A UDP-GalNAc:Polypeptide N-Acetylgalactosaminyltransferase Is Required for Epithelial Tube Formation*

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Epithelial tubes are essential for the proper function of a diverse array of eukaryotic organs. Here we present a novel class of genes required for maintaining epithelial cell shape, polarity, and paracellular barrier function in the Drosophila embryonic tracheal system. Mutations in one member of the UDP-GalNAc:polypeptide N-acetylgalactosaminyltransferase family (pgant35A) are recessive lethal and result in tracheal tubes that are irregular in diameter and morphology. Further analysis of the pgant35A mutants reveals diminished levels of the apical determinant Crbs and the luminal marker 2A12, concomitant with increased staining in cytoplasmic vesicles within tracheal cells. GalNAc-containing glycoproteins are severely diminished along the apical region of the tracheal system as well. Tracheal cells become irregular in size and shape, and septate junction proteins are mislocalized to a more apical position. Most notably, paracellular barrier function is lost in the tracheal system of the mutants. Overexpression of wild type pgant35A under control of the trachea-specific breathless (btl) promoter results in partial rescue of the lethality. We propose a model where pgant35A is required to establish proper apical composition of tracheal cells by influencing apical delivery of proteins/glycoproteins. Disruption of the normal apical content results in altered cell morphology and loss of paracellular barrier function. These studies demonstrate a previously unrecognized requirement for mucin-type O-glycosylation in epithelial tube integrity and have obvious implications for epithelial morphogenesis in higher eukaryotes, since a unique ortholog to pgant35A exists in mammals.

Epithelial tubes are crucial components of higher eukaryotic organs, such as the kidney, lung, salivary gland, and vascular system. Tube length and diameter control are critical, since tubes of irregular size or shape may be unable to transport fluids or gases efficiently and may lack the proper surface to volume ratio necessary for appropriate exchange of materials (1, 2). Additionally, the proper establishment of cell junctions to form a diffusion barrier across the tube surface is required to prevent loss of materials and inappropriate introduction of others from the surrounding environment.

The Drosophila tracheal system has served as a model system for epithelial tube formation and has aided in elucidating factors important for tube morphogenesis in mammalian systems (1–4). Through studies in the fly, genes that regulate cell polarity, tube diameter and length, and paracellular barrier formation have been identified (1, 5, 6). Genes encoding proteins such as Crumbs (Crbs) are necessary for establishing the apical regions of epithelial cells and coordinating formation of the zona adherens (ZA)2 in the subapical region, which then influences the position of other lateral components (7–12). Crbs also interacts with components of the apical membrane cytoskeleton (13), which is known to influence cell shape and epithelial morphogenesis (14, 15). Along the lateral surfaces, septate junctions are functionally analogous to vertebrate tight junctions and are responsible for establishing the transepithelial diffusion barrier of the tracheal tube (2, 16–19). Additionally, septate junctions influence cell shape and tube size, since some septate junction proteins have been found to limit the extent of apical cell surface formation (16, 20), and others severely alter tracheal tube morphology (2, 19, 21). All of these studies point to an incredibly complex interplay of many components to ensure proper cell polarity, morphology, and epithelial barrier formation.

Although many genes controlling tracheal tube morphogenesis have been identified, many more factors involved in these processes are still unknown. Here, we provide the first demonstration that mucin-type protein O-glycosylation is required for proper tracheal tube formation and function. The synthesis of mucin-type O-glycans is initiated by the UDP-GalNAc:polypeptide N-acetylgalactosaminyltransferase family (ppGalNAcTs or ppGnTases in mammals and pgants in Drosophila) (EC 2.4.1.41). This enzyme family is evolutionarily conserved and catalyzes the transfer of the sugar GalNAc onto thehydroxyl group of serine or threonine residues of secreted and membrane-bound proteins, laying the foundation for the formation of more complex sugar structures that can be further elaborated (Fig. 1, A and B) (reviewed in Refs. 22 and 23). Previous studies have shown that one member of this family is essential for viability in Drosophila (pgant35A) (24, 25). However, the developmental processes and cellular defects involved in the lethality were unknown. Surprisingly, we find that mucin-type O-glycans are abundant on the apical surface and

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

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lumen of tracheal tubes and are required for proper epithelial cell morphology, polarity, and paracellular barrier formation within the developing *Drosophila* tracheal system. This study will aid in elucidating the role of this posttranslational modification in mammalian systems, since a distinct mammalian ortholog to *pgant35A* exists and is expressed in tissues dependent upon epithelial function.

