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A Mucin-type *O*-Glycosyltransferase Modulates Cell Adhesion during *Drosophila* Development*[S]

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Cell-cell and cell-matrix adhesion are crucial during many stages of eukaryotic development. Here, we provide the first example that mucin-type *O*-linked glycosylation is involved in a developmentally regulated cell adhesion event in *Drosophila melanogaster*. Mutations in one member of the evolutionarily conserved family of enzymes that initiates *O*-linked glycosylation alter epithelial cell adhesion in the *Drosophila* wing blade. A transposon insertion mutation in *pgant3* or RNA interference to *pgant3* resulted in blistered wings, a phenotype characteristic of genes involved in integrin-mediated cell interactions. Expression of wild type *pgant3* in the mutant background rescued the wing blistering phenotype, whereas expression of another family member (*pgant35A*) did not, revealing a unique requirement for *pgant3*. *pgant3* mutants displayed reduced *O*-glycosylation along the basal surface of larval wing imaginal discs, which was restored with wild type *pgant3* expression, suggesting that reduced glycosylation of basal proteins is responsible for disruption of adhesion in the adult wing blade. Glycosylation reactions demonstrated that PGANT3 glycosylates certain extracellular matrix (ECM) proteins. Immunoprecipitation experiments revealed that PGANT3 glycosylates tiggrin, an ECM protein known to bind integrin. We propose that this glycosyltransferase is uniquely responsible for glycosylating tiggrin in the wing disc, thus modulating proper cell adhesion through integrin-ECM interactions. This study provides the first evidence for the role of *O*-glycosylation in a developmentally regulated, integrin-mediated, cell adhesion event and reveals a novel player in wing blade formation during *Drosophila* development.

Cell interactions and adhesion are critical in many diverse processes, from events occurring during embryogenesis and organogenesis to wound healing and the alterations in cell adhesion seen upon tumor formation and metastasis (1). *Drosophila* wing development has been used as a model system to identify factors responsible for regulating cell adhesion (2–5). Aberrant adhesion of the two epithelial cell layers that comprise the adult wing blade results in separation of the cell layers, creating a localized blister shortly after eclosion. Mutations in the integrin family of cell surface receptors (2, 3, 5–7) as well as proteins that interact with integrins (4, 5, 8–13) have been shown to produce wing blisters, highlighting the central role of integrin-mediated cell adhesion events during wing blade formation. Interestingly, many cell surface and extracellular matrix (ECM)[2] proteins that influence wing blistering also interact with integrins in other developmentally regulated cell adhesion events (2, 14, 15).

Cell surface, secreted, and ECM proteins undergo a number of post-translational modifications as they transit the secretory apparatus to their final destinations. Although the roles of classical *N*-linked glycans and proteoglycans are widely appreciated, recent studies have elucidated crucial roles for other types of glycans during development. The disaccharide GlcNAcβ1–3Fuc on the Notch receptor and its ligands has been shown to regulate receptor/ligand interactions and downstream signaling events (16–19). Protein *O*-fucosylation and the fucosyltransferase catalyzing this modification have roles in protein folding, trafficking (20, 21), and secretion (22). Most recently, *O*-linked glucose has been shown to have a modulatory role in Notch signaling events, influencing lateral inhibition and cell-fate specification (23). Taken together, these studies highlight the diversity of glycans found on proteins and the unique functional roles they play during development.

Studies from our laboratory and others have demonstrated the abundant presence of another type of glycosylation, known as mucin-type *O*-linked glycosylation (referred to as *O*-linked glycosylation) throughout development in diverse species (24–27). In contrast to the previously mentioned types of glycosylation, mucin-type *O*-glycosylation is initiated by a large family of enzymes known as the UDP-GalNAc:polypeptide *N*-acetylgalactosaminyltranferases (PGANTs in *Drosophila* or ppGalNAcTs in mammals) that transfer *N*-acetylgalactosamine (GalNAc) to serine or threonine residues of proteins destined to be membrane-bound or secreted (28, 29). Members of this family have unique but overlapping developmental expression patterns, and many show distinct substrate preferences *in vitro*, suggesting that each enzyme may be responsible for glycosylating a unique subset of proteins *in vivo* (25, 28, 29). *In vitro* data further indicate that there exists a hierarchy of activity within the family, with some members initiating the glycosylation of unmodified substrates and others acting only on previously glycosylated sub-

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[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental Table 1.

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[2] The abbreviations used are: ECM, extracellular matrix; ppGalNAcT or PGANT or *pgant*, UDP GalNAc:polypeptide *N*-acetylgalactosaminyltransferase; IR, inverted repeat; JNK, c-Jun NH_2-terminal kinase; RNAi, RNA interference; Ab, antibody.

strates, adding GalNAc at positions vicinal to sites previously modified by other family members (30–32). The unique spatial and temporal expression patterns, substrate preferences, and hierarchical action of members of this family suggest a highly regulated process governing the acquisition of this type of glycan.

Mucin-type *O*-glycans on secreted and membrane-bound proteins are uniquely positioned to mediate many events regulating homeostasis and development. Indeed, mutations in ppGalNAc-T3 are thought to be responsible for familial tumoral calcinosis, a rare human disease characterized by hyperphosphatemia and the development of calcified "tumors" in cutaneous and subcutaneous tissues (33). Mice deficient in ppGalNAc-T1, although viable and fertile, display reduced lymphocyte homing and bleeding disorders (34). Studies from our laboratory have demonstrated a role for another member of this family (*pgant35A*) in the proper formation of the embryonic tracheal system in *Drosophila* (35, 36). Finally, mice deficient in the core 1 β3-galactosyltransferase, which adds a galactose to the *O*-GalNAc of mucin-type *O*-glycans, die embryonically from defective angiogenesis resulting in fatal brain hemorrhages (37); hypomorphic mutations result in thrombocytopenia and kidney defects later in development (38). Altogether, these studies highlight the diverse consequences of alterations in *O*-glycosylation that have only recently been discovered due to the inherent complexity of the ppGalNAcT family.

Here, we examine the developmental role of another member of this family, *pgant3*. Previous work from our laboratory demonstrated that PGANT3 is one of the initiating glycosyltransferases, transferring GalNAc to previously unmodified substrates (25). Additionally, *pgant*3 gene expression is highly regulated during development (27). In this study we find that a transposon mutation in *pgant3* or RNA interference (RNAi) to *pgant3* results in wing blistering, implicating *O*-glycosylation in integrin-mediated cell adhesion occurring during wing blade formation. A reduction in *O*-glycoproteins was seen along the basal surface of the *pgant3* mutant wing imaginal discs along with altered disc morphology. Based on our studies, we propose that *O*-glycosylation of specific ECM proteins known to bind integrin is required for proper epithelial cell adhesion in the wing disc. This finding provides the first direct evidence for the role of mucin type *O*-glycans in a developmentally regulated cell adhesion event and identifies a novel protein modification required for proper wing blade formation in the fly.

EXPERIMENTAL PROCEDURES

Fly Strains Used—The stocks used in this study are as follows: Bloomington stocks #5138 (y^1, w*; P{w^{+mC} = tubP-GAL4}LL7/TM3, Sb^1) (the *tubulin*-Gal4 driver line); #8860 (w^{1118}, P{$w^{+mW.hs}$ = GawB}Bx^{MS1096}) (the wing-specific Gal4 driver line); #1561 (w*; P{$w^{+mW.hs}$ = Gal4-arm}4a, P{$w^{+mW.hs}$ = Gal4-arm}4b/TM3, Sb^1, Ser^1) (the *armadillo*-Gal4 driver line); #7748 (w^{1118}; Df(2R)Exel 6283, P^{XP-U}Exel6263); #8283 (w^{1118}; CyO, P{w^{+mc} = FRT (w^+)Tub-PBac™}2/wg^{Sp-1}); #8795 (w*; Tig^{A1}/CyO,P{lacZ-un3}276); #8796 (w*; Tig^X/CyO,P{lacZ-un3}276). Additionally, the following stocks from other sources were used: PBac{PB}*pgant3*^{c01318} from the Exelixis *Drosophila* Stock Collection (39); w; Dr/TM3, Sb^1, twi-2XGFP stock (the kind gift of D. Andrew); w; TM6C, cu, Sb, e, ca/Su(Tpl)^{s1}, red, e stock (the kind gift of J. Kennison).

