate *O*-GalNAc by adding galactose in a β1,3 linkage. *Drosophila* encode several putative core 1 GalTs, each expressed in distinct patterns. *CG9520* (*C1GalTA*) is expressed in the amnioserosa and central nervous system. A null mutation in *C1GalTA* is lethal, and mutant animals exhibit a striking morphogenetic defect in which the ventral nerve cord is greatly elongated and the brain hemispheres are misshapen. Lectin staining and blotting experiments confirmed that *C1GalTA* contributes to the synthesis of Gal-ß1,3-GalNAc in vivo. Our results identify a role for mucin-type *O*-glycosylation during neural development in *Drosophila*.

INTRODUCTION

O-linked glycosylation, in which glycans are attached to the hydroxyl groups of Ser or Thr residues, is a structurally and functionally diverse class of protein modifications (Haltiwanger and Lowe, 2004). One common class of *O*-linked glycosylation in eukaryotes encompasses glycans attached to extracellular protein domains via an *O*-linked N-acetylgalactosamine (*O*-GalNAc) (Hanisch, 2001; Hang and Bertozzi, 2005). This is commonly referred to as mucin-type glycosylation, because of its association with heavily glycosylated mucin proteins. In this context, mucin-type glycosylation is thought to form a protective barrier and extracellular lubricant. However, mucin-type glycosylation is also found on a wide variety of other proteins, and implicated in diverse functions, from lymphocyte homing to sperm-egg binding (Hanisch, 2001; Hang and Bertozzi, 2005). Additionally, aberrant mucin-type glycosylation is also often associated with tumor metastasis (Brooks et al., 2008). In this work, we describe a requirement for mucin-type glycosylation during the development of the *Drosophila* nervous system.

In mammals, *O*-GalNAc (Tn antigen) is most often modified by a β1,3 linked Gal to generate the core 1 disaccharide (T antigen), which is then further modified by the addition of other sugars. Several alternate core structures, in which different sugars are attached to *O*-GalNAc, have also been described, and the core *O*-linked glycans can then be elongated in diverse ways to generate a wide variety of mucin-type glycans (Hanisch, 2001; Hang and Bertozzi, 2005). The *Drosophila* genome does not appear to encode homologues of many of the enzymes required for the generation of the complex and diverse mucin-type glycans found in vertebrates (Adams et al., 2000), and mass spectrometry of *O*-glycans in *Drosophila* embryos identified mostly disaccharide (T

antigen) (North et al., 2006; Aoki et al., 2008). Nonetheless, some monosaccharide (Tn antigen) was detected, and recent analysis of *Drosophila O*-glycans has also resulted in the detection of smaller amounts of a number of more complex *O*-GalNAc glycans, including a set of glucouronylated core 1 *O*-glycans (Aoki et al., 2008; Breloy et al., 2008).

While glycoconjugate analysis suggests that synthesis of mucin-type glycans in *Drosophila* might be simpler than in vertebrates, in one respect *Drosophila* exhibit greater complexity than mammals. There exist a large family of enzymes that can catalyze the attachment of GalNAc to protein in mammals (ppGalNAcTs) (Ten Hagen et al., 2003a), but only a single core 1 Galactosyltransferase (core 1 GalT) has been identified (Ju et al., 2002a). Conversely, bioinformatics analysis suggests that *Drosophila* possess large families both of ppGalNAcTs (Ten Hagen et al., 2003b), and of core 1 GalTs (Correia et al., 2003). Biochemical studies have confirmed that several of these ppGalNAcTs and at least four of the core 1 GalTs possess the expected enzymatic activity (Ten Hagen et al., 2003b; Muller et al., 2005).

Although potential redundancy remains a challenge, genetic studies are beginning to inform our understanding of the biological requirements for mucin-type glycosylation. In mice, four different ppGalNAcTs have been mutated by gene targeting, but without apparent phenotypic effect, presumably due to redundancy among family members (Ten Hagen et al., 2003a). The existence of only a single core 1 GalT in mice is more favorable for genetic analysis, and a gene-targeted mutation in this gene is embryonic lethal with defective angiogenesis (Xia et al., 2004). However, it also remains possible that for some functions core 1 *O*-linked glycans are functionally redundant with other *O*-

linked or *N*-linked glycans. Although *Drosophila* also contain several ppGalNAcTs, expressed in overlapping patterns (Ten Hagen et al., 2003b; Tian and Ten Hagen, 2006b), mutation of at least one ppGalNAcT, *l(2)35a*, is lethal (Schwientek et al., 2002; Ten Hagen and Tran, 2002), and mutant animals have tracheal defects (Tian and Ten Hagen, 2006a).

In this work, we present a functional analysis of requirements for two of the core 1 GalTs in *Drosophila*. We report that the members of this gene family are expressed in diverse expression patterns, suggesting that genetic redundancy among them is limited. We created and characterized mutations in the core 1 GalT encoded by *GC9520 (C1GalTA)*, and identified a striking neural phenotypes in mutant animals, in which the ventral nerve cord fails to condense and the brain hemispheres are misshapen. This phenotype implicates mucin-type glycans in morphogenetic processes required for normal neural development.

RESULTS and DISCUSSION

Core 1 GalTs in *Drosophila*

When human core 1 GalT (C1GalT1) was first cloned, it was suggested that *Drosophila* contain two close homologues, encoded by *CG8708* and *CG9520* (Ju et al., 2002a). However, based on bioinformatic analysis, we suggested that *Drosophila* are likely to encode at least eight distinct core 1 GalT domains, which at that time were annotated as seven distinct genes (*CG9520*, *CG8708*, *CG2975*, *CG3119*, *CG7440*, *CG13904*, *CG18558*), one of which (*CG13904*) encoded two tandemly repeated core 1 GalT domains (Correia et al., 2003). Another gene, *CG2983*, shares a high degree of overall

sequence similarity with the other putative core 1 GalTs, but appears to lack critical amino acids within the most highly conserved sequence motifs (Correia et al., 2003). In a subsequent refinement of the *Drosophila* genome annotation, *CG13904* has been split into two adjacent genes, *CG34056* and *CG34057* (Drysdale and Crosby, 2005). We also note that four of the putative core 1 GalTs (*CG3119*, *CG2975*, *CG18558*, and *CG2983*) are located in close proximity to each other on the second chromosome (Table II-1), suggesting that they arose from relatively recent duplication events. By BLASTP (Correia et al., 2003) or ClustalW analysis, *CG9520* is both the closest *Drosophila* homologue of human core 1 GalT1, and the closest homologue of most of the putative *Drosophila* core 1 GalTs, and thus appears to represent the ancestral core 1 GalT in *Drosophila* (Fig. II-1).

The enzymatic activity of four putative *Drosophila* core 1 GalTs, encoded by *CG9520*, *CG8708*, *CG34056*, and *CG2975* has been reported (Muller et al., 2005). All four of them exhibit some glycosyltransferase activity on model substrates, transferring Gal onto the 3 position of GalNAc, implying that they can act as core 1 GalTs. *CG9520* in particular exhibited high activity on Tn and glycopeptide substrates, although it also exhibited weaker activity on glycolipids. We have independently confirmed the ability of *CG9520* to transfer Gal onto a simple GalNAc acceptor, pNp-GalNAc (not shown). We also attempted to detect core 1 GalT activity for *CG8708*, *CG7440* and *CG18558* in this same assay, but were unsuccessful. However, we note that Muller et al (2005) reported that the Gal transferase activity of *CG8708*, *CG34056*, and *CG2975* was very low, approximately a hundred fold less than that of *CG9520*, and only a few fold above background. The low or undetectable activity on simple model substrates for many

members of this gene family might suggest that they do not function as core 1 GalTs, and instead participate in the synthesis of alternative glycoprotein or glycolipid structures. It is also possible that full core 1 GalT activity for most family members can only be achieved on specific, as yet unidentified, substrates. Alternatively, the activity of some family members might require specific co-factors or chaperones (Ju and Cummings, 2002). Conventionally, *Drosophila* genes are named once genetic or biochemical functions for them have been determined, and on this basis we will refer to *CG9520* as *Core 1 Galactosyltransferase A* (*C1GalTA*), but because of uncertainty over their principal biochemical functions, continue to refer to other family members by their CG numbers.

Drosophila Core 1 GalT family members exhibit diverse, tissue-specific expression

To gain insight into the potential biological functions of known and putative core 1 GalTs, we examined their expression patterns throughout *Drosophila* development by in situ hybridization. These expression patterns are summarized in Figs II-2-4 and Table II-1. These studies revealed a striking diversity in expression patterns, as many of these genes are uniquely expressed in distinct epithelial tissues. The observation of distinct expression patterns suggests that they have distinct biological functions.

Four of the known and putative core 1 GalTs are expressed during embryonic development. As reported previously (Muller et al., 2005), we find that *C1GalTA* is expressed by amnioserosa cells (Fig. II-2B,C), and *CG8708* is expressed by salivary gland cells (Fig. II-4B,C). However, in contrast to Muller, we find that *CG13904/CG34056* is expressed specifically by tracheal cells rather than salivary gland

cells (Fig. II-3F), and we also found that during late embryonic development *C1GalTA* is expressed within the central nervous system (CNS) (Fig. II-2D). The tracheal expression of *CG13904/CG34056* is intriguing in light of the tracheal defects observed in a ppGalNAcT mutant (Tian and Ten Hagen, 2006a). We also report that *CG7440* is expressed by a limited number of cells in the anterior midgut and proventriculus (Fig. II-3E). The remaining core 1 GalTs were not detectably expressed during embryogenesis.

We also examined the expression of all family members at later stages of development. During larval development expression of core 1 GalTs was detected in parts of the digestive tract, imaginal discs, and CNS, (Table II-1, Figs II-2, 3). In the adult, several of the core1 GalTs exhibit distinct regionalized expression patterns in different parts of the male reproductive system (Fig. II-3A-C, Table II-1), and several are expressed in the nurse cells during oogenesis (Fig. II-2F, Table II-1).

Isolation of mutations in *C1GalTA* and *CG8708*

To investigate the biological requirements for mucin-type glycosylation in *Drosophila*, we sought to identify mutations in core 1 GalTs. We have focused on the two genes that are most closely related by sequence to mammalian *C1GalT1*, *CG8708* and *C1GalTA*.

In the case of *CG8708*, a P element insertion within the protein-coding second exon, *P[KG05736]*, was identified by the BDGP gene disruption project (Bellen et al., 2004) (Fig. II-4A). This insertion is located between conserved sequence motifs expected to be required for core 1 GalT function. Moreover, in situ hybridization to *Drosophila* embryos using a probe corresponding to portions of the gene downstream of the insertion site established that downstream sequences are not detectably transcribed in homozygous

mutant embryos (Fig. II-4D). Thus, this insertion likely corresponds to a null allele. Nonetheless, homozygous mutant animals are viable, fertile, and appear morphologically normal. Examination of salivary gland morphogenesis, using antibodies that stain salivary gland cells, failed to reveal defects in salivary gland development (Fig. II-4E,F and data not shown). *CG8708* might be genetically redundant, if, for example, other core 1 GalTs are indeed expressed in salivary gland cells (Muller et al., 2005). Alternatively, *CG8708* might have purely physiological rather than developmental roles.

The gene disruption project also identified a P-element insertion within *C1GalTA*, *P[KG02976]* (Fig. II-5A) (Bellen et al., 2004). Homozygous individuals are viable and appear morphologically normal. This insertion is located within the first intron, and in situ hybridization to mRNA using downstream probes in homozygous animals revealed no detectable effect on *C1GalTA* expression (not shown), suggesting that it does not impair gene expression. In order to create mutations in *C1GalTA,* we thus took advantage of the fact that transposase-mediated excision of P elements is often imprecise, resulting in deletion of flanking sequences. From 76 excision events, we isolated 20 homozygous lethal mutant chromosomes. However, genetic complementation tests with chromosomal deficiencies that delete the cytological location of *C1GalTA* (29F5) revealed that only two of the lethal mutations mapped to this region.

These two mutations, which we will refer to as $C1GalTA^{2.1}$ and $C1GalTA^{30.1}$, are also lethal in trans to each other, and subsequent molecular and genetic characterization confirmed that they are both alleles of *C1GalTA.* PCR and DNA sequencing revealed that $C1GalTA^{2.1}$ is associated with a large deletion of genomic DNA (Fig. II-5A), which removes much of the *C1GalTA* coding region, including motifs predicted to be essential

for its catalytic activity. *C1GalTA*$^{30.1}$ is associated with a smaller deletion (Fig. II-5A). This molecular analysis suggests that *C1GalTA*$^{2.1}$ is likely to be a null allele, whereas *C1GalTA*$^{30.1}$ might be a hypomorphic allele.

C1GalTA$^{2.1}$ homozygotes died during embryogenesis, but this was due to an unlinked lethal mutation, as hemizygous mutant animals, created using molecularly defined chromosomal deficiencies (*Df(2L)ED647* and *Df(2L)Exel7040*), can survive until pupal stages. *C1GalTA*$^{30.1}$ hemizygotes are also pupal lethal. Confirmation that the lethality is due to mutation of *C1GalTA*, rather than other genes, was provided by the observation that *C1GalTA*$^{2.1}$ or *C1GalTA*$^{30.1}$ hemizygotes can be rescued by a transgene (*P[C1GalTA*$^{+}$*]*) that comprises a genomic DNA fragment that only encodes *C1GalTA* (Fig. II-5A). These rescued animals are viable, fertile, and appear morphologically normal.

Glycosylation changes in *C1GalTA* mutants

The lethality of *C1GalTA* mutants, together with its normal enzymatic activity, implies that this gene participates in the synthesis of essential mucin-type *O*-glycans during *Drosophila* development. To visualize glycosylation changes in mutant animals, we took advantage of the availability of a lectin, peanut agglutinin (PNA), that specifically recognizes T antigen (Gal β1,3 GalNAc) (Wu et al., 1997). In wild-type embryos, PNA preferentially stains the amnioserosa, and at later stages, parts of the CNS (Fig. II-5B,C) (Burt and Anderson, 1985; Fredieu and Mahowald, 1994; D'Amico and Jacobs, 1995; Tian and Hagen, 2007). This is reminiscent of the expression of *C1GalTA*, consistent with the hypothesis that it represents bona fide detection of T antigen in *Drosophila*.