**EXPERIMENTAL PROCEDURES**

*Drosophila* Crosses—The mutants used in this study were described previously (24) and are as follows: *pgant35A*<sup>HG8</sup>, *pgant35A*<sup>SF32</sup>, and *pgant35A*<sup>3772</sup> (Fig. 1C). Mutants were balanced over CyO or CyO, P<sup>[w<sup>+</sup> = ActGFPI]MR1 as described previously (24). Crosses between heterozygotes carrying different allelic mutations and crosses to obtain germ line clones (GLCs) of *pgant35A*<sup>HG8</sup> and *pgant35A*<sup>SF32</sup> are described in the supplemental data.

The pUAST plasmid (26) was used to generate Gal4-inducible constructs that were then used to create transgenic flies. The complete coding region from the wild type *pgant35A* cDNA was removed from the plasmid, FlyH-2a, on Apal (blunt)/NotI ends and cloned into the EcoRI (blunt)/NotI sites of pUAST. Transformants were produced by Genetic Services Inc. (Cambridge, MA) using a methodology based on the procedure described previously (27, 28). Rescue experiments were performed using transgenic flies (w; P<sup>[UAS-pgant35A]<sup>5</sup>/P<sup>2</sup>UAS-pgant35A<sup>5</sup>)) with insertions on the third chromosome and the following stocks: w; btl-Gal4/TM3, Ser (29) (kind gift of D. Andrew); Bloomington stock #5138 (y<sup>1</sup>, w<sup>*</sup>; P<sup>tubP-GAL4</sup>LL7/TM6C, cu, Sb, e, ca males. The same crossing scheme was used with the *btl*-Gal4 driver line and P<sup>[UAS-pgant35A]</sup> transgenic lines heterozygous for *pgant35A*<sup>mutant</sup>/CyO. These heterozygous lines were then crossed as follows, and rescue of the *pgant35A* lethality was assessed by the presence of straight winged, Sb<sup>+</sup> progeny: *pgant35A*<sup>mutant</sup>/CyO; P<sup>[UAS-pgant35A]<sup>5</sup>/P<sup>2</sup>[UAS-pgant35A]<sup>5</sup>) females × *pgant35A*<sup>mutant</sup>/CyO; P<sup>[tubP-GAL4]<sup>LL7/TM6C, cu, Sb, e, ca males. The same crossing scheme was used with the btl-Gal4 driver line. The total population of stage 16–17 embryos from the *btl*-Gal4 crosses was stained with 2A12, and the ratio of those with straight (“normal”) to curved (“abnormal”) dorsal longitudinal tracheal trunks was calculated. Rescue of the *pgant35A* lethality by *btl*-driven *pgant35A* expression was assessed by the presence of straight winged, Sb<sup>+</sup> adult progeny.