Construction of Gal4-inducible pgant3 and pgant3IR Vectors and Transgenic Lines—The pUAST plasmid (40) was used to generate a Gal4-inducible construct expressing wild type *pgant3* cDNA that was then used to create transgenic flies. The complete coding region from the wild type *pgant3* gene was excised from the GH09147 cDNA clone (Invitrogen) using EcoRI and XbaI and cloned into the same sites of pUAST to generate the plasmid pUAS-*pgant3*. To generate the Gal4-inducible *pgant3*IR construct, sense (taatacctaggAAGGTGAATGTTACGGAGCGTGTGG) and antisense (taatacctaggCTGCGCCAGCATTACATTCGAAGTG) primers were used to amplify a 500-bp fragment from the catalytic region of *pgant3*. The PCR product was then cloned stepwise into the AvrII and NheI sites on either side of the *white* intron in the vector pWIZ (41) to generate a vector containing two inversely oriented *pgant3* fragments flanking the *white* intron. Transformants were produced by Genetic Services Inc. (Cambridge, MA) using methodology based on the procedure described previously (42, 43).

Fly Crosses—Rescue and overexpression experiments were performed using flies from a UAS-*pgant35A* transgenic line (36) or three independent UAS-*pgant3* transgenic lines (w; P{UAS-*pgant3*#3}, on the third chromosome) (w; P{UAS-*pgant3*#7}, on the X chromosome) (w; P{UAS-*pgant3*#10}, on the third chromosome) and the Gal4-driver stocks described herein. All stocks used in the rescue experiments were first crossed into the *pgant3*^{c01318} background to generate both Gal4 driver lines and P{UAS-*pgant*} transgenic lines heterozygous for *pgant3*^{c01318}. These heterozygous lines were then crossed as follows, and rescue of the wing blister phenotype was assessed by scoring straight winged progeny with or without Sb^1: *pgant3*^{c01318}/CyO; P{UAS-*pgant*}/P{UAS-*pgant*} females X *pgant3*^{c01318}/CyO;P{tubP-GAL4}LL7/TM3, Sb^1 males. Crosses to assess the effect of *pgant3* overexpression were performed as shown in Table 2.

Crosses to generate RNAi to *pgant3* were performed by crossing homozygous inverted repeat (IR) transgenic lines (w^{1118}; P{UAS-*pgant3*IR2#2} or w^{1118}; P{UAS-*pgant3*IR2#9}) to a tubulin-Gal4-driver line (P{tubP-GAL4}LL7/TM3, Sb^1, twi-2XGFP) and comparing progeny with and without Sb^1 and GFP. Crosses to the wing-specific driver (MS1096-Gal4) were performed using homozygous w^{1118}, P{$w^{+mW.hs}$ = GawB}Bx^{MS1096} females crossed to homozygous transgene-containing males. All *Drosophila* crosses were kept on MM media (KD Medical, Inc.) at 25 °C unless specified otherwise.

Mutant Sequencing and Quantitative Reverse Transcription-PCR—The genomic region flanking each transposon insertion was amplified and sequenced according to the previously described procedures (39). To examine the effect of the transposon on *pgant3* gene expression levels, *pgant3*^{c01318}/*pgant3*^{c01318} homozygotes, wild type, and transposon excision lines were used to isolate RNA and perform real-time PCR. Briefly, RNA was isolated using the FastRNA Pro Green kit (Q-BIOgene). cDNA synthesis was performed using the iScript cDNA synthesis kit (Bio-Rad). PCR primers were designed using Beacon Designer software (Bio-Rad).

Quantitative reverse transcription-PCR was performed on a MyiQ real time PCR thermocycler (Bio-Rad) using the SYBR-Green PCR Master Mix (Bio-Rad). Quantitative reverse transcription-PCR to determine expression levels of all *pgant* family members was performed using the PCR primers listed in supplemental Table 1 with cDNA prepared from wild type, Tub>*pgant3*IR2#2, and Tub>*pgant3*IR2#9 larval wing discs.

Wing Disc Fixation and Staining—Imaginal wing discs were stained according to standard procedures and analyzed by confocal microscopy. Mouse monoclonal anti-Tn antibody (Ca3638) (44) (dilution, 1:50) was the kind gift of Dr. Richard Cummings who had acquired the stocks of antibodies and hybridomas from the late Dr. Georg F. Springer. Immunopositive signals were developed using Cy3-conjugated donkey anti-mouse IgM antibody (dilution, 1:100) (Jackson ImmunoResearch Laboratories). Stained wing discs were analyzed using the Zeiss LSM 510 confocal laser scanning microscope. Images were processed using the LSM Imager Browser and Photoshop. Measurements of wing disc thickness and *O*-glycan staining in *x-z* cross sections were performed in the center of each disc. Values were averaged, and S.D. were calculated. Statistical significance was determined using a Student's *t* test.

Glycosylation Assays in Vitro—Assays for glycosyltransferase activity were performed as described previously (25). Briefly, media from COS7 cells expressing recombinant *pgant3* or *pgant35A* (25) was harvested and used in the *in vitro* reactions with [^{14}C]UDP-GalNAc, and the acceptor substrates denoted in Table 3. Reaction products were purified by anion exchange chromatography, and [^{14}C]GalNAc incorporation was measured. Reactions using media from cells expressing empty vector alone yielded background values that were subtracted from each experimental value. Adjusted experimental values were then averaged, and S.D. were calculated. All assays were performed in duplicate. Glycosyltransferase activity is expressed as dpm/h.

Western Blotting—Protein extracts were prepared from 3rd instar larval wing discs of wild type, *pgant3*^{c01318} homozygotes, and transposon excision lines (*pgant3*$^{c01318revertant\#7}$ homozygotes) as described (45). Samples were electrophoresed under reducing conditions in a 4–12% SDS-PAGE gradient gel. Gels were transferred to nitrocellulose; membranes were blocked with 1× blocking buffer (Sigma), incubated with Tn antibody (dilution, 1:500) or the tiggrin antibody (the kind gift of Drs. L. and J. Fessler) (9) (dilution, 1:500), and developed with horseradish peroxidase-conjugated secondary antibody (dilution, 1:10,000).

Immunoprecipitation—Equivalent amounts of protein extracts were prepared from 3rd instar larval wing discs from wild type and *pgant3*^{c01318} homozygotes. 50 μl of immobilized protein L suspension (Thermo Scientific) was added to 500 μl of protein extract and incubated at 4 °C for 1 h to preclear. The mixtures were centrifuged, and the precleared supernatant was collected. 10 μl of the tiggrin antibody were added to the precleared supernatant and incubated at 4 °C overnight. The following day 50 μl of washed immobilized protein L suspension was added, and incubation was performed at 4 °C overnight. Immunocomplexed proteins were collected by pulse centrifugation. Pellets were washed three times with lysis buffer. The final pellet was resuspended in sample loading buffer, heated to 95 °C for 5 min, and analyzed by reducing SDS-PAGE followed by immunoblotting with the Tn Ab as described.