Indeed, when *C1GalTA*$^{2.1}$ mutant embryos were stained with PNA, the amnioserosa staining was reduced, and the CNS staining was abolished (Fig. II-5D,E). Normal PNA staining was restored in animals with the genomic rescue construct (Fig. II-5F,G). These observations implicate *C1GalTA* as a major T synthase during embryonic development. The residual amnioserosa staining observed in *C1GalTA* mutants might derive from the maternal contribution of *C1GalTA* (Fig. II-2A,F), or from the activity of other core 1 GalTs. Further support for the conclusion that C1GalTA promotes synthesis of T antigen in vivo was provided by the observation that amnioserosa staining with an antibody that recognizes *O*-linked GalNAc (anti-Tn) increased in *C1GalTA*$^{2.1}$ mutant embryos (Fig. II-5H,I). Intriguingly, an obvious PNA staining of the salivary gland was observed *C1GalTA*$^{2.1}$ mutant embryos (Fig. II-5E), but not in wild-type embryos (Fig. II-5C). We suggest that in the absence of *C1GalTA*, either a *CG8708*-dependent synthesis of T antigen can now occur, or a pre-existing *CG8708*-dependent synthesis of T antigen in the salivary gland becomes recognizable by PNA.

We also examined glycosylation changes in mutant animals by separating proteins on SDS PAGE gels, transferring them to membranes, and then probing them with sugar binding proteins (lectin blots). PNA blotting identified several bands in wild-type embryos (Fig. II-5J,K). These bands correspond to *O*-glycosylated proteins, as PNA staining on blots was greatly reduced by treatment of embryo lysates with *O*-glycanase (Fig. II-5K), which specifically removes *O*-GalNAc glycans. Notably, these bands were also greatly reduced in *C1GalTA*$^{2.1}$ mutants (Fig. II-5J). Similar results were observed with another T-antigen binding lectin, Jacalin (not shown). These observations further

support the conclusion that *C1GalTA* is the principal T synthase during embryonic development.

C1GalTA is required for nervous system morphogenesis

In order to identify developmental processes that require *C1GalTA*–mediated glycosylation, we examined the external and internal morphology of mutant animals. Most embryonic and larval tissues were not visibly abnormal. However, the larval nervous system displayed a striking defect, in which the ventral nerve cord (VNC) was greatly elongated, and the larval brain hemispheres were distorted (Fig. II-6A-K); this phenotype was fully rescued by the wild-type transgene (Fig. II-6J). Thus, elongation of mucin type *O*-glycans is apparently required for normal development of the *Drosophila* nervous system.

In wild type, the VNC spans the length of the embryo when it first forms, but then towards the end of embryogenesis begins to condense, such that in the larva it spans only a few segments. The elongated length of the larval VNC suggested that this normal condensation process could be defective. To facilitate characterization of VNC condensation, we examined animals in which GFP was expressed in the VNC under *nrv2-Gal4* or *elav-Gal4* control. In wild type, condensation begins during stage 15, and continues through hatching (Olofsson and Page, 2005). In *C1GalTA* mutants, VNC condensation occurs, but fails to reach the same extent of condensation as in wild type, as the VNC is obviously elongated in first instar larvae (Fig. II-6A,B, Table II-2). To probe for defects in neural development during embryogenesis, we stained embryos with antibodies that specifically recognize neurons (anti-Elav), glia (anti-Repo), or axons

(anti-Fasciclin II, anti-Neuroglian), but none of these stains revealed obvious differences between wild-type and *C1GalTA* mutant embryos (Fig. II-6L-O and data not shown).

In wild-type animals the VNC remains short throughout larval development, but in *C1GalTA* mutants the VNC is even more elongated at third instar than it is at the end of embryogenesis. To examine the progression of the VNC morphology defect, we compared the relative length of the VNC at first instar, second instar, and early third instar. At each stage, the VNC in mutant animals is elongated compared to wild-type animals, and the VNC continues to increase in length throughout larval development (Fig. II-6, Table II-2). The continued requirement for *C1GalTA* during larval development is consistent with the observation that it is expressed in the CNS throughout larval development (Fig. II-2E, I). The elongated VNC of mutants is noticeably thinner (Fig. II-6C-H) and is also more fragile when dissected.

C1GalTA and processes implicated in VNC condensation

Condensation of the VNC occurs in many arthropods, but remains a poorly understood process. Some insight into genes and processes involved has come from genetic studies in *Drosophila*. Mutation of *worniu*, which encodes a snail family transcription factor, impairs VNC condensation (Ashraf et al., 2004). To investigate whether there was a regulatory link between *worniu* and the glycosylation effected by *C1GalTA* , we examined *C1GalTA* expression and PNA staining in $worniu^{1}$ mutants, but no differences were observed (not shown). Glia have been implicated in VNC condensation by the observation that VNC condensation is reduced in mutants that affect glial cells, including *prospero* and *repo* (Doe et al., 1991; Campbell et al., 1994; Halter et al., 1995). Using

anti-Repo staining as a marker, no difference in the number of glial cells was observed in *C1GalTA* mutant embryos (Fig. II-6L,M).

There is also evidence for a critical role of the extracellular matrix and cell-extracellular matrix interactions, as mutations in two different *Drosophila* integrin subunits have been reported to reduce VNC condensation (Brown, 1994). The secretion of extracellular matrix by hemocytes appears to be particularly important for VNC condensation, as disrupting hemocyte migration impairs both the deposition of extracellular matrix around the VNC and VNC condensation (Olofsson and Page, 2005). Intriguingly, in addition to the PNA reactivity associated with the amnioserosa and VNC, we noticed PNA staining on scattered cells whose number and distribution suggested that they correspond to hemocytes. This suggestion was confirmed by staining with antibodies against the hemocyte-specific protein Croquemort (Crq) (Franc et al., 1996) (Fig. II-7A). The PNA reactivity on hemocytes was abolished in *C1GalTA* mutants (Fig. II-7B).

Laminin is a component of the extracellular matrix and a ligand for integrins. *Drosophila* Laminin was first identified and purified as a PNA-binding protein (Montell and Goodman, 1988). *Drosophila* Laminin is a trimer of B1 (β), B2 (γ), and A (α) chains, and binding of Laminin trimers to PNA could be visualized by Western blotting of PNA-bound proteins using antibodies against Laminin B2 (Fig. II-7C). By contrast, lysates from *C1GalTA* mutant embryos lacked detectable association of Laminin with PNA (Fig. II-7C). These observations imply that *C1GalTA* is required for Laminin *O*-glycosylation. The detection of this requirement for *C1GalTA* in VNC condensation and Laminin *O*-glycosylation, together with prior studies implicating extracellular matrix deposition and

integrins in VNC condensation, suggest that Laminin could be a critical C1GalTA substrate in *Drosophila*.

As a number of distinct *O*-glycosylated proteins are detectable on PNA blots (Fig. II-5J,K), further studies will be required to clarify the significance and role of Laminin *O*-glycosylation. Nonetheless, our observations have identified the product of the *C1GalTA* gene as a major contributor to T antigen synthesis in the *Drosophila* embryo, and have clearly established a requirement for elongation of *O*-GalNAc glycans in the morphogenesis of the *Drosophila* nervous system.

EXPERIMENTAL PROCEDURES

Drosophila Genetics

Unless otherwise noted, stocks were obtained from the Bloomington *Drosophila* stock center. For nervous system labeling in larvae we used *elav-Gal4[C155]* and *UAS-mCD8:GFP*. Imprecise excisions were generated from a P-element insertion within *C1GalTA*, *P[KG02976]* (Bellen et al., 2004) by crossing to a transposase-expressing line (*Sp/CyO; ry*^{506} *Sb*^{1} *P{Δ2-3}99B /TM6B, Ubx*). 76 potential excision events were identified by the loss of the w^+ marker. From these w^- excisions, 20 homozygous lethal mutant chromosomes were isolated. Genetic complementation tests with chromosomal deficiencies (*Df(2L)N22-14*, *Df(2L)ED647*, and *Df(2L)Exel7040*) that delete the cytological location of *C1GalTA* (29F5), revealed that only two of the lethal mutations, *C1GalTA*$^{2.1}$ and *C1GalTA*$^{30.1}$, mapped to this region. PNA staining and rescue experiments displayed in the figures were performed using *Df(2L)ED647* and *Df(2L)Exel7040*. Breakpoints for the *C1GalTA*$^{2.1}$ and *C1GalTA*$^{30.1}$ alleles were obtained

by PCR amplification of genomic DNA from homozygous embryos using as primers DELCG9520US-1 5'AGAGAAACAGGCCATTGATAAAT and CG9520DS-1 5'TCAGCCGGCCCAAGGTC. PCR fragments were TA cloned into pGEMTeasy (Promega, Madison, WI) and then sequenced. In *C1GalTA*$^{2.1}$, a 6127 bp deletion was detected, which includes the sequences encoding the transmembrane domain and conserved sequence motifs required for core 1 GalT activity. This mutation also retains 1584 bp from the P element insertion. In *C1GalTA*$^{30.1}$, 4716 bp of genomic DNA are deleted, which includes most of the first intron of *C1GalTA*. In *CG8708*^{KG05736} (Bellen et al., 2004), the P element insertion is within the protein-coding second exon of *CG8708*, downstream of conserved motifs i and ii, but upstream of conserved motifs iii and iv (Correia et al., 2003).

Molecular Biology

In situ hybridization to mRNA was carried out as described previously (Irvine and Wieschaus, 1994). The templates for *C1GalTA, CG8708*, *CG7440* and *CG18558* were cDNAs SD07079 ,GH18356, CK00318, and AT24870 (Rubin *et al*., 2000), respectively. For *CG8708* and *C1GalTA*, we also created probes corresponding to portions of the gene downstream of the *P* element insertion sites by PCR using primer pairs including T3 promoter sequence (AATTAACCCTCACTAAAGGG) on one primer and T7 promoter sequence (TAATACGACTCACTATAGGG) on the other (David and Wedlich, 2001). Primer sequences used were: for *CG8708*, T7-CTATCCGTACAATCCCGAAACACC, T3-AGCCTGCCAAGTAAACTCAATAAA, and for *C1GalTA*, T7-

CCCCCGCAACGAAGAAAC, T3-TGTCCGAGCAGCAGTCAAGTC. The templates for *CG2975*, *CG13904*, *CG34056*, *CG34057*, *CG3119*, and *CG2983* were generated by PCR using primer pairs including T3 and T7 promoter sequences. Primer sequences used were: for CG2975, T7- GCTGATGCTAATGTTGCTGAT, T3-GTTCTGTAATTTGGTGTAGTGACG; for CG13904, T7-GTGCTGGGCCTCATCATTGG,
T3-CTGCAGGCGCTCGTTCATCT; for CG34056, T7-CCCCCGGCTCGTTTTGTAAG, T3-AGTCGGGGTGGCAGCGTTCTC; for CG34057, T7-TGCGCCAATTGGACACAGC, T3-CCCAAGCGCGACGGAAGAG; for CG3119, T7-ATTATCAGGTTTCTCGGACATTTG, T3-GGCGGAGCTTCGTATATTCTGAGT; for CG2983, T7-GAATACAACGTCGCCATCAAC, T3-ATTTTAGCGCATCTTTTTCAGT.

To confirm the enzymatic activity of *C1GalTA*, we cloned its C-terminal catalytic domain (amino acids 44-388) into the vector pMT(1B) (Haines and Irvine, 2005; Xu et al., 2007), resulting in the plasmid *pMT(1B)- C1GalTA:V5:His*. pMT(1B) contains the *Drosophila* metallothionein promoter (pMT) for inducible expression, the BiP signal sequence for secretion, a V5 epitope tag for detection, and a His-tag for purification. DNA encoding *C1GalTA* was amplified by PCR from cDNA clone SD07079 using primers YLCG9520-n' 5'-GGGGTACCGGAGCGAAGTGAATTCATG and YLCG9520-c'-tag 5'-GCTCTAGATTGCGTCTTTGTCTCGGCG. Restriction enzyme sites *Kpn*I and *Xba*I were included in the PCR primers to facilitate cloning. *pMT(1B)-C1GalTA:V5:His* was transfected into S2 cells, and concentrated media was used for

glycosyltransferase assays, as described previously (Ju et al., 2002b; Haines and Irvine, 2005). A similar strategy was used for *CG8708, CG7440,* and *CG18558*.

For genomic rescue, a DNA fragment containing the entire *C1GalTA* region, but none of the neighboring transcription units, was amplified from wild type (Oregon-R) genomic DNA by PCR using primers forward 5'-ATAAGAATGCGGCCGCGTTTTAATGCTTTTGTTGACTTTGTATAAGTG and reverse 5'-CCGCTCGAGCCGCTGGCTAAGACTATGCGATAATTC, and cloned into pCaSpeR4 using *NotI* and *Xho*I restriction sites included on the primers, to generate pCaSpeR4- $C1GalTA^{+}$. This construct was transformed into *Drosophila* using standard techniques, and insertions on the first $P[C1GalTA^{+}\text{-}X]$ and third chromosomes $P[C1GalTA^{+}\text{-}3]$were obtained.

Alignment of core 1 GalT sequences by BLASP has been reported previously (Correia et al., 2003). Alignment by ClustalW (Larkin et al., 2007) was performed using the EMBL-EBI server (http://www.ebi.ac.uk/Tools/clustalw2/), and a phylogenetic tree with proportional distances was constructed using the Trex server (http://www.trex.uqam.ca/index.php?action=newick&project=trex) (Makarenkov, 2001).

Immunohistochemistry and blotting

Antibody staining was performed essentially as described previously (Panin et al., 1997), using the primary antibodies: rat-Elav (1:67, 7E8A10, Developmental Studies Hybridoma Bank (DSHB)), mouse anti-Neuroglian (1:125, BP 104, DSHB), mouse anti-CNS axons (1:200, BP 102, DSHB), rat anti-Repo (1:1000, 8D12, DSHB), mouse anti-Fasciclin II (1:200, 1D4, DSHB), rabbit anti-crq antibody (1:2000, N. Franc), mouse anti-Tn (1:10,

Biomeda), and rabbit anti-GFP (1:400, Molecular Probes). Staining with biotinylated PNA lectin (Vector) was carried out as described previously (Tian and Hagen, 2007). Biotinylated PNA was used at 0.25ng/ml, and Streptavidin-Cy3 (Jackson ImmunoResearch) was used at 0.5ng/ml.

For lectin blotting 50 micrograms of lysates from both wild type and mutant embryos were run on SDS-PAGE gels and then transferred to membranes. Blots were probed with biotinylated PNA lectin (1:6000, Vector Laboratories) at 5mg/mL concentration followed by avidin and biotinylated HRP complexes using the ABC detection kit (Vector). Blots were then developed using a chemiluminiscence reagent (Perkin Elmer).

O-Glycanase treatment on embryo lysates was performed using GLYKO deglycosylation kit (Prozyme) according to the manufacturer's instructions. Briefly, 50 μg of embryo lysate was incubated with *O*-Glycanase in buffer at 37^0C for 3 hours. In control samples no enzyme was added. After the treatment samples were run on SDS-PAGE gels, Western blotted and analyzed by PNA lectin staining.

For co-immunoprecipitation experiments, embryo lysates in binding buffer (10 mM HEPES, pH 7.4, 150 mM NaCl) were passed through a PNA-agarose (Vector) column. After washing in high salt buffer (10 mM HEPES, pH 7.4, 500 mM NaCl) to remove unbound proteins, bound proteins were eluted with 0.2M galactose in binding buffer. Eluted proteins were subject to Western blotting using rabbit anti-Laminin B2 antisera (Kumagai et al., 1997)(1:3000).

