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

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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-β1,3-GalNAc in vivo. Our results identify a role for mucin-type *O*-glycosylation during neural development in *Drosophila*.

Keywords

Drosophila; galactosyltransferase; mucin; nervous system

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*-

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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 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. 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 Fig 2–Fig 4 and Table 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. 2B,C), and *CG8708* is expressed by salivary gland cells (Fig. 4B,C). However, in contrast to Muller, we find that *CG13904/CG34056* is expressed specifically by tracheal cells rather than salivary gland cells (Fig. 3F), and we also found that during late embryonic development *C1GalTA* is expressed within the central nervous system (CNS) (Fig. 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. 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 1, Fig 2, Fig 3). In the adult, several of the core1 GalTs exhibit distinct regionalized expression patterns in different parts of the male reproductive system (Fig. 3A–C, Table 1), and several are expressed in the nurse cells during oogenesis (Fig. 2F, Table 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. 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. 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. 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. 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. 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. 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. 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. 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. 5D,E). Normal PNA staining was restored in animals with the genomic rescue construct (Fig. 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. 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. 5H,I). Intriguingly, an obvious PNA staining of the salivary gland was observed *C1GalTA[2.1]* mutant embryos (Fig. 5E), but not in wild-type embryos (Fig. 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. 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. 5K), which specifically removes *O*-GalNAc glycans. Notably, these bands were also greatly reduced in *C1GalTA[2.1]* mutants (Fig. 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. 6A–K); this phenotype was fully rescued by the wild-type transgene (Fig. 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. 6A,B, Table 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. 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. 6, Table 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. 2E, I). The elongated VNC of mutants is noticeably thinner (Fig. 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. 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. 7A). The PNA reactivity on hemocytes was abolished in *C1GalTA* mutants (Fig. 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. 7C). By contrast, lysates from *C1GalTA* mutant embryos lacked detectable association of Laminin with PNA (Fig. 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. 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 *Not*I 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$^+$ -X]* and third chromosomes *P[C1GalTA$^+$ −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°C 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).