RESULTS

Transposon Insertion Decreases pgant3 Gene Expression and Causes Wing Blistering—pgant3 is one of 12 members of the *Drosophila* gene family encoding the polypeptide GalNAc glycosyltransferases that are responsible for initiating mucin-type O-linked glycosylation of secreted and membrane-bound proteins (25, 27). *pgant3* gene expression is highly regulated both spatially and temporally during development (27). We set out to examine the biological roles of *O*-glycosylation mediated by this glycosyltransferase using three putative transposon insertions in the *pgant3* gene from the Exelixis *Drosophila* stock collection (39). Genomic sequencing of the insertion sites revealed that only one transposon resided within the *pgant3* gene (PBac{PB}*pgant3*^{c01318}); the line containing this transposon is hereafter designated *pgant3*^{c01318}. *pgant3*^{c01318} contains a *piggyBac* transposable element in the fourth intron of *pgant3*, separating exons that encode the conserved catalytic region of the enzyme (Fig. 1*A*). Quantitative PCR was performed on wild type and homozygous transposon insertion mutants to assess the effect of the transposon on the expression levels of *pgant3* as well as the flanking *tetraspanin* genes (*Tsp42Ep* and *Tsp42Eq*) (Fig. 1*B*). As shown in Fig. 1*C*, *pgant3* gene expression 3′ to the transposon insertion site was significantly reduced in the transposon insertion line relative to wild type. Expression of the flanking *tetraspanin* genes was not affected. This result demonstrates that the transposon insertion specifically affects *pgant3* gene expression, thus supporting the use of this line to investigate *pgant3* gene function.

Heterozygous transposon insertion flies were crossed to assess the effect of the transposon on viability. Although a reduction in viability was seen relative to heterozygotes, most homozygous transposon insertion mutants did survive to adulthood. However, a significant number of *pgant3*^{c01318} homozygous adults displayed abnormal wings (Fig. 2 and Table 1). Specifically, wings of *pgant3*^{c01318} homozygotes developed blisters as they unfolded, which later collapsed into crumpled areas in the wing blade (Fig. 2). Wing blistering was not seen in wild type flies or flies heterozygous for the transposon insertion (Table 1). The extent of blistering varied, with some wings having small blisters and others showing blistering across the entire wing blade (Fig. 2, *B* and *C*).

The *pgant3*^{c01318} line was crossed to a deficiency line that uncovered the *pgant3* gene (Df(2R)Exel 6283, P^{XP-U}Exel6263) to verify that the blistering phenotype is the result of the transposon in *pgant3* and not due to mutations elsewhere on the chromosome. Progeny containing the transposon insertion over the deficiency displayed wing blistering (Table 1), lending further support for the role of *pgant3* in the wing blistering phenotype.

To conclusively demonstrate that the transposon insertion in *pgant3* is responsible for the wing blistering phenotype, the *piggyBac* transposon was precisely excised from the *pgant3* gene using the *piggyBac* transposase as described previously (39). Transposon excision lines (*pgant3*$^{c01318revertant\#7}$/

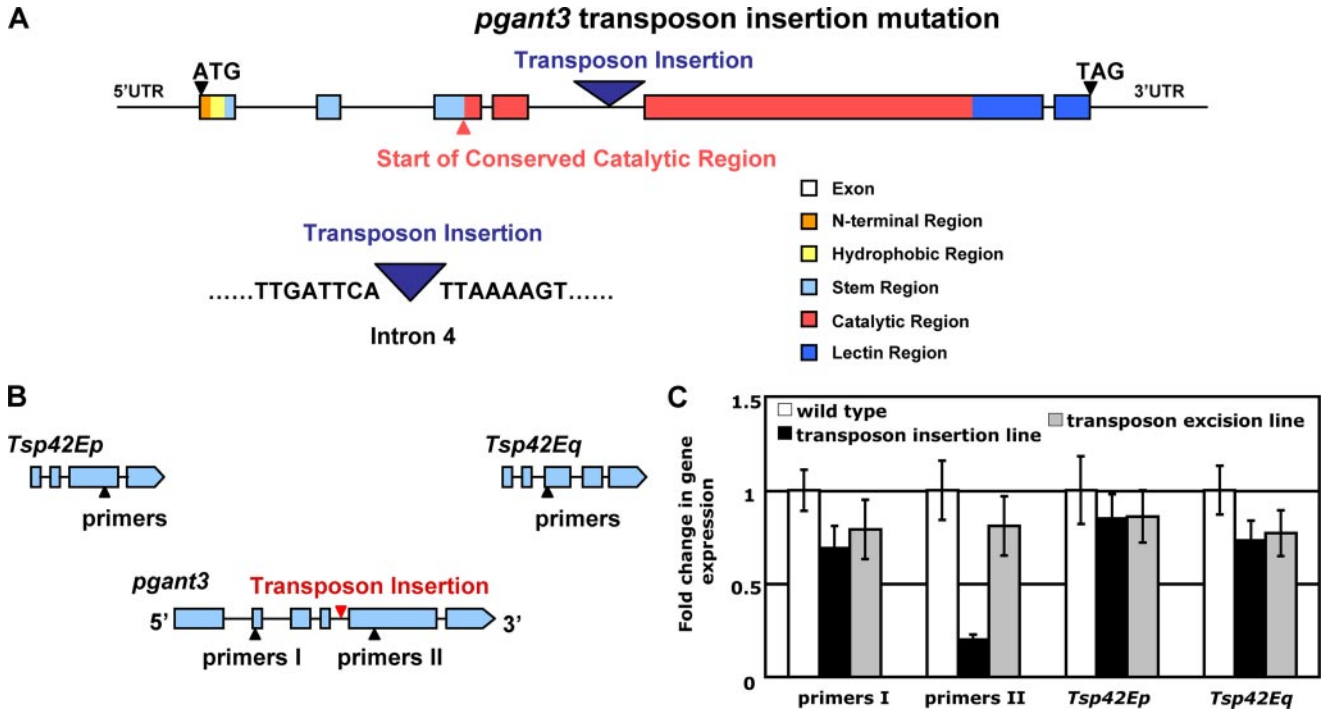

FIGURE 1. ***pgant3* gene expression is reduced in the *pgant3*^{c01318} transposon insertion stock.** *A*, position of the transposon in intron 4 of the *pgant3* gene is shown. Exons are represented as *boxes*, and introns are represented as *lines*. Functional elements of the *pgant3* coding region are shown in color. Nucleotide sequence of the transposon insertion site in the *pgant3*^{c01318} stock is shown. *B*, the genomic region of *pgant3* and flanking genes are shown. The positions of the primer pairs used for real-time PCR are shown as *triangles*. *C*, real-time PCR analysis of *pgant3* transcript levels using the primer pairs shown in *B* reveals a significant decrease in *pgant3* gene expression in *pgant3*^{c01318}/*pgant3*^{c01318} transposon insertion homozygotes relative to *pgant3*$^{c01318revertant\#7}$/*pgant3*$^{c01318revertant\#7}$ transposon excision homozygotes or wild type flies. Transcription from the flanking *Tsp42Ep* and *Tsp42Eq* genes was unaffected. RNA levels were normalized to 18 S rRNA.

pgant3$^{c01318revertant\#7}$) showed restoration of *pgant3* gene expression levels and wing integrity (Fig. 1*C* and Fig. 2*D*, and Table 1).

RNAi to pgant3 Causes Wing Blistering—To confirm that decreased levels of *pgant3* expression are responsible for the wing blistering observed, we constructed transgenic flies carrying Gal4-inducible *pgant3* IR constructs (UAS-*pgant3*IR) to specifically induce double-stranded RNA to *pgant3* and, thus, decrease *pgant3* expression via RNAi (Fig. 3). After crossing two independent UAS-*pgant3*IR transgenic lines to a tubulin-Gal4 driver line (which expresses Gal4 ubiquitously), we found that 95–97% of the progeny expressing *pgant3* double-stranded RNA displayed wing blistering (Table 1). Induction of *pgant3* RNAi exclusively in the wing disc by crossing UAS-*pgant3*IR lines to a wing-specific Gal4 driver line (MS1096-Gal4) resulted in 19–23% wing blistering (Table 1). Quantitative PCR verified that *pgant3* expression levels were specifically reduced in double-stranded RNA-expressing progeny, whereas expression of other *pgant* family members was unchanged (Fig. 3, *B* and *C*). Additionally, the degree of reduction in *pgant3* gene expression with each Gal4-driver correlated with the frequency of wing blistering observed. The tubulin-Gal4 driver resulted in a greater reduction in *pgant3* gene expression in wing discs relative to the MS1096-Gal4 driver and also gave a greater incidence of wing blistering (Table 1 and Fig. 3, *B* and *C*).