ACKNOWLEDGEMENTS

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Figure II-1. Phylogenetic relationship of Core 1 GalTs

A proportional, radial, phylogenetic tree was constructed to depict the relative sequence divergence among members of the Core 1 GalT family in *Drosophila* and human. The tree depicts graphically the results of ClustalW analysis.

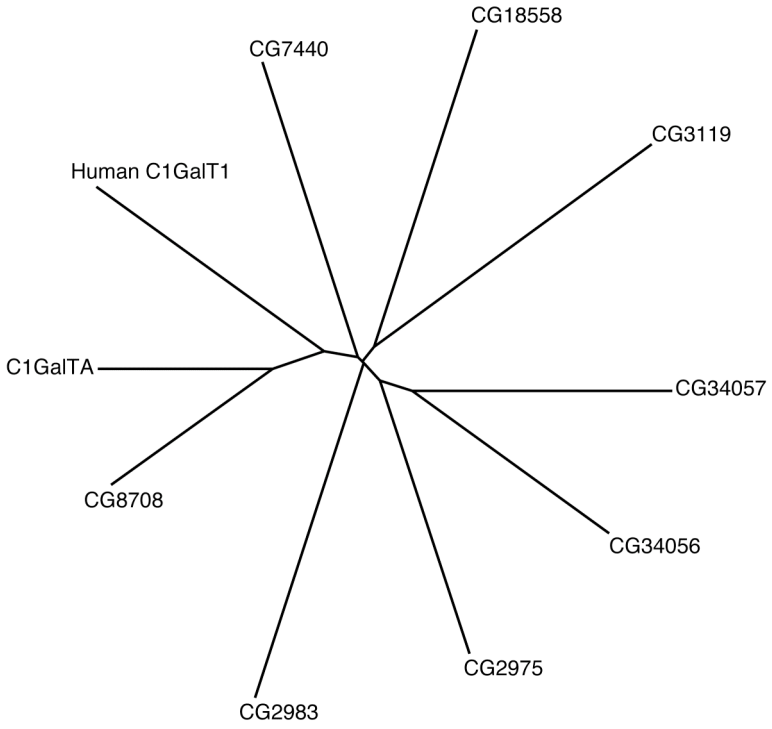

Figure II-1 Phylogenetic relationship of Core 1 GalTs

Figure II-2. Expression of *C1GalTA*

In situ hybridization to *Drosophila* tissues to detect expression of *C1GalTA* mRNA. A) Stage 6 embryo. Uniform expression above background is observed in early embryos, which likely includes maternally deposited mRNA. B) Stage 12 embryo. Strong expression is detected in the amnioserosa (as), and weaker expression elsewhere. C) Stage 14 embryo. Strong expression is detected in the amnioserosa. D) Stage 16 embryo. Expression is detected in the VNC and brain (br). E) CNS from first instar larva, expression is detected through out. F) Stage 10 follicle, with strong expression detected in the nurse cells (nc), but not in the oocyte (oo). G) Third instar wing imaginal disc, faint expression is detected. H) Third instar wing imaginal disc hybridized with a sense strand probe as a control, no staining is detected. I) Third instar CNS, *C1GaTA* is expressed in the brain and VNC.

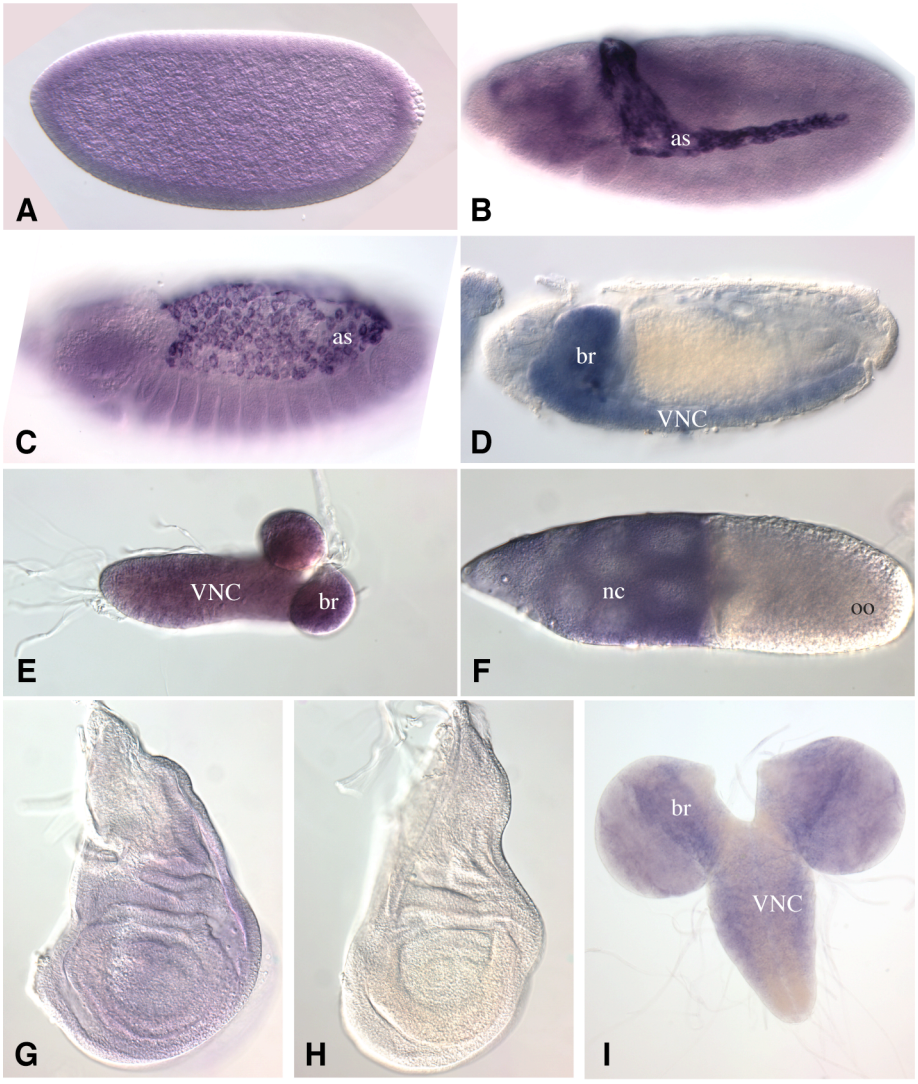

Figure II-2 Expression of C1GalTA

Figure II-3 Expression of Core 1 GalT family members

In situ hybridization to *Drosophila* tissues to detect expression of various members of the Core 1 GalT family. A-C) Male reproductive system, including ejaculatory duct (ed), testis (t), and accessory glands (ag). A) *CG2983* is expressed preferentially in the accessory glands. B) *CG18558* is expressed preferentially in the distal region of the testis C) *C1GalTA* is expressed preferentially in the ejaculatory duct. D) portion of the gut from a third instar larva, *CG7440* is expressed preferentially in the gastric cecae (gc) and parts of the proventriculus (pv). E) Stage 14 embryo, *CG7440* is expressed preferentially in the proventriculus. F) Stage 13 embryo, *CG13904* is expressed preferentially in the developing trachea.

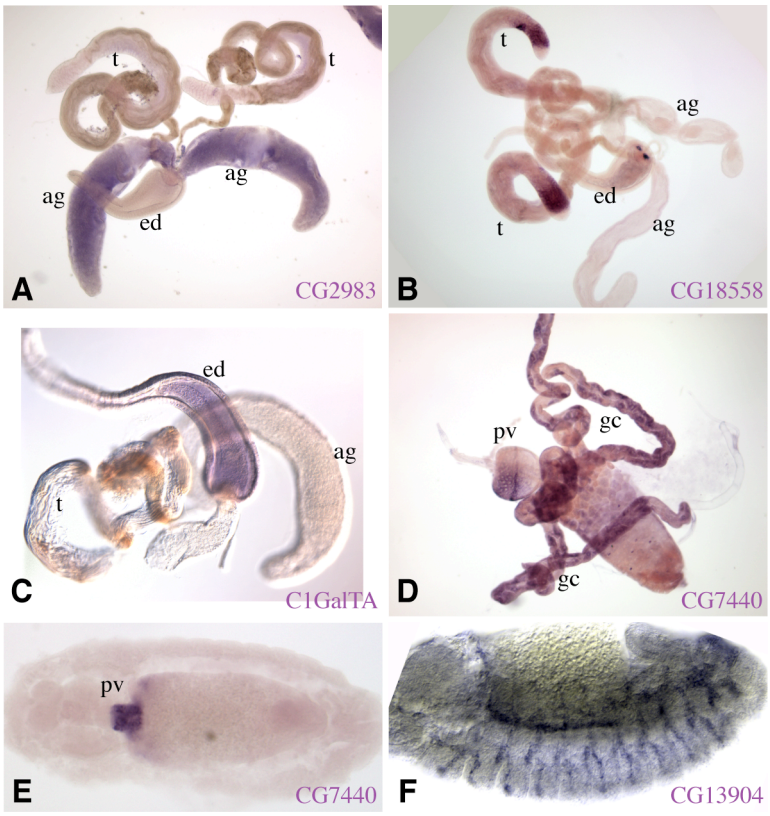

Figure II-3 Expression of Core 1 GalT family members

Figure II-4 A *CG8708* mutant does not affect salivary gland morphogenesis

A) Schematic of *CG8708* transcription unit shows introns as thin lines and exons as thick lines, with coding regions in black and non-coding regions in gray. The position of the *KG5736* insertion is indicated by the triangle. B) Stage 16 wild-type embryo, with *CG8708* expressed specifically in salivary glands (sg). C) Stage 12 wild-type embryo, with *CG8708* expressed specifically in the developing salivary glands (sg). D) Stage 12 $CG8708^{KG5736}$ homozygous embryo; *CG8708* expression is not detectable. E) Stage 12 wild-type embryo, anti-CREB staining marks the lumen of the developing salivary glands (sg). D) Stage 12 $CG8708^{KG5736}$ homozygous embryo; anti-CREB staining is not distinguishable from wild type.

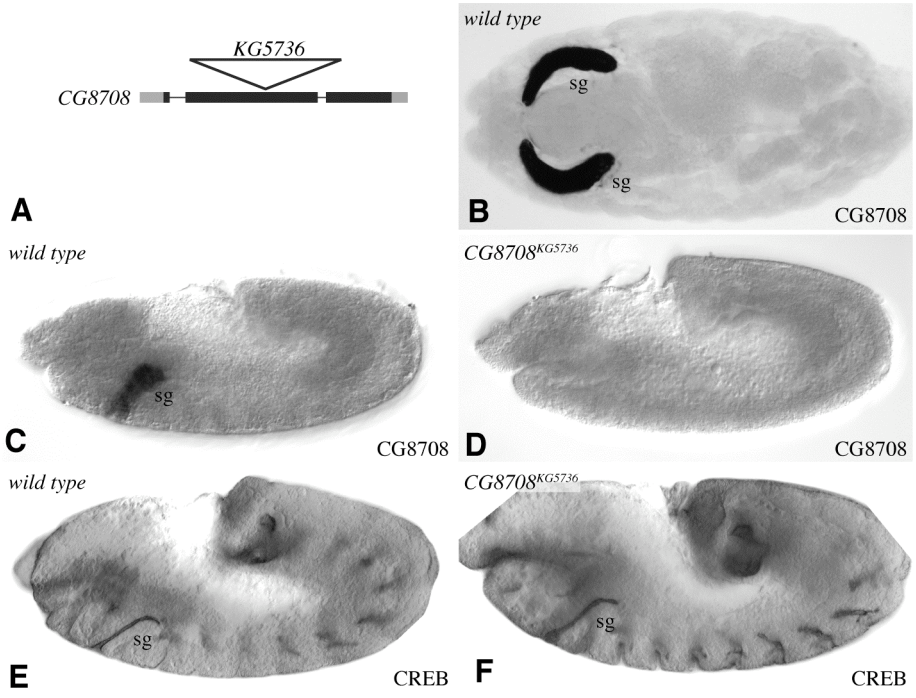

Figure II-4 A CG8708 mutant does not affect salivary gland morphogenesis.

Figure II-5 Influence of *C1GalTA* mutants on PNA and anti-Tn staining

A) Top line shows a schematic of the *C1GalTA* transcription unit, with introns as thin lines, exons as thick lines, coding regions in black and non-coding regions in gray. The position of the *KG2976* insertion is indicated by the triangle. Lower lines show DNA retained in mutant alleles, and DNA included in the rescue construct. B) Stage 13 *wild-type* embryo showing PNA staining (red) in the amnioserosa (as). C) Stage 16 *wild-type* embryo showing PNA staining in the CNS; the CNS is marked by anti-Elav staining (blue). D) Stage 13 $C1GalTA^{2.1}$ mutant embryo showing reduced PNA staining in the amnioserosa. E) Stage 16 $C1GalTA^{2.1}$ mutant embryo lacks PNA staining in the CNS, but PNA staining is now detected in the salivary gland (sg). F) Stage 13 $C1GalTA^{2.1}$ mutant embryo with the P[C1GalTA^{+}] rescue construct, PNA staining is indistinguishable from wild type. G) Stage 16 $C1GalTA^{2.1}$ mutant embryo with the P[C1GalTA^{+}] rescue construct, PNA staining is indistinguishable from wild type. H) Stage 13 wild-type embryo showing very low anti-Tn staining (yellow) in the amnioserosa. C) Stage 13 mutant embryo showing anti-Tn staining in the amnioserosa. J) PNA lectin blot on lysate from wild-type and $C1GalTA^{2.1}$ mutant embryos. Two prominent bands detected in both wild-type and mutant lanes are actually background bound by the detection reagent (Avidin-hrp) as revealed by the control blot in which PNA was omitted. K) PNA lectin blot on lysate from wild-type embryos, where indicated (+) lysate was treated with *O*-glycanase.