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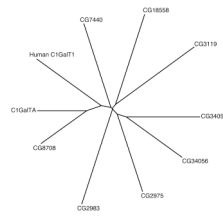
Figure 1. Phylogenetic relationship of Core 1 GalTs
A proportional, radial, phylogenetic tree was constructed to depict the relative sequence divergence among members of the Core 1 GalT family in *Drosophila* and human. The tree depicts graphically the results of ClustalW analysis.
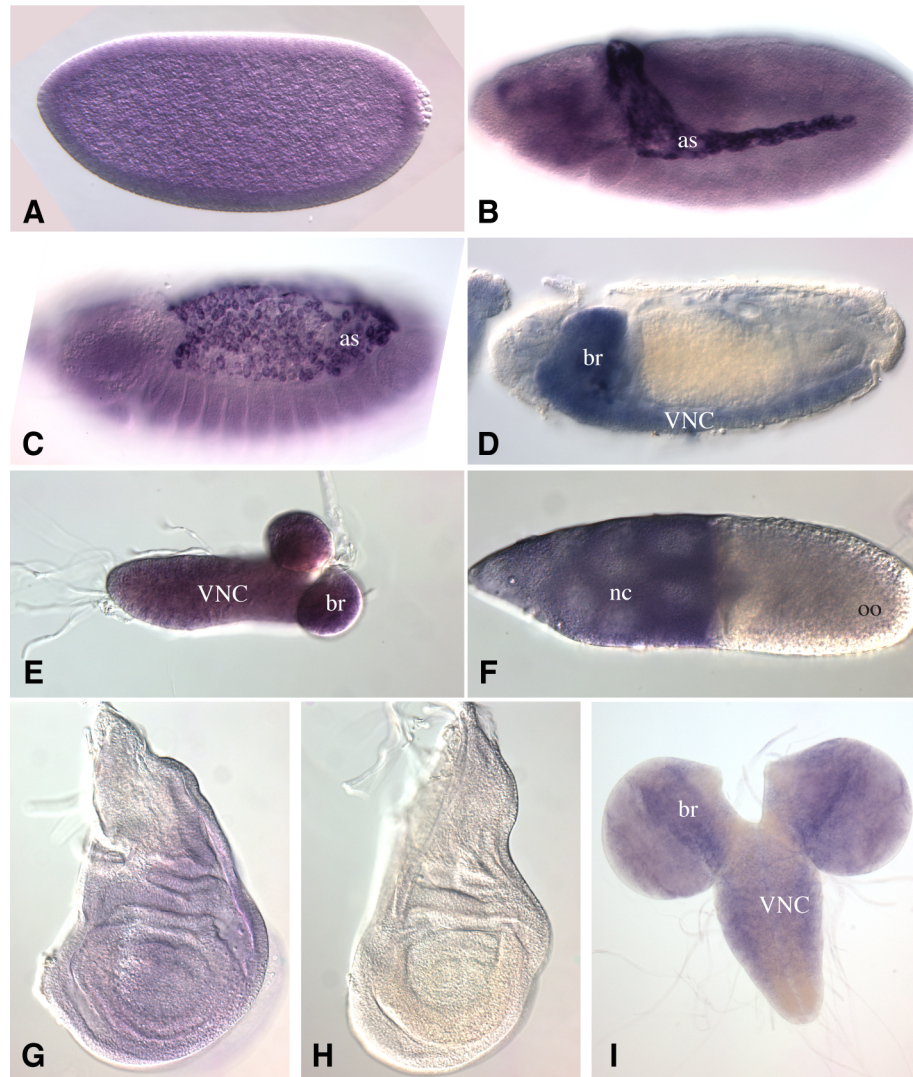