Wild Type pgant3 Expression Restores Wing Integrity but Expression of Another pgant Family Member Does Not—To demonstrate that *pgant3* expression is crucial for wing integrity, we constructed Gal4-inducible transgenic lines carrying wild type *pgant3* cDNA (UAS-*pgant3*) to perform rescue experiments. The presence of the UAS-*pgant3* expression construct in the homozygous *pgant3*^{c01318} transposon background resulted in significant reduction or elimination of wing blistering (Table 1 and Fig. 2*E*) even in the absence of a Gal4 driver. This was seen with multiple independent transgenic lines (Table 1). However, transgenic lines overexpressing another *pgant* family member under the control of the tubulin-Gal4 driver (*pgant35A*) (27, 36) failed to completely rescue wing blistering in the homozygous *pgant3*^{c01318} transposon background (Table 1). These results indicate that the restoration of wing integrity is specific to *pgant3* gene expression and cannot be achieved by overexpression of another family member. This suggests that PGANT3 is uniquely responsible for glycosylating a subset of proteins required for proper wing blade formation.

Overexpression of pgant3 Results in Lethality—While performing the aforementioned rescue studies, we noticed that progeny containing both UAS-*pgant3* and certain Gal4 drivers were not viable. This was seen with multiple independent UAS-*pgant3* transgenic lines (Table 2 and data not shown). Overexpression of wild type *pgant3* using the tubulin-Gal4 driver was lethal, with most animals dying during embryogenesis and larva stages (Table 2 and data not shown). UAS-*pgant3* expression under the control of the *armadillo* driver (*arm*-Gal4, whose expression level is less than that of Tub-Gal4) resulted in partial lethality, with 50% of the animals surviving to adulthood. Expression of *pgant3* under the control of a wing-specific driver had no effect on viability or wing blade formation (Table 2 and data not shown). These results indicate that although overex-

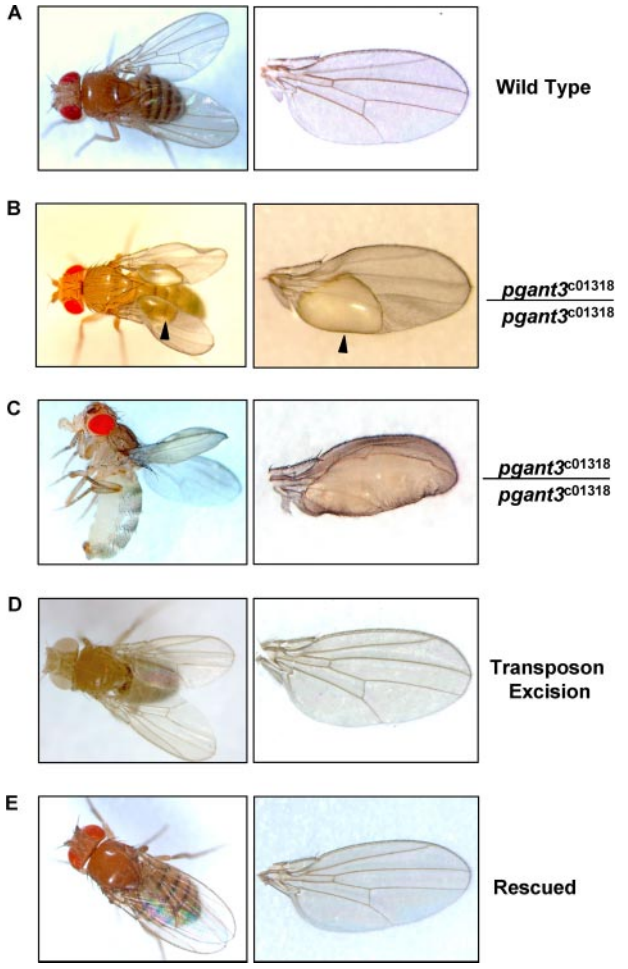

FIGURE 2. ***pgant3* transposon insertion results in wing blistering.** Wild type (*A*) *pgant3*^{c01318}/*pgant3*^{c01318} transposon insertion homozygotes (*B* and *C*), *pgant3*$^{c01318revertant\#7}$/*pgant3*$^{c01318revertant\#7}$ transposon excision homozygotes (*D*), and rescued homozygous mutants expressing a wild type *pgant3* transgene (*pgant3*^{c01318}/*pgant3*^{c01318}; *UAS-pgant3*) (*E*) are shown. Close up views of wings are shown in the *panels* on the *right*.

TABLE 1
Frequency of flies displaying blistered wings

Genotype	% Blistered[a]	*n*[b]
+/+	0	150
pgant3^{c01318}/+	0	114
pgant3^{c01318}/+ @ 18 °C	2	279
pgant3^{c01318}/*pgant3*^{c01318}	95	129
pgant3^{c01318}/*pgant3*^{c01318} @ 18 °C	99	147
pgant3^{c01318}/Df(2R)Exel6283, P$^{XP\text{-}U}$Exel6283	96	190
pgant3$^{c01318revertant\#7}$/*pgant3*$^{c01318revertant\#7}$	0	150
pgant3$^{c01318revertant\#7}$/*pgant3*^{c013180}	0	213
Tub>*pgant3*IR2#2	97	168
Tub>*pgant3*IR2#9	95	133
MS1096>*pgant3*IR2#2	23	126
MS1096>*pgant3*IR2#9	19	145
pgant3^{c01318}/*pgant3*^{c01318}; UAS-*pgant3*$^{\#3}$/+	0	173
pgant3^{c01318}/*pgant3*^{c01318}; UAS-*pgant3*$^{\#10}$/+	9	163
UAS-*pgant3*$^{\#7}$/Y;*pgant3*^{c01318}/*pgant3*^{c01318}	4	122
pgant3^{c01318}/*pgant3*^{c01318}; UAS-*pgant35A*$^{\#5}$/Tub-Gal4	83	153
pgant3^{c01318}/*pgant3*^{c01318}; Tub-Gal4/+	90	168
pgant3^{c01318}/*pgant3*^{c01318}; UAS-*pgant35A*$^{\#5}$/+	94	121
pgant3^{c01318}/*tig*^{X} @ 18 °C	8	115
pgant3^{c01318}/*tig*^{A1} @ 18 °C	6	91
tig^{X}/+ @ 18 °C	0	119
tig^{A1}/+ @ 18 °C	0	94

[a] (number of flies displaying blistered wings/total number of flies of denoted genotype) × 100.
[b] *n* = total number of flies of denoted genotype scored.

pression of *pgant3* is tolerated in the wing, specific regulation of *pgant3* levels in other tissues and stages is necessary for development to proceed properly. This may be the reason for the highly restricted pattern of expression seen for *pgant3* during embryonic development (27).