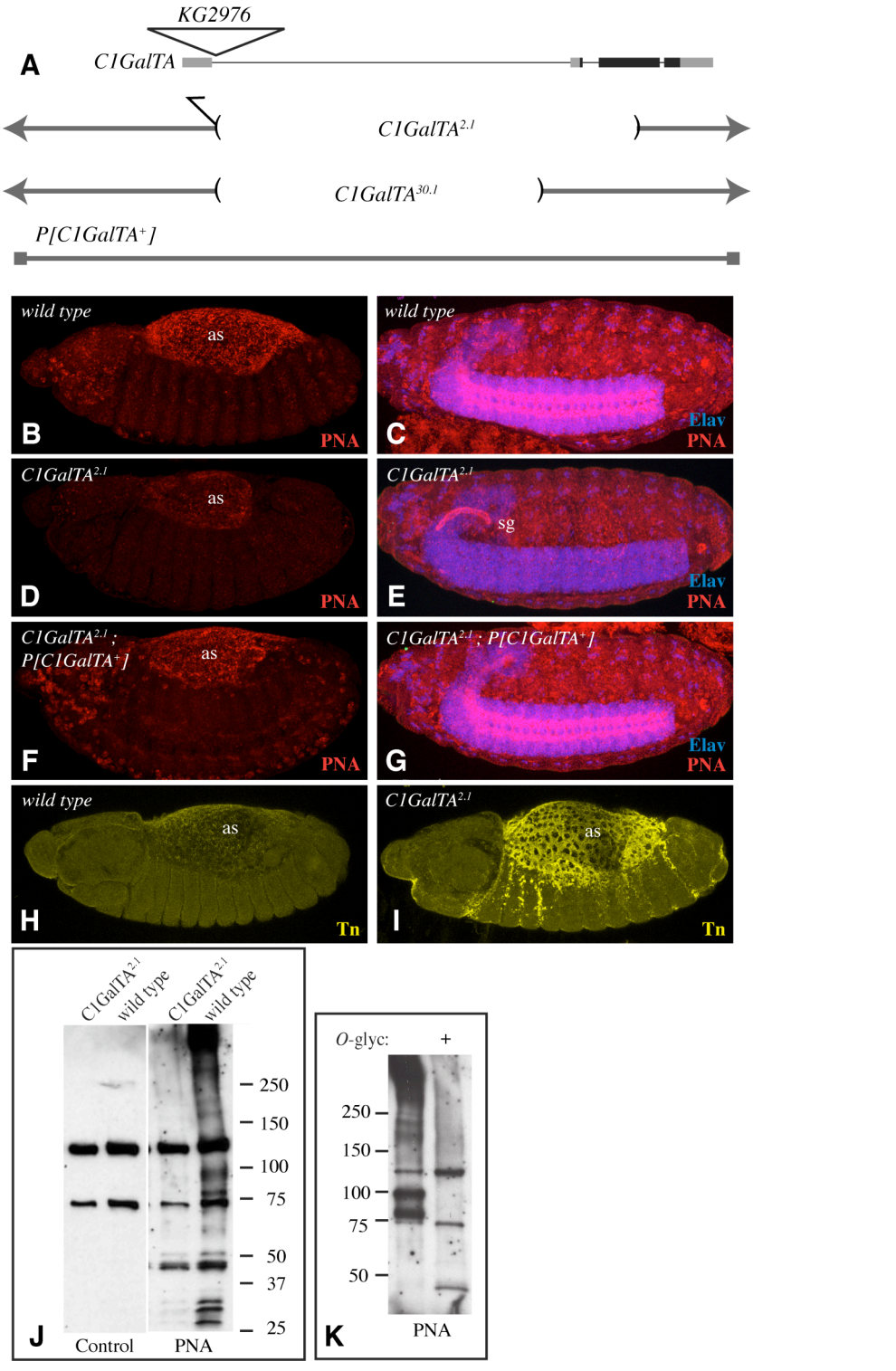

Figure II-5 Influence of C1GalTA mutants on PNA and anti-Tn staining

Figure II-6 VNC Phenotype of *C1GalTA* mutants

A-H) Composite confocal images of larvae with *Elav-Gal4* and *UAS-GFP* transgenes. GFP is expressed in the nervous system and salivary glands, arrow points to the posterior end of the VNC. A-D, G, H are saggital views, E,F is a ventral view. A) *wild-type* first instar. B) $C1GalTA^{2.1}$ mutant first instar. C) *wild-type* second instar. D) $C1GalTA^{2.1}$ mutant second instar. E) *wild-type* second instar. F) $C1GalTA^{2.1}$ mutant second instar. G) *wild-type* third instar. H) $C1GalTA^{2.1}$ mutant third instar. I) Dissected late third instar CNS from *wild type*, stained with anti-Elav. J) Dissected late third instar CNS from $C1GalTA^{2.1}$ mutant with $P[C1GalTA^{+}]$ rescue construct, stained with anti-Elav. K) Dissected late third instar CNS from $C1GalTA^{2.1}$ mutant, stained with anti-Elav. L,M) Stage 16 wild-type (L) and $C1GalTA^{2.1}$ mutant (M) embryos stained with anti-Repo to detect glial cells. N,O) Stage 16 wild-type (N) and $C1GalTA^{2.1}$ mutant (O) embryos stained with anti-FasII to detect a subset of axon fasicles.

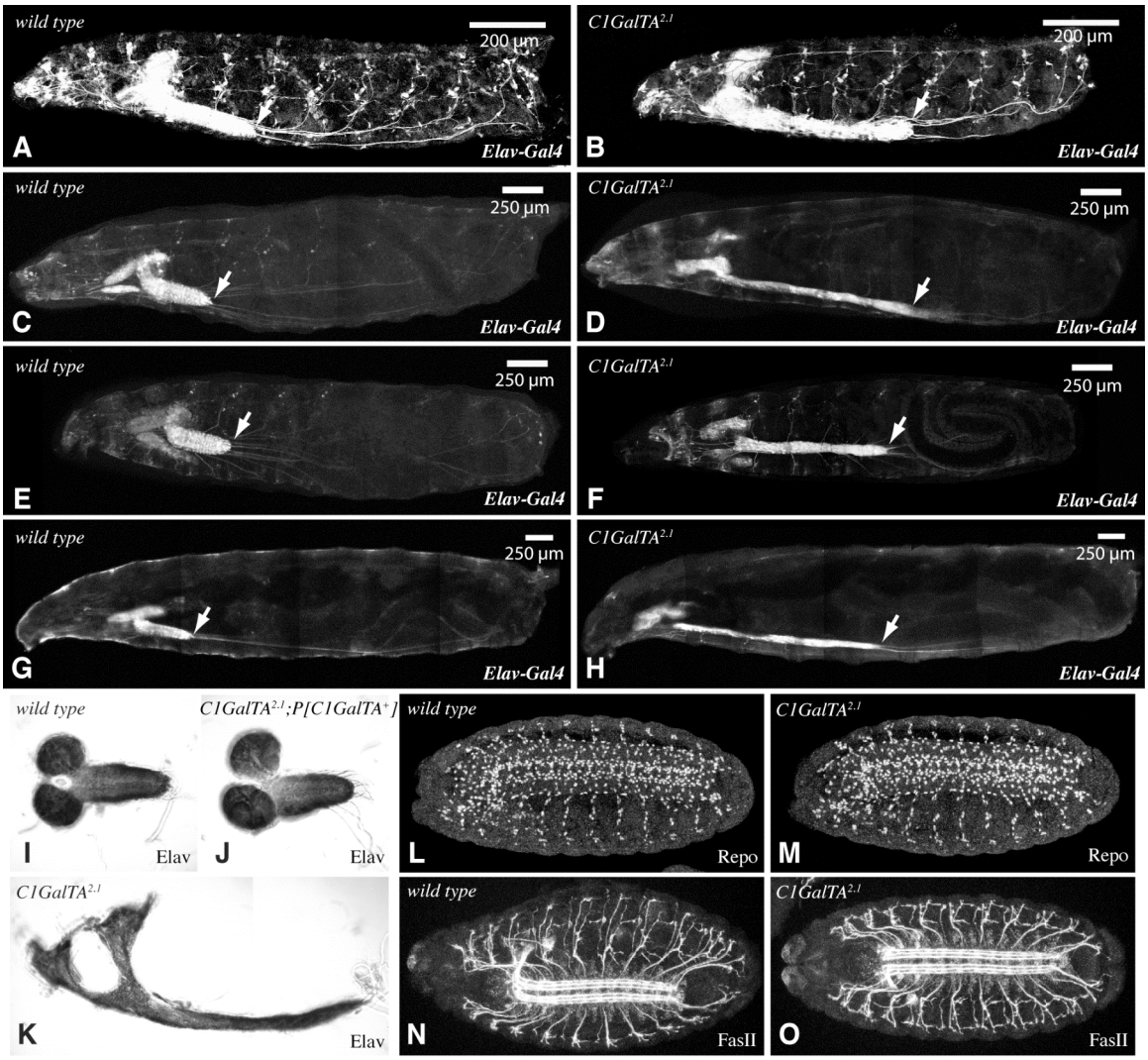

Figure II-6 VNC Phenotype of C1GalTA mutants

Figure II-7 Influence of *C1GalTA* mutants on PNA staining of hemocytes and Laminin

A,B show stage 15 embryos stained with PNA (red), anti-Elav (blue), and anti-Crq (green). Panels marked prime show single channels of the embryo above. A) Wild type. PNA staining is detected on hemocytes; inset shows a close-up of the boxed region. B) $C1GalTA^{2.1}$ mutant; normal PNA staining is lost, although PNA staining is now detected on the salivary gland (sg). C) Western blot using anti-Laminin B2 on lysates from wild-type or $C1GalTA^{2.1}$ mutant embryos and on material precipitated using PNA-beads. The anti-B2 antibodies cross-react with both Laminin B1 (β, upper band) and Laminin B2 (γ, lower band) (Kumagai et al., 1997).

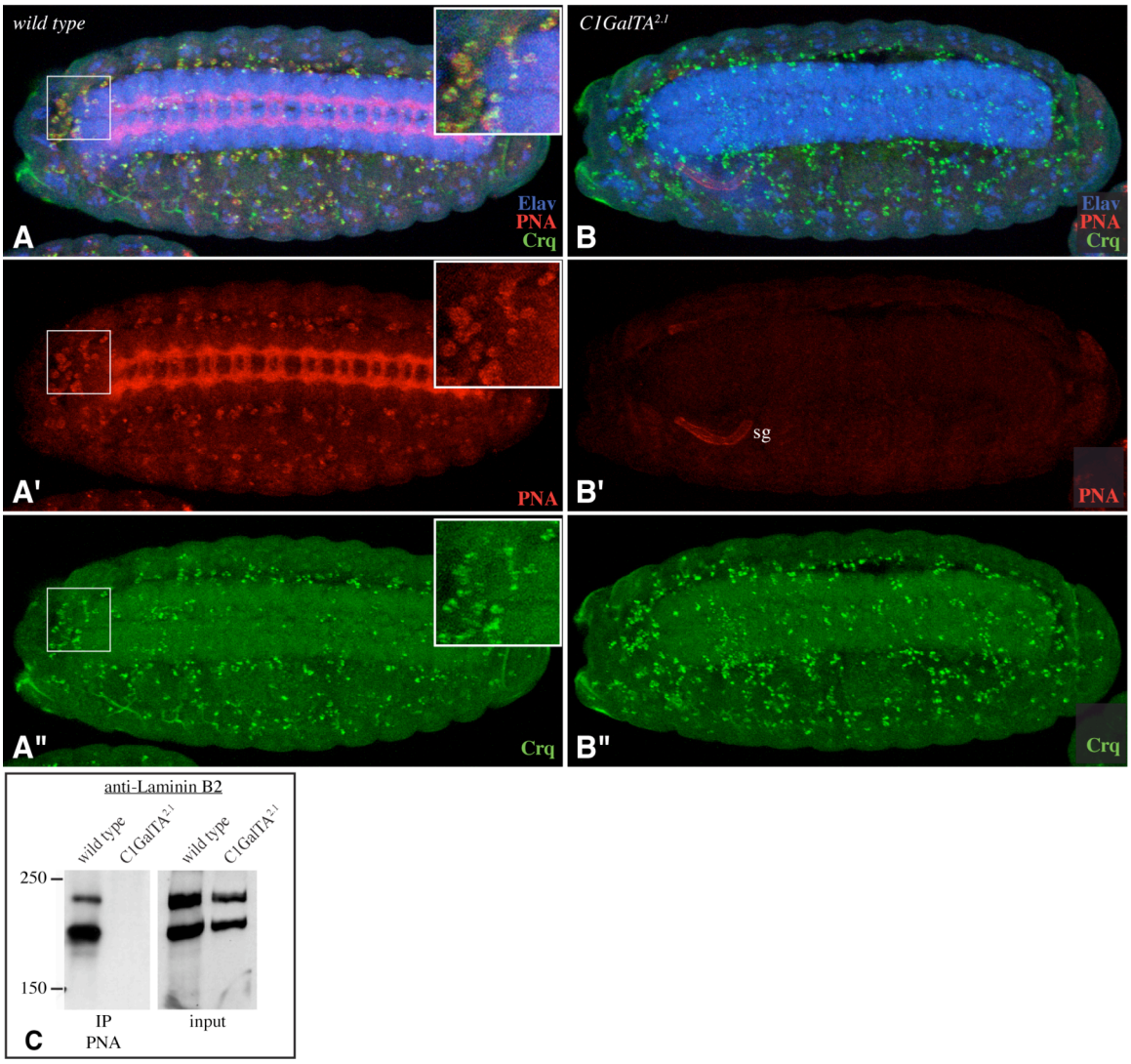

Figure II-7 Influence of C1GalTA mutants on PNA staining of hemocytes and Laminin

Table II-1 Expression of the Core1 GalT family in *Drosophila*

Gene	Cytological Location	Embryo	Larva	Adult Female	Adult Male
C1GalTA	29F5	Amnion serosa, CNS	Gut, nervous system, imaginal discs	Nurse cells	Ejaculatory duct
CG8708	44B5-7	Salivary gland	Salivary gland	Not detected	Ejaculatory duct, accessory gland
CG7440	18A3	Proventriculus, midgut	Gastric caeca, Proventriculus, nervous system, imaginal discs	Nurse cells	Testis
CG13904/ CG34056	61D4	Trachea	Imaginal discs and brain	Follicle cells	Accessory gland
CG34057	61D4	Not detected			
CG2975	23B4	Not detected	Proventriculus	Nurse cells	
CG18558	23B4	Not detected		Nurse cells	Testis
CG3119	23B3	Not detected	Brain		
CG2983	23B5	Not detected	Imaginal discs	Not detected	Accessory gland

Tissues in which prominent expression of the listed gene was detected are noted, blank cells indicate that the expression was not examined.

Table II-2 Length of the VNC in wild-type and mutant larvae

Genotype	**Instar**	**Number measured**	**Larval length** (μm)	**VNC length** (μm)	**Ratio** (%)
Wild type	1st	20	1403 +/- 114	351 +/- 36	25
C1GalTA$^{2.1}$	1st	15	1231 +/- 125	530 +/- 69	43
Wild type	2nd	24	2752 +/- 343	350 +/- 46	13
C1GalTA$^{2.1}$	2nd	29	2582 +/- 511	923 +/- 201	36
Wild type	early 3rd	11	4103 +/- 464	437 + / - 50	11
C1GalTA$^{2.1}$	early 3rd	15	4202 +/- 519	1554 +/- 253	37

Average lengths as measured from confocal micrographs of larvae expressing GFP under *elav-Gal4* control (Fig. 5), the variation (+/-) indicates one standard deviation. Ratio indicates VNC length / Larval length.