Figure 2. Expression of *C1GalTA*
In situ hybridization to *Drosophila* tissues to detect expression of *C1GalTA* mRNA. A) Stage 6 embryo. Uniform expression above background is observed in early embryos, which likely includes maternally deposited mRNA. B) Stage 12 embryo. Strong expression is detected 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.
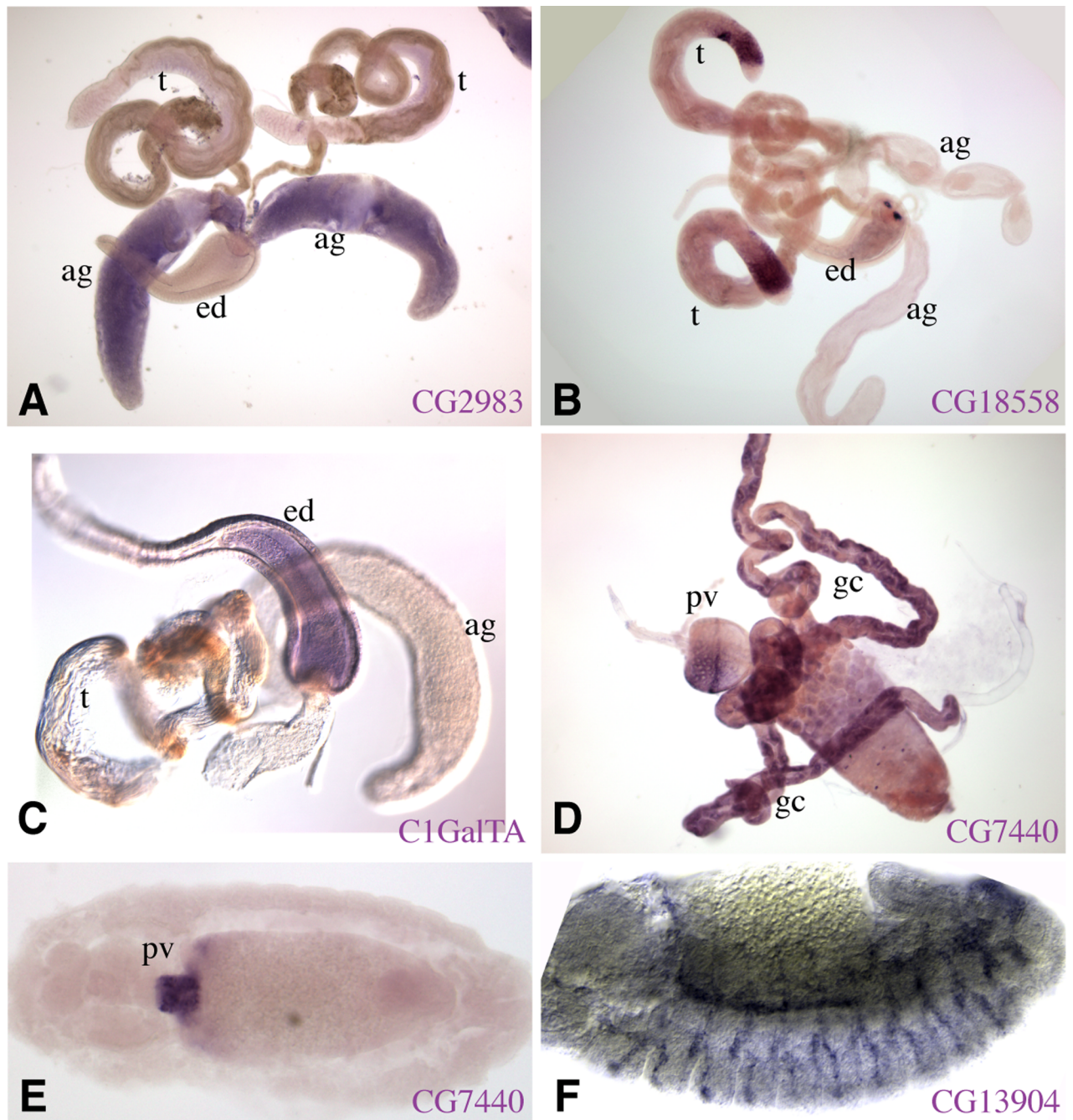
Figure 3. Expression of Core 1 GalT family members