Altered O-Glycan Staining and Wing Disc Morphology is Seen in pgant3^{c01318}/*pgant3*^{c01318} *Wing Discs*—Wing discs from *pgant3*^{c01318} homozygotes were stained with an antibody (Tn Ab) that detects the Tn antigen, GalNAcα-S/T (44), to visualize alterations in protein *O*-glycosylation patterns in the mutant discs. Wing discs from *pgant3*^{c01318} homozygotes did not show global changes in *O*-glycosylation relative to wild type discs (Fig. 4, *A* and *B*). However, examination of optical cross-sections through the mutant wing discs revealed a decrease in *O*-glycoproteins specifically along the basal surface of the columnar epithelial cells (Fig. 4, *D* and *E*). The average height of *O*-glycan staining along the basal surface of *pgant3*^{c01318} homozygous mutant wing discs was 3-μm *versus* 7-μm for wild type discs (Fig. 4*G*); no significant decrease in *O*-glycans along the apical surface was observed. Additionally, mutant discs were decreased in thickness relative to wild type (Fig. 4*H*), with mutants having an average thickness of 30 μm, whereas wild type had an average of 44 μm. Furthermore, basal *O*-glycan staining and disc thickness were restored in mutant discs expressing wild type *pgant3* (Fig. 4, *F–H*), concomitant with restoration of adult wing blade integrity (Fig. 2*E*). These results demonstrate that decreased *pgant3* gene expression results in localized reductions in *O*-glycans along the basal region of wing discs followed by separation of epithelial cell layers in the adult wing, both of which can be rescued with wild type *pgant3* expression. Because the basal surface of larval wing discs will mediate contacts between epithelial cell layers in the adult wing, this implicates basal *O*-glycoproteins modified by PGANT3 in these cell adhesion events. Additionally, these studies provide evidence that reduced *O*-glycosylation results in altered columnar epithelial cell morphology, as mutant discs were significantly thinner than wild type discs.

PGANT3 Glycosylates ECM Proteins in Vitro—To identify potential substrates for PGANT3, we compiled a list of secreted and membrane-bound proteins expressed in wing discs that are predicted to have potential sites of *O*-glycosylation (Table 3) using the NetOGlyc server. This software employs a neural network prediction program based on confirmed sites of *O*-glycosylation in many diverse proteins. We then performed *in vitro* glycosylation reactions using recombinant PGANT3 and peptides derived from these proteins to determine which substrates PGANT3 is capable of glycosylating. As shown in Fig. 5*A*, PGANT3 transfers a substantial amount of GalNAc to tenM1, a specific peptide derived from the tenascin-major protein, and tigA, a peptide derived from the tiggrin protein. No glycosylation was seen for tenM2 or tigB, additional peptides derived from tenascin-major and tiggrin, respectively, suggesting that PGANT3 activity is specific to certain subregions within these proteins. A modest amount of glycosylation was also seen with thrombospondin. No significant transfer was seen to the other peptides tested in this assay, indicating that PGANT3 glycosylates specific regions of certain extracellular matrix proteins *in vitro*. To demonstrate specificity of the PGANT3 peptide

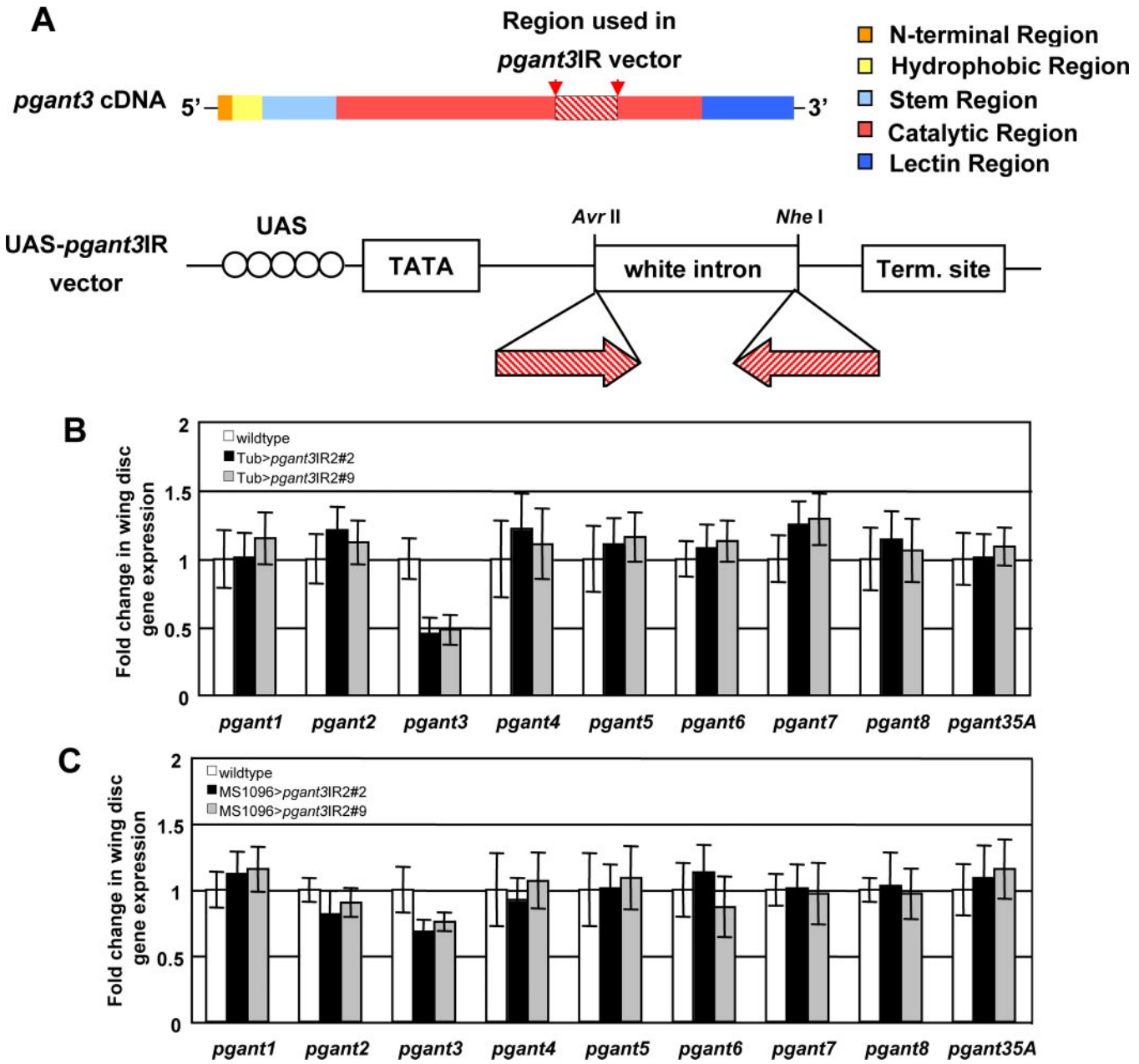

FIGURE 3. **RNAi to *pgant3* results in a specific decrease in *pgant3* gene expression and induces wing blisters *in vivo*.** *A*, the segment of the *pgant3* coding region used to construct the UAS-*pgant3*IR vector (below) is shown as a *diagonally striped box*. Functional elements of the *pgant3* coding region are shown in color. Quantitative PCR was employed to assess *pgant* family member gene expression in wing discs from wild type and two independent UAS-*pgant3*IR transgenic lines (UAS-*pgant3*IR2#2 and UAS-*pgant3*IR2#9) under the control of the tubulin-Gal4 driver (*B*) or the wing-specific MS1096-Gal4 driver (*C*). In both instances induction of *pgant3* double-stranded RNA resulted in a specific reduction in *pgant3* expression without significantly affecting the expression of other family members.

TABLE 2

Over-expression of *pgant3* results in lethality

UAS-*pgant3#3* and UAS-*pgant3#10* are independent transgenic lines generated as described under "Experimental Procedures."