Chapter III Other Studies on *C1GalTA*

INTRODUCTION

O-GalNAc glycans have been implicated in diverse processes, from lymphocyte homing, to sperm-egg binding, to providing a protective coating along epithelia (Van den Steen, Rudd et al. 1998; Hanisch 2001; Dube and Bertozzi 2005). Characterizing *C1GalTA* mutants, we learned mutant animals exhibit a striking morphogenetic defect in which the ventral nerve cord (VNC) is greatly elongated and the brain hemispheres are misshapen. This result indicates *C1GalTA* is required during the VNC condensation at the end of embryogenesis.

In this chapter, I will present the other studies that I have done on core 1 GalTs. This would help to understand the biological functions of normal mucin-type *O*-glycans in *Drosophila*.

Overexpression of *C1GalTA* and *CG8708* induced a wing blistering phenotype. Mutations in a ppGalNAcT, *pgant3*, also cause wing blistering (Zhang et al., 2008). Both results implicate mucin-type *O*-glycans in epithelial cell adhesion in the *Drosophila* wing blade.

The PNA reactivity was associated with hemocytes and the VNC. Examining stage 12 embryos showed the PNA reactivity on hemocytes appears before that in the VNC. Prior study suggests hemocyte-deposited extracellular matrix is important for VNC condensation (Olofsson and Page, 2005). These results together suggest expression of T-antigen on hemocytes seems essential for CNS development.

RESULTS AND DISCUSSION

C1GalTA over-expression results in wing-blistering phenotype

Among the core 1 β1, 3-galactosyltransferases in Drosophila, each of them is expressed in distinct tissues.

The restricted expression raises the question whether it is essential to express one galactosyltransferase in one tissue not the others. To answer this question, I take advantage of the Gal4/UAS system. To make *UAS-C1GalTA* and *UAS-CG8708*, their cDNAs were placed downstream of a UAS regulatory element. I ectopically expressed them using broadly expressed Gal4 drivers, e.g. *da-Gal4*, *ap-Gal4*, and examined them for abnormal morphology. Both *da-Gal4* and *ap-Gal4* gave a wing blister phenotype with *UAS-C1GalTA* or *UAS-CG8708*. (Figure III-1) This result suggests the ectopic expression of *C1GalTA* or *CG8708* would modify a substrate in the wing where it normally would not be modified. Abnormal mucin-type *O*-glycosylation might alter the adhesion between the wing blades and influence wing growth and development.

Overexpression of *C1GalTA* and *CG8708* induced a wing blistering phenotype. Mutations in *pgant3,* a ppGalNAcT responsible for the initial step of mucin-type *O*-glycosylation, also causes wing blistering (Zhang et al., 2008). Both results implicate proper formation of mucin-type *O*-glycans is crucial for the wing blade development in *Drosophila*.

Wing blistering is also commonly seen in integrin mutants (Bunch et al., 1998; Prokop et al., 1998; Walsh and Brown, 1998; Brown et al., 2002). Since the wing blade development involves integrins, it is presumably mediated by cell-basement membrane

adhesion. Altogether, these results suggest a role of mucin-type *O*-glycosylation in cell adhesion during the formation of the *Drosophila* wing blade.

Expression of mucin-type *O*-glycans coincides with hemocyte migration at the embryonic ventral midline

Examining the mutant animals, I found that *C1GalTA* is required for VNC condensation and T-antigen expression on hemocytes and CNS. A prior study showed blocking hemocyte migration would disrupt deposition of extracellular matrix components around the VNC and prevent VNC condensation (Olofsson and Page, 2005).

Hemocytes of the *Drosophila* embryo are derived from the head mesoderm (Tepass et al., 1994). From this origin, they migrate extensively along specific pathways to disperse throughout the embryo (Cho et al., 2002). A majority of hemocytes will migrate, from anterior to posterior, along the ventral midline, where hemocytes come into close contact with the cells of the CNS midline and the neighboring ventral epidermis. Following their alignment along the ventral midline, these hemocytes undergo a rapid lateral migration (Wood et al., 2006).

Hemocyte migration can be followed by PNA staining. Examining stage 13 *wild-type* embryos (Figure III-2A), PNA reactivity showed hemocytes align along the ventral midline next to the CNS. In stage 14 embryos (Figure III-2B), PNA staining starts to appear along the ventral midline in the CNS. In stage 15 embryos (Figure III-2C), PNA staining is in the CNS and on hemocytes, which starts to migrate laterally from the ventral midline. The PNA reactivity in the CNS starts to appear right after the hemocyte alignment along the ventral midline. The fact that PNA staining in CNS appears after

hemocyte alignment along ventral midline, together with prior studies implicating extracellular matrix deposition in VNC condensation, suggests the PNA recognizable mucin-type *O*-glycans might be expressed on extracellular matrix components and deposited on the CNS by hemocytes. Or, the PNA recognizable mucin-type *O*-glycans might be induced in the CNS when hemocytes align along the ventral midline.

C1GalTA expression in CNS is not sufficient for VNC condensation

C1GalTA mutations are lethal and cause defective VNC condensation. *C1GalTA* is required for nervous system morphogenesis, which is consistent with its expression within the central nervous system (CNS) during late embryonic development.

To investigate where C1GalTA expression is required, rescue experiments were performed using the Gal4/UAS system. I placed the *C1GalTA* cDNA downstream of a UAS regulatory element. Using the broadly expressed *da-Gal4*, *UAS-C1GalTA* was able to rescue *C1GalTA* mutant animals ($C1GalTA^{2.1}/C1GalTA^{30.1}$)(Table III-1). Almost 90% of the *C1GalTA* mutant animals could be rescued by over-expressing *C1GalTA* with *da-Gal4*. And, the rescued animals are viable, fertile and have normal VNC. However, when using a neuron-specific Gal4 driver, *elav-Gal4*, the extended VNC was not significantly rescued. The results from these rescue experiments suggest the control of *C1GalTA* expression is very critical.

To analyze if another core 1 β1, 3-galactosyltransferase could function interchangeably with *C1GalTA*, I tested the salivary gland-specific core 1 β1, 3-galactosyltransferase, *CG8708*. Using broadly expressed *da-Gal4*, *UAS-CG8708* was able to rescue *C1GalTA* mutant, $C1GalTA^{2.1}/C1GalTA^{30.1}$ (Table III-1). About 67% of the

C1GalTA mutant animals could be rescued by over-expressing *CG8708* with *da-Gal4*. And, the rescued animals are viable, fertile and have normal VNC. Over-expressing *CG8708* with *elav-Gal4* did not rescue. This result implies that *CG8708* can catalyze glycosylation with the same substrates as *C1GalTA*, although with less activity.

In the beginning, it was surprising to learn that a *C1GalTA* mutant could be rescued by overexpressing *C1GalTA* or *CG8708* with *da-Gal4*, and not by *elav-Gal4*. The PNA-recognizable mucin-type *O*-glycans in the CNS start to appear after the hemocyte alignment along the ventral midline. The PNA-recognizable mucin-type *O*-glycans in the CNS might occur because of the extracellular matrix deposition by hemocytes. And, the expression of *C1GalTA* in neural cells is not sufficient to rescue its normal biological functions.

There are also other cell types in the VNC, e.g. glial cells, which do not express elav. Thus, the expression of *C1GalTA* in glial cells could be responsible for synthesizing the PNA-recognizable mucin-type O-glycans in CNS.

EXPERIMENTAL PROCEDURES

Molecular Biology

To over-express CG9520 and CG8708, plasmid pUAST-CG9520 and pUAST-CG8708 were made by cloning their cDNA into pUAST, respectively. To make pUAST-CG9520, CG9520 cDNA was cloned from SD07079. The ~1.9 kb fragment from SD07079, treated with EcoRV and XhoI, was isolated and cloned into the backbone of pUAST, treated with BglII, klenow fragment, and XhoI. To make pUAST-CG8708, the cDNA of

CG8708 was cloned from GH18356 into pUAST. The ~1.6kb fragment from GH18356, treated with BglII and XhoI, was isolated and cloned into pUAST, treated with BglII and XhoI.

Immunohistochemistry

Antibody staining was performed essentially as described previously (Panin et al., 1997), using the primary antibodies: rat-Elav (1:67, 7E8A10, Developmental Studies Hybridoma Bank (DSHB)). Staining with biotinylated PNA lectin (Vector) was carried out as described previously (Tian and Hagen, 2007). Biotinylated PNA was used at 0.25ng/ml, and Streptavidin-Cy3 (Jackson ImmunoResearch) was used at 0.5ng/ml.

Drosophila Genetics

Unless otherwise noted, stocks were obtained from the Bloomington *Drosophila* stock center. To rescue *C1GalTA* mutant, $C1GalTA^{2.1}/C1GalTA^{30.1}$, by overexpressing *C1GalTA* or *CG8708, da-Gal4* and *elav-Gal4* were employed. For *da-Gal4,* $C1GalTA^{2.1}$*/CyOtwiGFP; da-Gal4/Tm6b* was crossed with *UAS-C1GalTA/Fm7c KrGFP;* $C1GalTA^{30.1}$*/CyOtwiGFP* and *UAS-CG8708/Fm7c KrGFP;* $C1GalTA^{30.1}$*/CyOtwiGFP*. For *elav-Gal4, elavGal4/ Y;* $C1GalTA^{2.1}$*/CyOtwiGFP* was crossed with *UAS-C1GalTA/Fm7c KrGFP;* $C1GalTA^{30.1}$*/CyOtwiGFP* and *UAS-CG8708/Fm7c KrGFP;* $C1GalTA^{30.1}$*/CyOtwiGFP*.

Figure III-1 *C1GalTA* overexpression causes wing blistering phenotype

A) *wild-type* adult wing of w^- fly. B). Overexpression of *UAS-C1GalTA* driven by *da-GAL4* induces wing blistering phenotype.

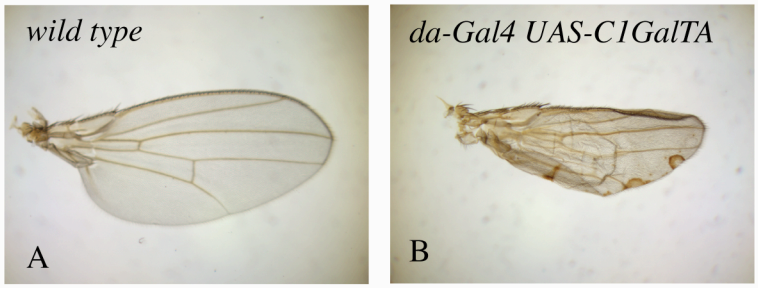

Figure III-1 C1GalTA overexpression causes wing blistering phenotype

Figure III-2 Expression of mucin-type *O*-glycans coincides with the hemocyte migration at the embryonic ventral midline

Embryos were stained with PNA (red). The central nervous system (CNS) is marked by anti-Elav (blue) staining. Panels marked prime show the PNA staining of each embryo. A) Stage 13 *wild-type* embryo. PNA staining is detected on hemocytes, which tend to align along the CNS at this stage. B). Stage 14 *wild-type* embryo. In addition to hemocytes, PNA staining starts to appear in the CNS. C) Stage 15 *wild-type* embryo. PNA staining is in the CNS and on hemocytes, which starts to migrate laterally from the ventral midline.

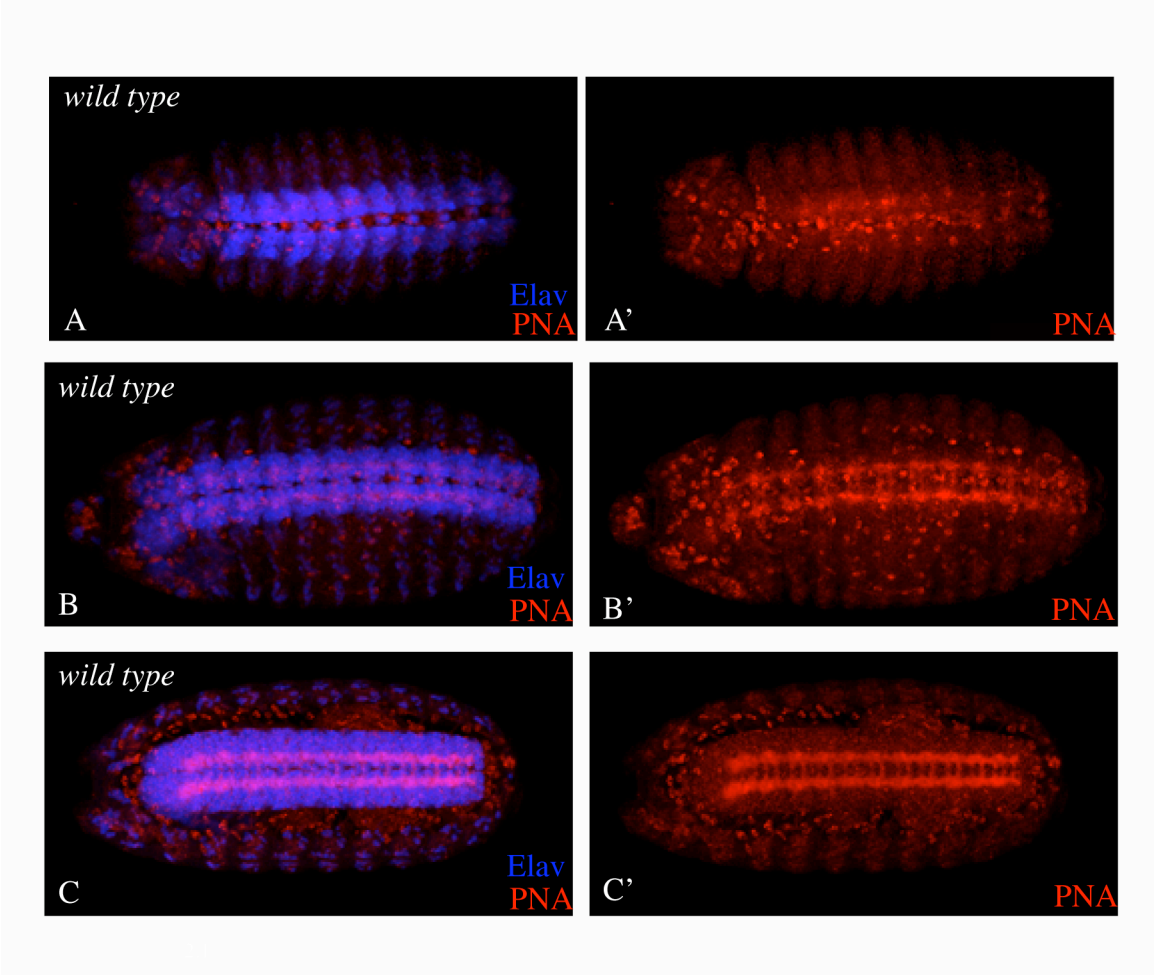

Figure III-2 Expression of mucin-type *O*-glycans coincides with the hemocyte migration at the embryonic ventral midline

Table III-1 Rescue experiment with Gal4/UAS system.