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.
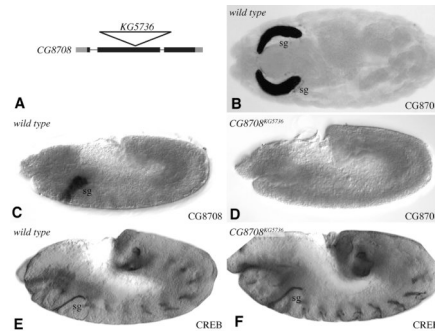
Figure 4. *CG8708* mutant does not affect salivary gland morphogenesis
A) Schematic of *CG8708* transcription unit shows introns as thin lines and exons as thick lines, with coding regions in black and non-coding regions in gray. The position of the *KG5736* insertion is indicated by the triangle. B) 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.
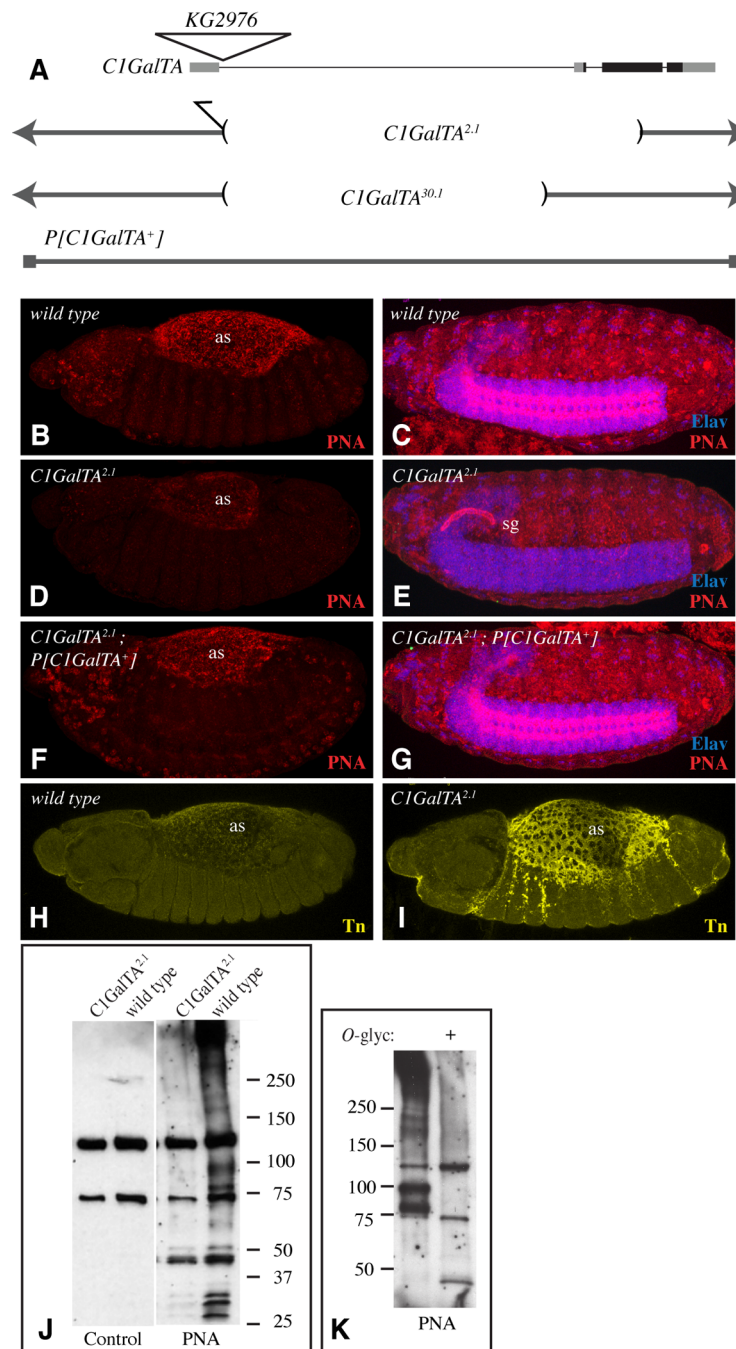
Figure 5. Influence of *C1GalTA* mutants on PNA and anti-Tn staining
A) Top line shows a schematic of the *C1GalTA* transcription unit, with introns as thin lines, exons