*Whole Mount Antibody Staining and Lectin Staining*—Embryos homozygous for *pgant35A* mutations were selected by lack of GFP fluorescence and fixed as previously described (30). Immunostaining was according to standard procedures. The following antibodies from the Developmental Studies Hybrids Bank were used at the indicated concentrations: 2A12 (1:5) (31), anti-Crumbs (Cq4) (1:100), anti-Dlg (4F3) (1:50), anti-DE-cadherin (DCAD2) (1:25), anti-Armadillo (N2 7A1) (1:100), and anti-α-spectrin (3A9) (1:25). Mouse monoclonal anti-Tn antibody (Ca3638) (32) (1:50) was a kind gift of Dr. Richard D. Cummings, who had acquired the stocks of antibodies and hybridomas from the late Dr. Georg F. Springer. The rabbit anti-Sinuous serum (1:500) was a generous gift of G. Beitel; heat-fixed embryos were used for Sinuous staining (19). All secondary antibodies (Biotin-, fluorescein isothiocyanate-, and Cy3-conjugated) were purchased from Jackson ImmunoResearch Laboratories and used at concentrations of 1:2000 (Biotin-conjugated) and 1:100 (fluorescence-conjugated). The fluorescence-conjugated lectins, *Artocarpus integrifolia* (Jacalin), *Arachis hypogaea* (peanut agglutinin), *Vicia villosa* agglutinin, and *Triticum vulgare* (wheat germ agglutinin) were purchased from EY Laboratories. Alexa Fluor 568-conjugated *Glycine maximus* (soybean agglutinin) was purchased from Molecular Probes. All lectins were used at 10 μg/ml. Fluorescein-conjugated chitin-binding probe was purchased from BioLabs and used at a 1:500 dilution. Stained embryos were staged according to Hartenstein (33) and analyzed with the Zeiss LSM 510 confocal laser-scanning microscope. Images were processed using the LSM Image Browser and Photoshop.

*Whole Mount in Situ Hybridization*—Probe synthesis and whole mount RNA in situ hybridizations were performed as described previously (30), except proteinase K (Sigma; 3 μg/ml) was used to penetrate embryos for 2 min at room temperature and 1 h on ice. Primary antibody against the protein of interest was added along with the anti-digoxigenin antibody. Fluorochrome-conjugated secondary antibody was used to detect the primary antibody, and horseradish peroxidase-conjugated digoxigenin was amplified by tyramide signal amplification (PerkinElmer Life Sciences) (1:50). Stained embryos were analyzed by confocal microscopy as described above.

*Transepithelial Barrier Dye Permeability Assay*—Alexa Fluor 568-conjugated 10-kDa dextran (Molecular Probes) was injected into the posterior cavity of stage 16–17 dechorionated embryos as described previously (17). The embryos were examined by confocal microscopy.

**RESULTS**

*pgant35A Is Required during Early Pupation and Embryogenesis*—To examine the stage at which homozygous *pgant35A* mutants die, we crossed two heterozygous strains containing distinct *pgant35A* allelic mutations. Two of these previously characterized mutants (*pgant35A*<sup>HG8</sup> and *pgant35A*<sup>3772</sup>) contain point mutations in the coding region of *pgant35A*, resulting in stop codons that delete all or most of the catalytic region and render the enzymes inactive (Fig. 1C). The third mutant (*pgant35A*<sup>SF32</sup>) contains a missense mutation, changing a conserved arginine to a tryptophan and severely diminishing enzymatic activity (Fig. 1C) (24). Each *pgant35A* allele was balanced over a wild type chromosome carrying the green fluorescent protein (GFP) under the control of the actin promoter (Act5C-GFP), allowing us to identify homozygous mutant *pgant35A* progeny by lack of GFP fluorescence. Results from three crosses using the previously characterized *pgant35A* mutants demonstrated that substantial portions of *pgant35A*/ *pgant35A* homozygous mutants arrest during embryogenesis (13–27%) and larval development (27–36%) (Table 1). Those homozygous mutants that continue to pupation never proceed past pupal stage P4ii (when the head sac is everted), with most arresting during prepupal stages P1–P4 (when histolysis is
O-Glycosylation in Epithelial Tube Formation

occurring, prior to imaginal leg and wing disc eversion). These results indicate that pgant35A is required to complete pupation and also plays a role during embryonic and larval development.