Genotype	Number analyzed	Number of normal VNC	Number of Elongated VNC	Ratio (%)
Wild type	100	100	0	-
C1GalTA$^{2.1}$/C1GalTA$^{30.1}$	100	0	100	-
UAS-C1GalTA/+; C1GalTA2$^{.1}$/C1GalTA$^{30.1}$; da-Gal4/+	113	101	12	89.4
UAS-CG8708/+; C1GalTA$^{2.1}$/C1GalTA$^{30.1}$; da-Gal4/+	48	32	16	66.7
UAS-C1GalTA/elav-Gal4; C1GalTA$^{2.1}$/C1GalTA$^{30.1}$	61	1	60	1.6
UAS-CG8708/elav-Gal4; C1GalTA$^{2.1}$/C1GalTA$^{30.1}$	53	0	53	0

To rescue *C1GalTA$^{2.1}$/C1GalTA$^{30.1}$* by overexpressing *C1GalTA* or *CG8708, da-Gal4* and *elav-Gal4* were employed. 3rd instar larvae of each genotype were dissected, and their ventral nerve cord (VNC) was examined. Ratio indicates the percentage of larvae, in which VNC was rescued. *C1GalTA* mutant can be rescued by overexpressing *C1GalTA* or *CG8708* with *da-Gal4*. However, *C1GalTA* mutant can hardly be rescued by *elav-Gal4*.

Chapter IV General Discussion

Mucin-type O-glycans modulate cell adhesion during *Drosophila* development

Cell adhesion is crucial during many diverse developmental stages. Mutations in many integrins results in a wing blistering phenotype, which implicates a loss of adhesion between the dorsal and ventral surfaces of the wing blade. Both overexpression of *C1GalTA* and mutations in a ppGalNAcT, *pgant3* (Zhang et al., 2008), cause a wing blistering phenotype, which implies that they have a role in cell adhesion during wing development.

C1GalTA and *CG8708* encode the core 1 β1, 3-galactosyltransferases, which elongate *O*-GalNAc by adding galactose in a β1,3 linkage and are responsible for core 1 mucin-type *O*-glycan synthesis in *Drosophila*. *PGANT3 is a ppGalNAcT* responsible for the first step of mucin-type *O*-glycosylation. These two kinds of glycosyltransferses functions together to properly form the mucin-type *O*-glycans, which involve cell adhesion during the *Drosophila* wing blade formation.

Epithelial cell adhesion in the *Drosophila* wing blade is regulated primarily by integrin-ECM interactions (Bunch et al., 1998; Prokop et al., 1998; Walsh and Brown, 1998; Brown et al., 2002). An integrin binding ECM protein, tiggrin, has been shown to involved in integrin-mediated cell adhesive events during *Drosophila* development, including muscle-tendon cell adhesion and wing blade adhesion (Bunch et al., 1998).

Interestingly, tiggrin can be glycosylted by PGANT3 (Zhang et al., 2008). Mutations in *pgant3* result in a loss of tiggrin glycosylation, and expression of wild type *pgant3* restores tiggrin glycosylation and wing integrity. Thus, *O*-glycans on tiggrin might play a role in integrin-ECM adhesion between dorsal and ventral surfaces of the wing blade.

Examination of wing discs from *pgant3* mutants showed a specific reduction in *O*-glycans along the basal surface of the columnar epithelial cells. Furthermore, basal surface glycosylation was restored in mutants expressing wild type *pgant3* (Zhang et al., 2008). These glycosylation changes are significant, since integrins and ECM proteins localized along the basal surface mediate the adhesive contacts when the disc everts and opposing basal surfaces come in contact with each other (Brower, 2003).

In contrast to *pgant3* mutations, overexpressing *C1GalTA and CG8708* may increase core 1 mucin-type *O*-glycosylation on the potential substrates (e.g. tiggrin-*O*-GalNAC) made by PGANT3. Aberrant mucin-type *O*-glycosylation on proteins present along the basal surface of wing discs might interfere with adhesion and cause wing blistering in the adult.

The studies on overexpressing *C1GalTA* and *pgant3* mutations suggest that *O*-glycosylation of ECM proteins (e.g. tiggrin) could play a role in the wing blade adhesion. This implicates the requirement of mucin-type *O*-glycans in integrin-dependent cell adhesion processes during development. The widespread presence of mucin type *O*-glycans suggests a potential for involvement of *O*-glycans in cell adhesion in many developmental processes.

Requirement of mucin-type *O*-glycosylation during neural development in *Drosophila*

A null mutation in *C1GalTA*, $C1GalTA^{2.1}$, is lethal. $C1GalTA^{2.1}$ hemizygotes can survive until pupal stages. The mutant animals display a morphogenetic defect in which the ventral nerve cord (VNC) is greatly elongated and the brain hemispheres are distorted;

this phenotype could be fully rescued by the genomic rescue construct (*P[C1GalTA^{+}]*). Thus, *C1GalTA* is required for normal development of the *Drosophila* nervous system.

Lectin staining and blotting experiments showed that T antigen is normally expressed on CNS and hemocytes during *Drosophila* embryogenesis, and that C1GalTA contributes to the synthesis of T antigen in *Drosophila melanogaster*. In the *C1GalTA* mutants, *C1GalTA$^{2.1}$* or *C1GalTA$^{30.1}$* hemizygotes, the T antigen expression on both CNS and hemocytes was abolished. Normal T antigen expression was restored in animals with the genomic rescue construct (*P[C1GalTA^{+}]*). These observations implicate elongation of mucin-type *O*-glycans by C1GalTA is essential for neural development in *Drosophila*.

Recently, Yoshida et al. (2008) identified a hypomorphic allele, *CG9520^{EY13370}*, which resulted from insertion of a P-element into the first intron of the *C1GalTA* gene. In third instar larvae homozygous for *CG9520^{EY13370}*, the level of the *C1GalTA* transcript was reduced to 15% of that of wild-type third instar larvae (Yoshida et al., 2008), and the galactosyltransferase activity was reduced to 44% of that of wild type. The expression level of T antigen in *CG9520^{EY13370}* embryos, compared to that of wild-type embryos, was lower on hemocytes and abolished on the CNS. These results also demonstrated that C1GalTA synthesizes T antigen on the CNS and hemocytes in *Drosophila* embryos.

Although lectin staining showed T antigen disappeared from the *CG9520^{EY13370}* CNS, there was no observable difference in VNC condensation compared to wild-type embryos(Yoshida et al., 2008). It is possible that the C1GalTA galactosyltransferase activity remained in *CG9520^{EY13370}* is sufficient to make mucin-type *O*-glycans required for neural development in Drosophila.

Previous studies showed that embryonic hemocytes have a role during CNS development in the *Drosophila* embryo. Hemocytes of the *Drosophila* embryo migrate extensively along specific pathways to disperse throughout the embryo (Tepass et al., 1994; Cho et al., 2002; Wood et al., 2006). Blocking hemocyte migration would disrupt deposition of extracellular matrix components around the VNC and prevent VNC condensation (Olofsson and Page, 2005).

Following hemocyte migration by PNA staining, we found that PNA staining in the CNS appears after hemocyte alignment along the ventral midline. Prior studies implicates extracellular matrix deposition in VNC condensation(Olofsson and Page, 2005). Taken together, these observations imply that hemocytes deposit extracellular matrix when they align along the ventral midline. Thus, PNA recognizable mucin-type *O*-glycans might be expressed on extracellular matrix components and deposited on the CNS by hemocytes.

Potential role of mucin-type *O*-glycans in VNC condensation

Genetic and biochemical studies on *C1GalTA* have identified a requirement for mucin-type O-glycans for VNC condensation during neural development in *Drosophila*. However, the mechanism of how mucin-type *O*-glycans contributes to VNC condensation remains unclear.

Mutations in two different *Drosophila* integrin subunits have been reported to reduce VNC condensation, which indicates a critical role of the extracellular matrix and cell-extracellular matrix interactions during VNC condensation (Brown, 1994). Furthermore, the secretion of extracellular matrix by hemocytes appears to be crucial for

VNC condensation, as disrupting hemocyte migration impairs both the deposition of extracellular matrix around the VNC and VNC condensation (Olofsson and Page, 2005).

ECM and basement membrane have important roles in cell migration and tissue morphogenesis (Brown et al., 2000; Geiger et al., 2001). Integrin receptors and their ligands mediate most interaction between cells and the ECM. The extracellular domain of integrins recognizes and binds to ECM proteins, whereas the intracellular domain associates with cytoskeleton elements. It has been shown previously that alterations in integrin-ECM interactions can affect cell size and shape in the wing disc (Dominguez-Gimenez et al., 2007). The reorganization of cell shape is commonly associated with epithelial morphogenesis and organ formation, which requires a precise and coordinated remodeling of the cytoskeleton and the adhesive properties of cells.

Laminin is a component of the extracellular matrix and a ligand for integrins. Our studies on C1GalTA demonstrate the requirement for *C1GalTA* in VNC condensation and Laminin *O*-glycosylation. Prior studies implicates extracellular matrix deposition and integrins in VNC condensation(Brown, 1994; Olofsson and Page, 2005). Altogether, these observations suggest that Laminin could be a critical C1GalTA substrate in *Drosophila*.

We hypothesize altered mucin-type *O*-glycans on laminin molecules affect the integrin-laminin interaction. This might disturb the structural integrity of ECM around CNS and influence the cell shape. Or, the bulky *O*-glycans might distort the integrin-laminin signaling pathway to the cytoskeleton. In either case, the morphological changes seen in the *C1GalTA* mutants are the result of alterations in integrin-laminin interactions

influencing cytoskeletal architecture and cell shape, which in turn affects VNC condensation.

Mucin-type *O*-glycans on extracellular matrix proteins in development

Laminin and tiggrin are both integrin binding proteins secreted by the hemocytes. Interestingly, the *O*-glycosylation on Laminin and tiggrin appears to correlate with the requirement of their respective glycosyltransferase in *Drosophila* development. C1GalTA is required for Laminin *O*-glycosylation and VNC condensation. PGANT3 is required for tiggrin *O*-glycosylation and wing blade formation. The mucin-type *O*-glycans could affect the integrin-ECM interaction and mediate the morphological changes in developmental processes. Building upon these studies, it will be interesting to investigate the role of *O*-glycans on the other ECM proteins in other developmental contexts.

Future work

During embryogenesis, hemocytes are responsible for the production and secretion of several extracellular matrix (ECM) molecules. In late embryogenesis, these ECM molecules are secreted and basement membranes develop, covering all cell surfaces that are in contact with the hemolymph. (Wood and Jacinto, 2007)

Hemocytes express several extracellular matrix (ECM) components such as the enzyme Peroxidasin (Nelson et al., 1994), papilin, glutactin, tiggrin, basement membrane associated dSPARC (Martinek et al., 2002), and the proteoglycan MDP-1 (Hortsch et al., 1998). Hemocytes also produce structural components of the basement membrane such as LamininA (Kusche-Gullberg et al., 1992), and the two Collagen IV molecules identified

in *Drosophila*: Cg25C and Viking (Knibiehler et al., 1987, Le Parco et al., 1989, Mirre et al., 1988 and Yasothornsrikul et al., 1997).

Prior studies indicate that the secretion of extracellular matrix (ECM) by hemocytes appears to be particularly important for VNC condensation (Olofsson and Page, 2005). Our results of PNA blotting showed *C1GalTA* is required for a number of distinct *O*-glycosylated proteins (Fig. II-5J,K). Several ECM molecules are also glycoproteins, which could be O-glycosylated by *C1GalTA*. In the future, we could initiate the identification of *C1GalTA* substrates by characterizing the requirement of *C1GalTA* for O-glycosylation on the ECM proteins secreted by hemocytes. For example, we could examine the PNA binding ability of an ECM protein in the *C1GalTA* mutant animals, which could be done by PNA blotting of proteins immunoprcipitated by antibodies against that ECM protein.

Appendix *CG8668* is not essential for *Drosophila* development

ABSTRACT

CG8668 is an UDP-galactose beta-*N*-acetylglucosamine beta-1, 3-galactosyltransferase, transferring a galactose moiety from UDP-Gal to p-nitrophenyl-*N*-acetylglucosamine (pNp-GlcNAc). In situ hybridization showed *CG8668* is expressed during embryogenesis and in adult reproductive organs. At early embryogenesis, CG8668 is expressed in the pole cells from stage 5 to stage 7 and as pair-rule stripes at stage 6. Later on, at stage 11 to 12, there is some scattered dot-staining appearing; whose distribution suggests that those cells may be hemocytes. In adult reproductive organs, *CG8668* is expressed in the testis of male and in the follicle cells at anterior and posterior parts of eggs. A gene targeting screening was performed to make *CG8668* null mutants through homologous recombination. Two alleles were made. In $CG8668^{GT1}$, CG8668 protein was deleted from a.a.323 to the end of the protein. In $CG8668^{GT2}$, CG8668 protein was deleted from a.a.323 to 524. In both alleles, the transmembrane motif and the catalytic domain of CG8668 were removed and replaced by a marker gene, mini-white. These *CG8668* mutants are viable. They do not show obvious morphological phenotype and are perfectly fertile. These results suggest *CG8668* is not essential for Drosophila development.

INTRODUCTION

Glycosyltransferases

Glycosyltransferases catalyze the transfer of sugar moieties from activated donor molecules to specific acceptor molecules, forming glycosidic bonds. Each distinct linkage is usually the product of a different glycosyltransferase (Spiro, 2002; Narimatsu, 2006).

Fringe and brainiac are β1, 3 N-acetylglucosaminyltransferases involved in the regulation of Notch activity in *Drosophila* (Moloney et al., 2000b; Muller et al., 2002a). Based on the sequence similarity, Fringe, brainiac and 18 other putative glycosyltransferases are categorized into a family of β1, 3-glycosyltransferases in *Drosophila* (Correia et al., 2003). These β1, 3-glycosyltransferases are related to human β-1, 3-glycosyltransferases (Muller et al., 2002b; Togayachi et al., 2008). Among these β1, 3-glycosyltransferases, *CG8668* was my first focus for its pair-rule expression pattern during embryogenesis (Figure A-1).

Immune system

The immune system is responsible for identifying and destroying aberrant cells and infectious organisms. Vertebrate animals defend themselves against infections by two systems known as adaptive immunity and innate immunity. The adaptive immune system produces receptors by somatic gene rearrangement that recognize specific antigens and that allow organisms to develop an immunological memory, whereas the innate immune system relies on germ line-encoded factors for recognition and killing of foreign invaders.

Insects lack an adaptive immune system, but they do have a well-developed innate response. Consequently, the insect immune response can be used as a model system to study innate immunity (Hoffmann et al., 1999). The *Drosophila* innate immune system includes three responses: humoral responses, cellular responses, and proteolytic cascades.