as thick lines, coding regions in black and non-coding regions in gray. The position of the *KG2976* insertion is indicated by the 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.
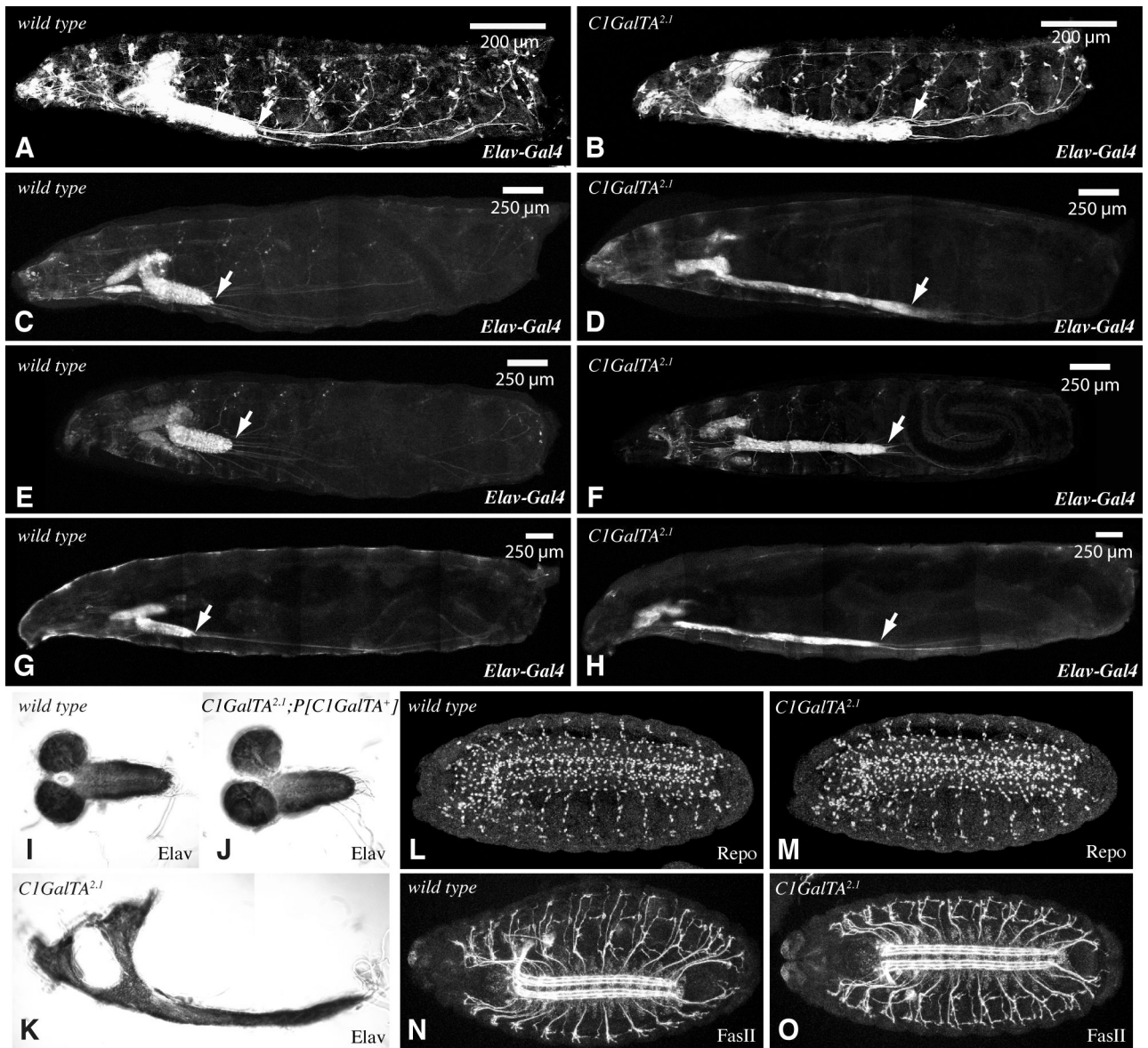
Figure 6. VNC Phenotype of *C1GalTA* mutants
A–H) Composite confocal images of larvae with *Elav-Gal4* and *UAS-GFP* transgenes. GFP is expressed in the nervous system and salivary glands, arrow points to the posterior end of the VNC. A–D, G, H are saggital views, E,F is a ventral view. A) *wild-type* 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 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).
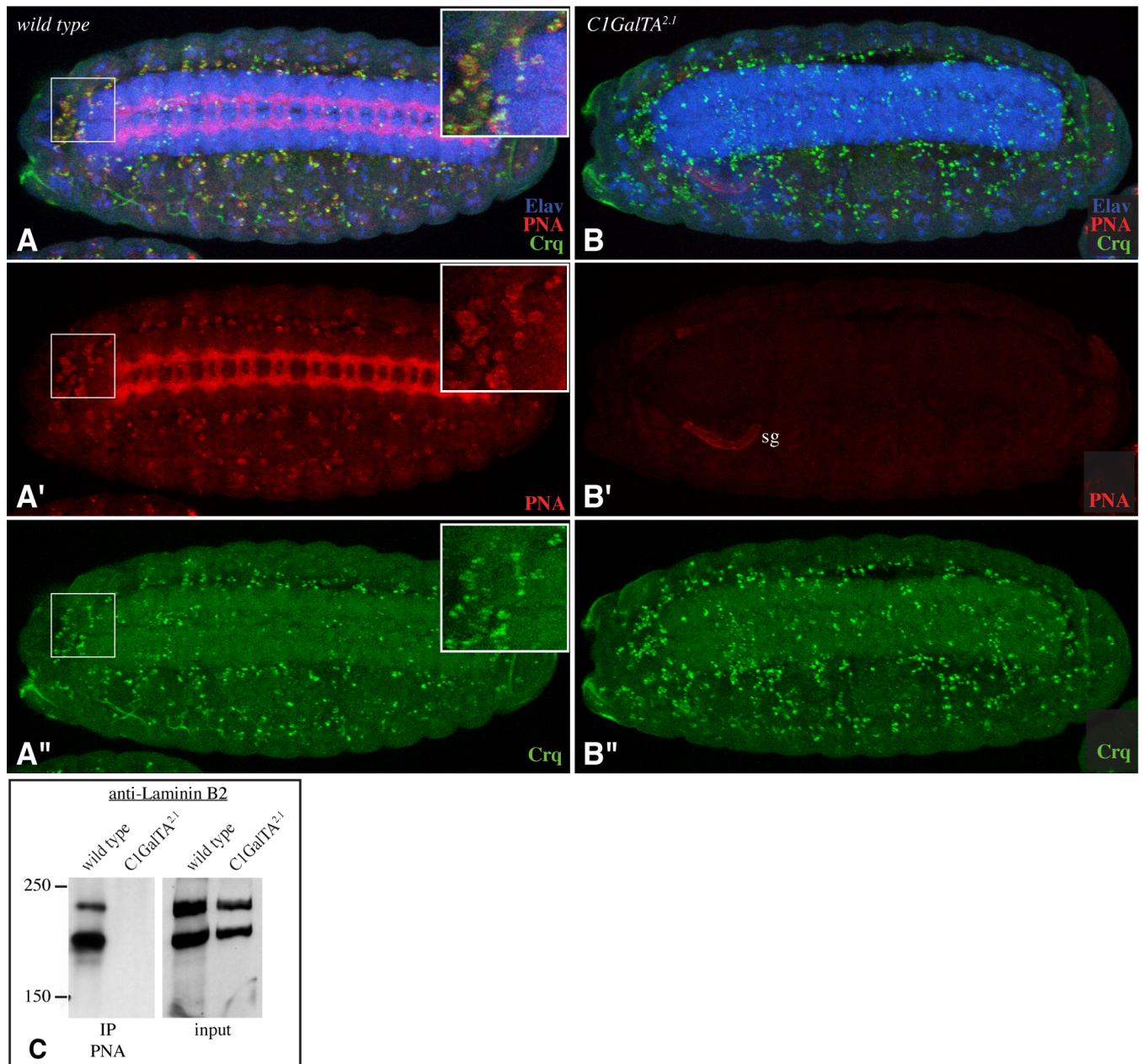
Table 1

Expression of the Core1 GalT family in *Drosophila*

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

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

Table 2

Length of the VNC in wild-type and mutant larvae

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.

Genotype	Instar	Number measured	Larval length (μm)	VNC length (μm)	Ratio (%)
Wild type	1st	20	1403 +/− 114	351 +/− 36	25
ClGalTA2.1	1st	15	1231 +/− 125	530 +/− 69	43
Wild type	2nd	24	2752 +/− 343	350 +/− 46	13
ClGalTA2.1	2nd	29	2582 +/− 511	923 +/− 201	36
Wild type	early 3rd	11	4103 +/− 464	437 + /− 50	11
ClGalTA2.1	early 3rd	15	4202 +/− 519	1554 +/− 253	37