Crosses	Progeny overexpressing *pgant3*[a]	Progeny without *pgant3* overexpression	Survival of *pgant3* overexpressing flies
			(%)
UAS-*pgant3*#3/UAS-*pgant3*#3 × *Tub*-Gal4/TM3, Sb^1	0	312	0
UAS-*pgant3*#10/UAS-*pgant3*#10 × *Tub*-Gal4/TM3, Sb^1	6	171	4
UAS-*pgant3*#3/UAS-*pgant3*#3 × *arm*-Gal4/TM3, Sb^1	126	213	50
UAS-*pgant3*#3/TM6C, Sb^1 × MS1096-Gal4/MS1096-Gal-4	214	211	100

[a] Assessed by the absence of Sb^1.

glycosylation observed, similar assays were performed using recombinant PGANT35A. Although PGANT35A was able to transfer GalNAc to tenM1, no glycosylation above background was seen for the other substrates tested (Fig. 5*B*). This *in vitro*

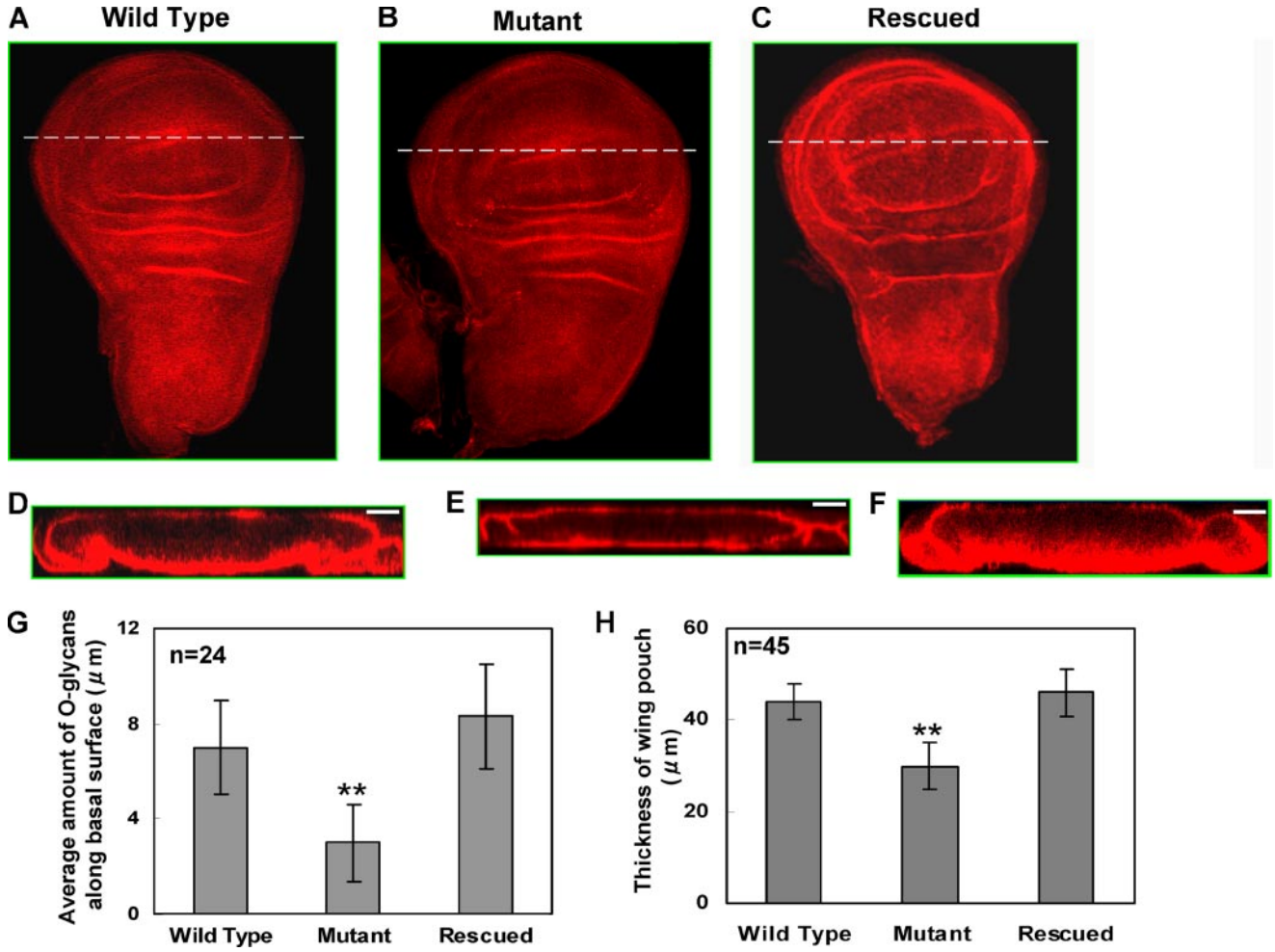

FIGURE 4. ***O*-Glycan-specific antibody staining in wing imaginal discs reveals decreased basal O-glycans and altered morphology in *pgant3*^{c01318} mutants.** Wing imaginal discs from wild type (*A* and *D*), *pgant3*^{c01318}/*pgant3*^{c01318} transposon insertion homozygotes (*Mutant*) (*B* and *E*), and transposon homozygotes expressing wild type *pgant3* (*pgant3*^{c01318}/*pgant3*^{c01318}; UAS-*pgant3*) (*Rescued*) (*C* and *F*) were stained with the Tn antibody and visualized by confocal microscopy. *D–F* represent optical X-Z cross-sections of each disc in the region of the *white line* shown in *A–C*. Anterior is to the *left*, and posterior is to the *right* in *A–C*. Apical is at the *top*, and basal is at the *bottom* in *D–F*. *G*, average amount of *O*-glycan staining seen in wild type, mutant, and rescued discs along the basal surface of the wing pouch (measured as described under "Experimental Procedures"). *H*, average thickness of the wing pouch in wild type, mutant, and rescued discs. n = total number of wing discs of each genotype analyzed. S.D. are shown. *Scale bar* = 20 μm. **, $p < 0.01$.

TABLE 3

Peptide substrates derived from proteins expressed in wing discs

The bold residues are sites of potential *O*-glycosylation based on predictions performed by the NetOGlyc server. NA, no evidence from references.

Peptide	Gene	Protein	Peptide sequence	Wing blisters in mutants
if	*if*	αPS2 integrin	EPQVNQTSF**TT**YSTSSSSSG	yes
lanA1	*LanA*	Laminin A	**T**LPP**TT**P**TTTTTTTTT**	yes
mew	*mew*	αPS1 integrin	VGFFKRIRP**T**DPTLSGNLE	yes
scab	*scb*	αPS3 integrin	VDPVEVTT**T**LSGGLERTV	yes
tigA	*tig*	Tiggrin	LEGE**T**ARPRPPNPAPIVS**T**PKP	yes
tigB	*tig*	Tiggrin	QQATKVEVEA**T**SEPSFWEKLK	yes
tenM1	*ten-m*	Tenascin-major	FLLEGVTP**T**APPDVPPRNPT	NA
tenM2	*ten-m*	Tenascin-major	TSNSGTAQGLQS**T**SASAEATSS	NA
tsp	*tsp*	Thrombospondin	IQIKLVNS**T**EGPGPMMRNS	NA

specificity is in accord with the *pgant3*-specific *in vivo* rescue of wing blistering, supporting a unique role for PGANT3 in the wing disc that cannot be compensated for by the activity of another glycosyltransferase family member. The *in vitro* data presented here suggests that tiggrin is one of the unique candidate targets for PGANT3.

Loss of Specific O-Glycoproteins Is Seen in pgant3^{c01318} Mutant Wing Discs—Protein extracts from wing discs of wild type, *pgant3*^{c01318} homozygotes, and transposon excision lines were run on SDS-PAGE gels, Western-blotted, and probed with the Tn Ab to detect changes in the presence of *O*-glycosylated proteins. No differences were seen between the majority of bands present in wild type and mutant lanes. However, glycoproteins of ~68 and ~200 kDa that were present in wild type and transposon excision samples were absent or severely reduced in staining intensity in *pgant3*^{c01318} mutant samples; additionally, there was a slight reduction in intensity within the high molecular weight region at the top of the gel in the

pgant3^{c01318} mutant sample (Fig. 6*A*). Western blots further demonstrated that glycosylation of the ~200-kDa band is restored in rescued *pgant3*^{c01318} mutant wing discs (expressing wild type *pgant3*) (Fig. 6*B*).