Humoral defenses involve the rapid synthesis by the fat body of antimicrobial peptides that are released into the hemolymph (Meister et al., 2000). Cellular defenses refer to hemocyte-mediated responses including phagocytosis and encapsulation. The rapid induction of proteolytic cascades results in melanization, hemolymph (the insect blood) coagulation, and production of signaling molecules. The activated melanization process, carried out by the crystalline inclusion-containing crystal cells, locally generates toxic oxygen intermediates and culminates in the production of melanin at wound sites or around microorganisms (Ashida and Brey, 1995).

Abnormal immune responses in *Drosophila*

In many mutations in *Drosophila*, an immune response against self- tissues occurs. Their mutant phenotypes can be categorized into two groups: abnormal development of certain blood cells or other tissues and hyperactive immune response (Watson et al., 1991). The characteristic phenotype of these mutations is melanization, which forms black masses of tissue by aggregates of hemocytes or hemocytes surrounding cells from other tissues. The hemocytes form capsules and deposit melanin as they normally would during an immune response, but in this case the encapsulation is directed against the organism itself.

These melanotic mutations have been studied in *Drosophila* for about forty years. Over 165 genes have been identified that can be mutated to give a melanization phenotype. Only half of them are well characterized. An RNAi targeted against *CG8668* resulted in melanization, which led me to study its potential involvement in immune response.

RESULTS AND DISCUSSION

Expression of *CG8668*

CG8668 was picked for its tissue-specific expression pattern. Based on in situ hybridization, *CG8668* is expressed during embryogenesis and in adult reproductive organs. At early embryogenesis, *CG8668* is expressed uniformly by maternal contribution. It is expressed in the pole cells from stage 5 to stage 7 and as pair-rule strips at stage 6. (Figure A-1) Later on, at stage 11 to 12, there is some scattered dot-staining appearing; whose distribution suggests that those cells may be hemocytes. And it is also expressed in some cells along the anterior mid gut tissue at stage 14. In larval imaginal discs, there is no specific expression pattern of *CG8668*. Though, comparing with negative control, *CG8668* seems weakly expressed uniformly in wing imaginal disc. In adult reproductive organs, *CG8668* is expressed in testis of male and follicle cells at anterior and posterior part of eggs (Figure A-2). The developmentally regulated expression of *CG8668* indicates it might be important in a variety of processes.

Mutations of *CG8668*

To investigate the biological function of *CG8668*, I used double-stranded RNA interference (RNAi) to reduce the expression of *CG8668*. dsRNA was generated using the *UAS/GAL4* technique from yeast system in which the Gal4 protein domain will bind the *UAS* regulatory element and stimulate the transcription of the downstream sequence (Duffy, 2002).

To generate an expressed RNAi construct, I fused genomic DNA containing the second and third exons of *CG8668* and the second intron to its second exon in inverted orientation. This design is according to the observation that following splicing the hairpin dsRNA molecules formed will effectively suppress expression of the target genes (Kalidas and Smith, 2002). This RNAi construct was cloned into the pUAST vector whose DNA will be randomly inserted into fly genome, from which dsRNA molecules can be formed in specific tissues under the control of different *Gal4* drivers.

Because of chromosomal position effects, independent *UAS* insertions direct different levels of expression. Some insertion lines, which express much more dsRNA molecules, may give rise to more severe phenotypes.

Eleven transgenic flies were obtained. I tested their RNAi phenotypes in combination with *daughterless* (*da*)-Gal4, which drives ubiquitous expression. Experiments were performed at 25ºC and 29ºC to take advantage of the temperature dependent gene expression—higher temperature gives higher RNAi expression, which would results in more severe phenotype. Four lines do not give rise to any phenotype. Two lines, $CG8668^{RNAi9\text{-}6}$ and $CG8668^{RNAi9\text{-}6}$, result in melanization at 29ºC, not at 25ºC. Five lines give a melanization phenotype at 25ºC and 29ºC (Figure A-3). One of these

five lines, *CG8668*$^{RNAi29\text{-}1}$, is lethal and gives more severe and more consistent melanization phenotypes, and at early pupal stages and sometimes in late third instar larva.

The RNAi strategy does not completely deplete the expression of *CG8668*, so the RNAi phenotype might reflect only part of *CG8668*'s biological functions. This could be even worse in the case of *CG8668*, which can act catalytically. Thus, it was important to create *CG8668* null mutants and analyze their phenotypes. In addition, RNAi constructs sometimes have non-specific, "off-target" effects.

In the P-element insertion/ Gene Disruption project of the *Drosophila* genome, a P-element insertion line, *P[KG01020]*, was identified right before the transcription start site of *CG8668*. This insertion is viable and shows no phenotype. Flanking sequences are sometimes removed when a P-element excises. To remove *CG8668* sequences and create a mutation, I used *KG1020* to pursue a P-element excision screening. Among 120 lines analyzed, the only deletion line obtained, *CG8668*^{KG1020del}, is viable and deletes about 1.4 kb upstream of *CG8668* (Figure A-4). The P-element excision screening was not successful. And, the imprecise excision tended to delete in the other direction from *CG8668*.

Besides the P-element excision screening, I also performed a gene targeting screening to make *CG8668* mutants. Two alleles, *CG8668*^{GT1} and *CG8668*^{GT2}, were made using gene targeting constructs GT8668-1 and GT8668-2, respectively (Figure A-4). In *CG8668*^{GT1}, about 1.1kb deleted, CG8668 protein was deleted from a.a.323 to the end of the protein. In *CG8668*^{GT2}, about 600 bp deleted, CG8668 protein was deleted from a.a.323 to 524. In both alleles, the transmembrane domain and motifs predicted to be

essential for its catalytic activity were removed and replaced by a marker gene, mini-white. These *CG8668* mutants are viable. They do not show obvious morphological phenotype and are perfectly fertile. These results suggest *CG8668* is not essential for Drosophila development. Furthermore, the melanization phenotype caused by the expressed RNAi is a non-specific, "off-target" effect.

Overexpression of *CG8668*

I also expressed *CG8668* under the control of a heterologous promoter to test if this can cause any abnormal phenotypes. I cloned a *CG8668* cDNA into pUAST. Seven transgenic flies were obtained. Again, using the UAS-GAL4 technique, I tested six of them at 29ºC under the control of da-Gal4, which expressed uniformly. One line did not show any obvious phenotype. Three lines showed slower growth at larval stages and died before pupariation. Two lines died during embryogenesis.

The phenotypes of *CG8668* overexpression show that controlled expression of *CG8668* is essential in *Drosophila*.

***CG8668* is an UDP-Gal: GlcNAc galactosyltransferase**

Being most closely related to mammalian β3GalT1, 2, and 5 (Amado et al., 1999; Isshiki et al., 1999), *CG8668* is predicted to be an UDP-galactose beta-N-acetylglucosamine beta-1, 3-galactosyltransferase, transferring a galactose moiety from UDP-Gal to p-nitrophenyl-N-acetylglucosamine (pNp-GlcNAc).

To characterize the enzymatic activity of CG8668, I cloned its C-terminal catalytic domain into the pMT (1B) vector (made by Nicola Haines), which contains the

Drosophila metallothionein promoter (pMT) for inducible expression, the BiP signal sequence for secretion, the V5 epitope for detection, and the His-tag for purification.

From this pMT (1B)-*CG8668*-Tag vector, a truncated CG8668 can be expressed in S2 cells and secreted into the media. Labeling assays of the concentrated media were performed using radiolabeled UDP-sugar and measuring the transfer of radioactivity to various sugar acceptors (Figure A-5). The enzymatic reaction showed CG8668 is able to transfer a galactose moiety from UDP-Gal to *p*-nitrophenyl-N-acetylgalactosamine (pNp-GlcNAc). This pNp modification is to facilitate purification of the enzymatic products by the Sep-Pak C_{18} reverse-phase chromatography. Regarding its donor specificity, CG8668 uses UDP-Gal rather than UDP-Glucose, UDP-N-acetylgalactosamine, or UDP-N-acetylglucosamine. Considering its acceptor specificity, pNp-GlcNAc is better than pNp-Galactose or pNp-Mannose.

Although CG8668 can glycosylate a simple monosaccharide acceptor in vitro (pNp-GlcNAc), under physiological conditions it may have more restricted substrates. For example, it may normally recognize GlcNAc in the context of a specific oligosaccharide, and only when attached to a particular protein or lipid. Identifying the actual biological substrates of CG8668 will be essential to defining its function.

CG8668 in immune response system

Before we realized the RNAi-induced melanization is a non-specific, “off-target” effect, another RNAi experiment in S2 cells was performed. I decided to keep this part of experiment as a record here.

The melanization phenotype caused by CG8668 RNAi leads us to examine the roles of *CG8668* in immune responses. The best-studied defense effectors in innate immunity are antimicrobial peptide genes (AMPs) that are rapidly induced following microbial infection(Hoffmann, 2003). In *Drosophila*, AMPs are primarily induced via activation of the Toll (mainly responding to fungi or gram-positive bacteria) and/or Imd (mainly responding to gram-negative bacteria) pathways (Hoffmann, 2003).

Upon immune challenge with fungus or Gram-positive bacteria, the Toll pathway will be activated, which will stimulate the transcription of downstream target genes, e.g. the antimicrobial peptides Metchnikowin and defensin, respectively. When challenging with Gram-negative bacteria, the Imd pathway will be activated and the antimicrobial peptides, e.g. diptericin, will be induced.

To characterize the nature of immune response in *CG8668* RNAi, I used real-time PCR to look at the expression level of *defensin* (*def*), *diptericin* (*dipt*), and *Metchnikowin* (*Mtk*), representing the infection of Gram-positive, Gram-negative bacteria and fungus, respectively.

Assume the expression level the translation initiation factor, *elfa*, is affected by CG8668 RNAi treatment. I analyze the relative expression level of these AMPs with *elfa* (Table A-2). Though to different extent, the relative expression of these three AMPs are all decreased in CG8668 RNAi treated-S2 cells. On the contrary, their relative expressions are all increased when over-expressing CG8668. It seems immune response was induced in the CG8668 RNAi treated-S2 cells.

However, the experiments of CG8668 RNAi treatment in S2 cells does not agree with the immune response raised by CG8668 RNAi in larvae. CG8668 seems to play

different roles in inducing AMPs expression in S2 cells and melanization in larval tissues. The biological mechanism of CG8668 in melanization and induction of AMPs expression remain unclear. In the future, the identification of CG8668 substrate(s) might help to understand its biological functions.

EXPERIMENTAL PROCEDURES

Drosophila Genetics

Unless otherwise noted, stocks were obtained from the Bloomington *Drosophila* stock center. Imprecise excisions were generated from a P-element insertion near *CG8668*, *P[KG01020]* (Bellen et al., 2004) by crossing to a transposase-expressing line (*Sp/CyO; ry506 Sb1 P{Δ2-3}99B /TM6B, Ubx*). 120 potential excision events were identified by the loss of the w^+ marker. Among these w^- excisions, the only deletion line obtained, $CG8668^{KG1020del}$, deletes about 1.4 kb upstream of *CG8668*.

Molecular Biology

In situ hybridization to mRNA was carried out as described previously (Irvine and Wieschaus, 1994). The template for *CG8668* was cDNA SD02482 (Rubin et al., 2000).

To confirm the enzymatic activity of CG8668, we cloned its C-terminal catalytic domain (amino acids 44-388) into the vector pMT(1B) (Haines and Irvine, 2005; Xu et al., 2007), resulting in the plasmid pMT(1B)-CG8668-Tag. pMT(1B) contains the Drosophila metallothionein promoter (pMT) for inducible expression, the BiP signal sequence for secretion, a V5 epitope tag for detection, and a His-tag for purification.

DNA encoding CG8668 was amplified by PCR from cDNA clone SD02482 using primers CG8668-n'
5'-GGGGTACCAACGACTAGCACCCTCACAGCG
and CG8668-c'-tag 5'-GCTCTAGATTACACTTGGTGGTCTGATCCAGCA. Restriction enzyme sites KpnI and XbaI were included in the PCR primers to facilitate cloning. pMT(1B)- C1GalTA:V5:His was transfected into S2 cells, and concentrated media was used for glycosyltransferase assays, as described previously (Ju et al., 2002b; Haines and Irvine, 2005).

To over-express *CG8668*, plasmid *pUAST-CG8668* was made by cloning *CG8668* cDNA into pUAST. The 2.9 kb fragment from SD02482, treated with EcoRI and SpeI, was isolated and cloned into the backbone of pUAST, treated with EcoRI and XbaI.

For *CG8668* RNAi experiment, plasmid *pUAST-CG8668RNAi* was made by cloning part of *CG8668* genomic DNA, in inverted orientation, into pUAST. Two DNA fragments were amplified from genomic DNA. One, containing part of the second and third exons of CG8668 and its first intron, was amplified using primers CG8668RNAi-1 5'-GAAGATCTCCTAACCATTTTCATCTACGCCTCG and CG8668RNAi-2 5'-GGAATTCGAGCTGTTGTTGTTGGTGCTACTGTTG. The other DNA fragment, containing only the portion of the second exon in the first DNA fragment, was amplified using primers CG8668RNAi-3
5'-GGAATTCCTGTGATGCCATCAACACTCTGACC and CG8668RNAi-4 5'-CCGCTCGAGCCTAACCATTTTCATCTACGCCTCG. These two fragments were first fused with EcoRI, and cloned into pUAST with BglII and XhoI.

For gene targeting of *CG8668*, *GT8668-1* and *GT8668-2* were made with pW25 (Gong and Golic, 2004). In both constructs, the upstream fragment (US) was amplified by primers GT8668USF 5'-TACCCGTACGTCCGCTTTCTCGTTAGTTCCTTAG and GT8668USR 5'-AGCAGGCGCGCCCGTGGCCTGGCTCGTAGA, and cloned into pW25 by BsiWI and AscI. In GT8668-1, the downstream fragment (DS1) was amplified by primers GT8668DS1F 5'-TACCGGTACCAAGCGCTTCCCAAATAATACCTCT and GT8668DS1R 5'-AGCAGCGGCCGCGACGACACCGCAACCAATCTA, and cloned by Acc65I and NotI. In GT8668-2, the downstream fragment (DS2) was amplified by primers GT8668DS2F 5'-TACCGGTACCTGTTCACCACGGGCATAGTAGC and GT8668DS2R 5'-AGCAGCGGCCGCGCGTTGAAGTTTTGCCCATCTCGT, and cloned by Acc65I and NotI.

The best-studied defense effectors in innate immunity are antimicrobial peptide genes (AMPs) that are rapidly induced following microbial infection(Hoffmann, 2003). Since inducing RNAi of *CG8668* results in melanization, I focused on the expression level of AMPs [i.e. *defensin* (*def*), *diptericin* (*dipt*), and *Metchnikowin*(*Mtk*)]. I used real-time quantitative RT-PCR to measure their RNA levels in larval extracts. For *defensin,* I used primers defensin5 5'-TCTCGTGGCTATCGCTTTTGC and defensin3 5'-TCGTTGCAGTAGCCGCCTTT. For *diptericin*, I used primers dipt5 5'-TGCCGTCGCCTTACTTTGCT and dipt3 5'-GCTAGACTCGGATACCAATCG. For *Metchnikowin*, I used primers mtk5 5'-GCCACCGAGCTAAGATGCAA and mtk3 5'-CGACATCAGCAGTGTGAATTTCC.

Table A-1 Antimicrobial peptide production (AMP) induction by CG8668 RNAi treatment in S2 cells

Q (AMP/*elfa*)	*Defensin* (G^+)	*Diptericin* (G^-)	*Metchnikowin* (fungus)	*CG8668*
S2	9.52	2.16	3.26	0.4
S2/pMT(1B)8668Tag	15.27	3.13	4.42	22.6
S2/CG8668 RNAi	1.34	0.72	2.84	0.0

Relative expression Q (AMP/elfa)

$$= \frac{\text{RNA level of each antimicrobial peptide or } \textit{CG8668}}{\text{RNA level of translation initiation factor a (}\textit{elfa}\text{)}}$$

Figure A-1. Expression of *CG8668*

In situ hybridization was performed to *Drosophila* tissues to detect expression of *CG8668* mRNA. *CG8668* is expressed in the pole cells from stage 5 to stage 7 and as pair-rule strips at stage 6. Later on, at stage 11 to 12, there is some scattered dot-staining appearing; whose distribution suggests that those cells may be hemocytes. And it is also expressed in some cells along the anterior mid gut tissue at stage 12.

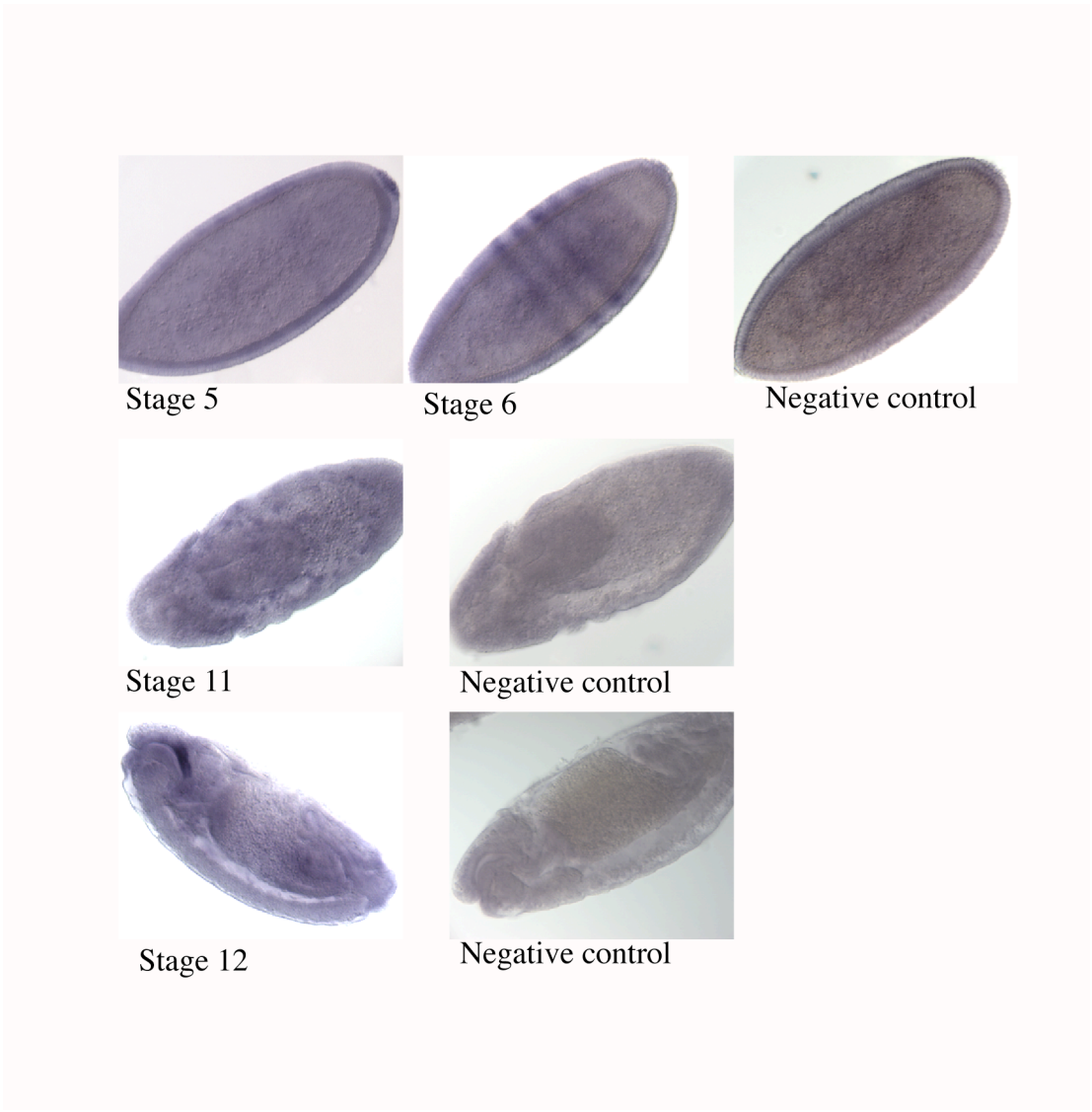

Figure A-1 Expression of *CG8668* during embryogenesis

Figure A-2. Expression of CG8668

In situ hybridization was performed to *Drosophila* tissues to detect expression of *CG8668* mRNA. In larval imaginal discs, there is no specific expression pattern of *CG8668*. Though, comparing with negative control, *CG8668* seems weakly expressed uniformly in wing imaginal disc. In adult reproductive organs, *CG8668* is expressed in testis of male and follicle cells at anterior and posterior part of eggs.

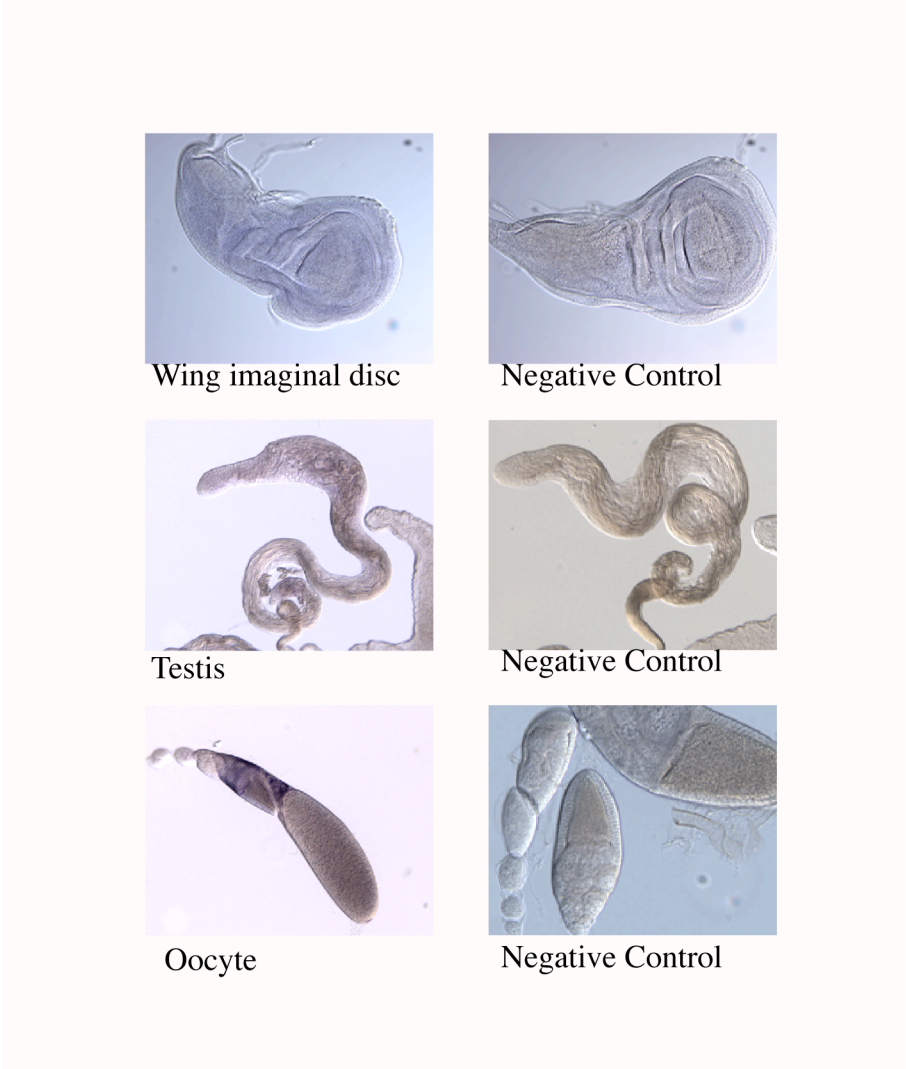

Figure A-2 Expression of *CG8668* in larval and adult tissues

Figure A-3 Melanization phenotypes of UAST-RNAi lines

Melanization phenotypes of UAST-RNAi lines: *CG8668*$^{RNAi9\text{-}6}$, *CG8668*$^{RNAi21\text{-}3}$, and *CG8668*$^{RNAi29\text{-}1}$ under the control of *daughterless-Gal4*.

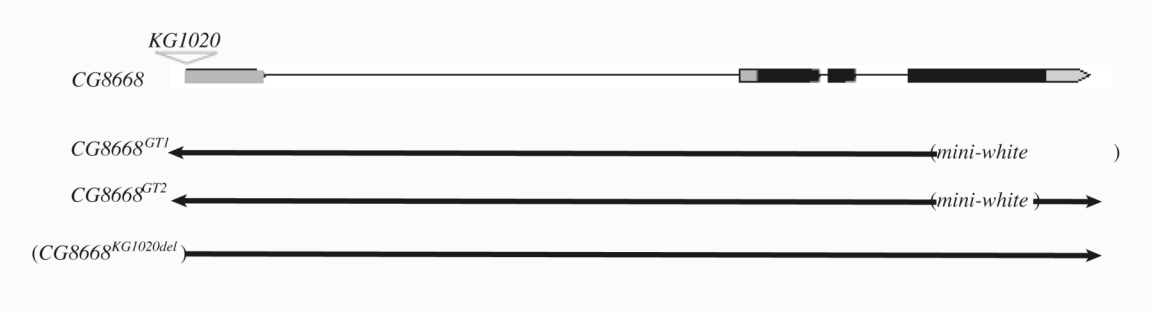

Figure A-4 Mutations of *CG8668*

Top line shows a schematic of the *CG8668* transcription unit, with introns as thin lines, exons as thick lines, coding regions in black and non-coding regions in gray. The position of the *P[KG01020]* insertion is indicated by the triangle. Lower lines show DNA retained in mutant alleles. Both $CG8668^{GT1}$ and $CG8668^{GT2}$ were created by homologous recombination. In $CG8668^{GT1}$, about 1.1kb deleted, CG8668 protein was deleted from a.a.323 to the end of the protein. In $CG8668^{GT2}$, about 600 bp deleted, CG8668 protein was deleted from a.a.323 to 524. In both alleles, the transmembrane domain and motifs predicted to be essential for its catalytic activity were removed and replaced by a marker gene, mini-white. $CG8668^{KG1020del}$ was isolated from a P-element excision screening of *P[KG1020]*. In $CG8668^{KG1020del}$, the genomic DNA deletes about 1.4 kb upstream of *CG8668*.

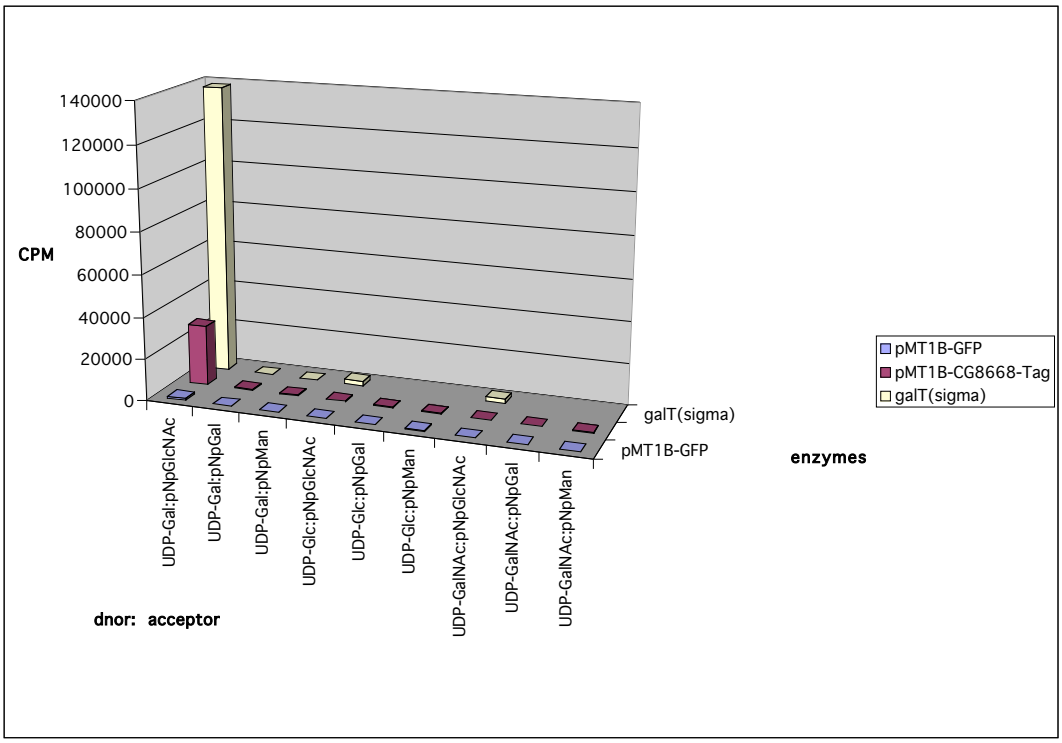

Figure A-5 CG8668 enzymatic activity

To characterize CG8668 enzymatic activity, a truncated CG8668 was expressed in S2 cells and secreted into the media. Labeling assays of the concentrated media were performed using radiolabeled UDP-sugar and measuring the transfer of radioactivity to various sugar acceptors. The enzymatic reaction showed CG8668 is able to transfer a galactose moiety from UDP-Gal to *p*-nitrophenyl-N-acetylgalactosamine (pNp-GlcNAc).

pMT(1B)-GFP is a negative control expressing GFP. galT(Sigma) is a galactosyltransferase manufactured by Sigma.

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