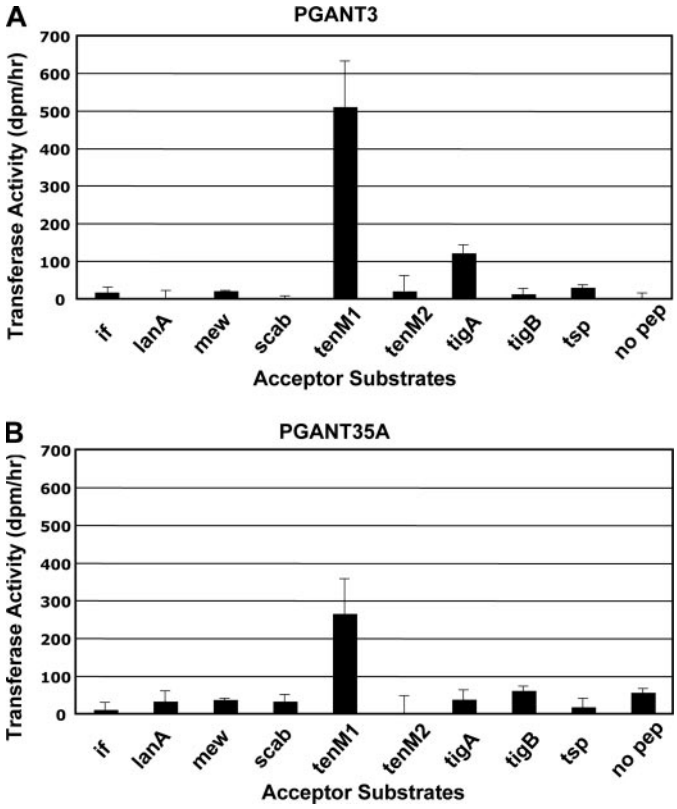

FIGURE 5. **Glycosylation of ECM peptides by PGANT3.** Peptides derived from secreted and membrane-bound proteins expressed in wing discs were incubated with recombinant PGANT3 (*A*) or PGANT35A (*B*) enzymes in *in vitro* glycosylation reactions, and transfer of labeled GalNAc was measured. Both PGANT3 and PGANT35A glycosylate the tenM1 peptide, but glycosylation of the tigA peptide was only observed with PGANT3. Rates of GalNAc incorporation (dpm/h) are shown on the *vertical axes*, and peptide substrates tested are shown on the *horizontal axes*. *Error bars* indicate S.D. Background values (*no pep* = no peptide acceptor present in the reaction) are shown in each graph.

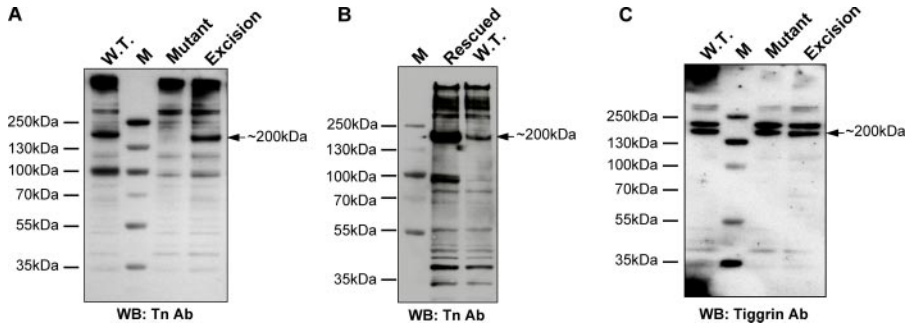

FIGURE 6. **Altered patterns of *O*-glycosylation of wing imaginal disc proteins from *pgant3* transposon insertion mutants.** *A*, protein extracts from the wing imaginal discs of wild type (*W.T.*), *pgant3*^{c01318}/*pgant3*^{c01318} transposon insertion homozygotes (*Mutant*), and *pgant3*$^{c01318revertant\#7}$/*pgant3*$^{c01318revertant\#7}$ transposon excision homozygotes (*Excision*) were subject to SDS-PAGE electrophoresis, blotted, and probed with the Tn antigen antibody (*Tn Ab*) to detect *O*-linked glycoproteins carrying the Tn antigen structure (GalNAcα-S/T). Loss or severe reduction in ~68- and ~200-kDa bands in the mutant sample relative to the wild type or transposon excision sample is seen. *WB*, Western blot. *B*, Western blot of protein extracts from wild type and transposon homozygotes expressing wild type *pgant3* (*pgant3*^{c01318}/*pgant3*^{c01318}; UAS-*pgant3*) (*Rescued*) wing discs probed with the Tn Ab. *C*, Western blot from *panel A* was probed with the tiggrin antibody. The position of the ~200-kDa band is denoted on the *right side* of each blot. Size markers (*lane M*) are shown to the *left* of each *panel*.

Based on our *in vitro* data and the size of the predominant band absent in the mutant discs (~200 kDa), we hypothesized that this band may be the tiggrin protein. Western blots probed with the tiggrin antibody revealed that the lower tiggrin band runs in the exact position of the ~200 kDa band detected with the Tn Ab (Fig. 6*C*), strongly suggesting that the differentially glycosylated band is tiggrin. Collectively, these results support the notion that *pgant3* is responsible for glycosylating specific proteins within the wing discs, one of which may be the ECM protein tiggrin.

Tiggrin Is an in Vivo Substrate for PGANT3—To conclusively address whether tiggrin represents the ~200-kDa *O*-glycosylated band that is lost in *pgant3*^{c01318} mutants, we performed immunoprecipitation experiments from wild type wing disc extracts using the tiggrin antibody (Fig. 7). Western blots of immunoprecipitated tiggrin blotted with the Tn Ab revealed that tiggrin is *O*-glycosylated in wild type wing discs and runs at the same position as the predominant ~200-kDa band seen on previous Western blots (Fig. 7*A*). Nonspecific bands detected by the secondary antibody are shown in Fig. 7*B*. No Tn Ab reactivity was seen in the immunoprecipitated sample from mutant wing discs (Fig. 7*A*), suggesting that tiggrin is either not *O*-glycosylated or is unstable/degraded. Although the data from Fig. 6 indicate that tiggrin from mutant discs is present but not glycosylated, we cannot rule out the possibility that the lack of *O*-glycans may also result in altered protein stability. Nonetheless, these results conclusively demonstrate that tiggrin is a *bona fide* substrate for PGANT3 *in vivo*.

Genetic Interactions between pgant3 and tiggrin Increase Wing Blistering—To demonstrate an *in vivo* interaction between *tiggrin* and *pgant3* in mediating the wing blistering phenotype, we performed genetic interaction experiments. Two distinct *tiggrin* mutants (*Tig*^{X} and *Tig*^{A1}) (14) showed no blistering heterozygously at 18 °C (Table 1); *pgant3*^{c01318} heterozygotes displayed 2% wing blistering at 18 °C (Table 1). However, transheterozygotes consisting of *pgant3*^{c01318} and *Tig*^{X} or *pgant3*^{c01318} and *Tig*^{A1} both resulted in an increased wing blistering frequency (Table 1). These genetic interactions were only seen at 18 °C. We did not observe genetic interactions between *pgant3* and other genes known to be involved in various aspects of wing blade adhesion (*if*, *by*, *bsk*, *wb*, *mys*, *mew*, *Ten-m*, *bs*) (data not shown). Thus, *pgant3* and *tiggrin* mutations, when combined, function to exacerbate the same morphological phenotype, further demonstrating that the glycosylation of tiggrin by PGANT3 has a functional consequence in modulating cell adhesive events in the adult wing.

DISCUSSION

Cell adhesion and the factors that regulate it are crucial during many diverse developmental stages. Here

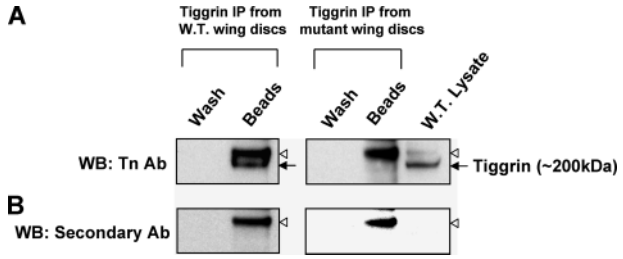

FIGURE 7. **Tiggrin is *O*-glycosylated by PGANT3 *in vivo*.** Immunoprecipitation (*IP*) of tiggrin from wild type and *pgant3*^{c01318}/*pgant3*^{c01318} mutants was performed as described under "Experimental Procedures." *A*, the nonspecific wash (*Wash*) and immunoprecipitated tiggrin (*Beads*) from each sample were subject to SDS-PAGE electrophoresis, blotted, and probed with the Tn Ab. Tiggrin from wild type discs is detected with the *O*-glycan specific antibody, but tiggrin from mutant discs shows no reactivity. Wild type wing disc lysate (*W.T. Lysate*) was included as a control for detection of *O*-glycosylated tiggrin. *B*, Western blots (*WB*) were reprobed with the secondary antibody alone to identify the nonspecific band present in each sample (denoted with a *white triangle* to the *right* of each blot). The position of the specific tiggrin band is denoted with a *black arrow*.

we describe for the first time the role of mucin-type protein *O*-glycosylation in cell adhesion during development. Mutations in the *pgant3* gene, which encodes an enzyme responsible for initiating protein *O*-glycosylation, resulted in aberrant adhesion between the cell layers comprising the wing blade. Epithelial cell adhesion in the *Drosophila* wing blade is regulated primarily by integrin-ECM interactions (2–5, 8, 14–15). However, a role for *O*-glycosylation of these proteins in modulating their cell adhesive functions during development has not been previously described. Our data elucidate a novel role for *O*-glycosylation in cell adhesion during wing development.

Here we show that a known integrin binding ECM protein, tiggrin, is specifically glycosylated by PGANT3 and that the activity of PGANT3 is functionally significant in mediating phenotypic consequences in the developing wing. PGANT3 glycosylates tiggrin both *in vitro* and *in vivo*; mutations in *pgant3* result in a loss of tiggrin glycosylation (accompanied by wing blistering), whereas expression of wild type *pgant3* restores tiggrin glycosylation and wing integrity. Additionally, genetic interaction experiments reveal that the combination of *pgant3* and *tiggrin* mutations resulted in an increased blistering frequency, demonstrating that these genes are acting in the same phenotypic pathway. Prior studies have shown that tiggrin binds integrin (9) and is involved in integrin-mediated cell adhesive events during *Drosophila* development, including muscle-tendon cell adhesion and wing blade adhesion (14). Indeed, the wing blistering seen in *tiggrin* mutants (along the posterior portion of the wing blade) (14) is similar to what was often observed in *pgant3* mutants. Given this, we propose that *O*-glycans on tiggrin mediate some aspect of integrin-ECM adhesion in the wing blade. Our Western blots demonstrate that the tiggrin protein is present in *pgant3* mutants, suggesting that the loss of *O*-glycans does not result in significant tiggrin degradation. However, *O*-glycans on tiggrin may influence more subtle aspects of protein stability as well as transport, secretion, localization, or binding interactions. Indeed, prior work from our laboratory suggests that *O*-glycosylation by another member of this glycosyltransferase family is involved in protein transport during *Drosophila* development (36). We are currently investigating the specific mechanistic function of *O*-glycans on tiggrin.

To address the developmental origin of the wing defect in *pgant3* mutants, we examined developing wing imaginal discs. Previous studies have shown that defects in JNK signaling related to integrin function in the developing wing can result in wing blistering (11). However, we did not detect changes in JNK phosphorylation or downstream signaling in *pgant3* mutants (data not shown). Detailed morphological examination of mutant wing discs revealed that although global patterns of glycosylation were unchanged, *pgant3* mutants displayed a specific reduction in *O*-glycans along the basal surface of the columnar epithelial cells. Additionally, basal surface glycosylation was restored in mutants expressing wild type *pgant3*, as was wing integrity and tiggrin glycosylation. These regional alterations in glycosylation are significant, as integrins and ECM proteins present along the basal surface mediate the adhesive contacts that will form as the disc everts and opposing basal regions come in contact with one another (2). Thus, we hypothesize that the wing blisters that form in the adult are most likely the result of an initial reduction in *O*-glycosylation of proteins normally found along the basal surface of larval wing discs.

Reduced basal glycosylation in larval wing discs was also accompanied by a reduction in the height of the columnar cells comprising the wing disc. Again, expression of wild type *pgant3* restored glycosylation and columnar cell height. It has been shown previously that alterations in integrin-ECM interactions can affect cell size and shape in the wing disc (46). We hypothesize that the morphological changes seen in the *pgant3* mutants are the result of alterations in integrin-ECM interactions influencing cytoskeletal architecture and cell shape.

Although tiggrin appears to be the major *O*-glycosylated protein affected in *pgant3* mutants, our *in vitro* and Western blot data suggest that PGANT3 may glycosylate other proteins in addition to tiggrin. *In vitro* biochemical assays revealed that other wing disc ECM proteins, such as tenascin-major (~300 kDa) and thrombospondin (~120 kDa), are glycosylated by PGANT3. No significant glycosylation was seen for the integrin peptides tested. Tenascin-major was also glycosylated by another PGANT family member (PGANT35A), suggesting that its glycosylation is not PGANT3-specific. Western blots further revealed an additional glycosylated band (~68 kDa) that is decreased in *pgant3* mutants. These results suggest that PGANT3 may glycosylate a subset of basal ECM proteins (including tiggrin) in the developing wing disc. The aforementioned ECM proteins are also known to specifically interact with αPS2 integrin to mediate cell adhesion in a number of developmental contexts (9, 10, 13, 14). We postulate that glycosylation of ECM proteins in addition to tiggrin could also play a role in the wing blade adhesion. We are currently trying to identify the ~68-kDa band.

Our studies indicate that *pgant3* is uniquely responsible for glycosylating certain proteins such as tiggrin, which are required for proper wing development and that the activity of another family member is not compensatory. This unique requirement for *pgant3* in the developing wing disc is demonstrated by *in vivo* rescue experiments where wild type *pgant3* can restore wing integrity, but overexpression of another

related family member (*pgant35A*, which is normally expressed in the wing disc and is also an initiating peptide transferase) cannot. This result is likely due to isoform-specific substrate preferences that were also observed *in vitro*. These data highlight the significance of substrate preferences seen for PGANT family members *in vitro* and illustrate the functional consequences of this specificity *in vivo*.

The notion of unique roles for *pgant3* during development is further supported by the observation that overexpression of *pgant3* is lethal, whereas *pgant35A* overexpression has no obvious effect. Although we currently do not know the specific nature of this lethality (most *pgant3* overexpressing animals died during embryonic and larval stages), these results suggest that control of PGANT3 enzymatic levels during development is critical. Indeed, we know from previous *in situ* experiments that *pgant3* gene expression is specifically regulated during embryonic development, being found in a very restricted subset of tissues, including the wing disc (27). The unique substrate preferences of PGANT3 may necessitate very strict spatial and temporal regulation of enzymatic activity during development. The role of *pgant3* in other tissues and stages is currently under investigation.

In summary, we have identified a novel role for mucin-type *O*-glycosylation during development. Here, we present evidence for the requirement of *O*-glycans in cell adhesion processes during development. Previous work has demonstrated the role of *O*- and *N*-linked carbohydrates and sugar-binding proteins in cell adhesion mediating lymphocyte homing and extravasation within the mammalian immune system (34, 47–49). The presence of mucin type *O*-glycans on secreted and membrane-bound proteins across many species suggests a more widespread role for *O*-glycans in cell adhesive events in many developing organ systems. Building upon the studies presented here, it will be interesting to investigate the role of *O*-glycans in other developmental contexts.

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