Expression of Wild Type pgant35A Rescues Lethality—To conclusively demonstrate that mutations in the pgant35A gene are responsible for the lethality observed, we constructed transgenic flies containing a wild type pgant35A cDNA under the control of the UAS promoter (UAS-pgant35A transgenic line) and crossed these with the tubulin promoter-Gal4 driver transgenic line in the mutant backgrounds. As mentioned earlier, homozygous mutants derived from the heterozygous cross die throughout development but never proceed past pupation. Expression of wild type pgant35A cDNA under the control of the tubulin-Gal4 driver (which is expressed ubiquitously) resulted in rescue of the lethality in 86% of the homozygous mutant progeny. Rescued progeny were viable and fertile. Additionally, the expected number of wild type heterozygous progeny expressing the tubulin-Gal4-driven pgant35A cDNA were also seen, indicating that overexpression of pgant35A did not have any obvious effect on viability or fertility. These experiments conclusively demonstrate that the pgant35A gene is responsible for the lethality observed.

pgant35A Germ Line Clones Arrest during Embryogenesis and Have Tracheal Defects—Recent work by our group has shown that pgant35A has very strong maternal RNA expression in the embryo (30). To examine when the product of the pgant35A gene is first required during development, we generated pgant35A mutant germ line clones (GLCs) (which contain no wild type maternal pgant35A transcripts in the oocyte). Homozygous mutant pgant35A GLCs were crossed to heterozygotes containing a different pgant35A mutant allele over a wild type chromosome carrying Act5C-GFP. For each allelic combination, most homozygous maternal and zygotic (m/z) pgant35A mutants arrested during embryogenesis (88%); only 12% hatched to larvae (Table 2). Of those larvae, none proceeded to pupation. Some progeny that received a wild type pgant35A allele from their fathers were able to

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**TABLE 1**

Percentage survival of progeny from pgant35A heterozygous mutant crosses

| Genotype | Embryos hatching to larvae | Larvae that pupate | Pupae that eclose |
|----------|----------------------------|--------------------|-------------------|
| w; pgant35A<sup>1608</sup>/pgant35A<sup>16775</sup> | 73% (n = 176) | 73% (n = 218) | 0% (n = 161) |
| w; pgant35A<sup>1608</sup>/pgant35A<sup>16775</sup> | 80% (n = 134) | 64% (n = 239) | 0% (n = 155) |

**TABLE 2**

Percentage survival of progeny from pgant35A mutant germ line clone crosses

| Genotype | Embryos hatching to larvae | Larvae that pupate | Pupae that eclose |
|----------|----------------------------|--------------------|-------------------|
| w; pgant35A<sup>1608</sup>/pgant35A<sup>16775</sup> | 12% (n = 570) | 0% (n = 110) | NA* |
| w; pgant35A<sup>1608</sup>/pgant35A<sup>16775</sup> | 45% (n = 397) | 17% (n = 176) | 93% (n = 30) |
| w; pgant35A<sup>1608</sup>/pgant35A<sup>16775</sup> | 12% (n = 405) | 0% (n = 107) | NA |
| w; pgant35A<sup>1608</sup>/pgant35A<sup>16775</sup> | 76% (n = 269) | 77% (n = 205) | 96% (n = 157) |

*NA, not applicable. n = total number of progeny scored for each genotype at each stage.
complete embryogenesis (76% for pgant35A\textsuperscript{SF32/3775} GLC crosses and 45% for pgant35A\textsuperscript{H10/3775} GLC crosses), pupate (77% for pgant35A\textsuperscript{SF32/3775} GLC crosses and 17% for pgant35A\textsuperscript{H10/3775} GLC crosses), and continue to adulthood, indicating that zygotic 2A12 protein expression is required during embryogenesis.

pgant35A homozygous m/z mutant progeny have highly irregular tracheal systems, as seen when staining for the trachea-specific luminal marker, 2A12 (Fig. 2). Most mutants display normal tracheal development until stage 13–15 (Fig. 2, G–I) but acquire obvious tracheal defects by stages 15–17 (Fig. 2, A–F). In particular, the dorsal longitudinal trunca is convoluted and follows a circuitous path (Fig. 2, B, C, E, and F). Additionally, it is highly irregular in diameter, with many regions being much wider or narrower than in wild type embryos. Secondary and tertiary branches show irregularities in branch formation and diameter as well (Fig. 2, B, C, E, and F). Staining with the 2A12 marker is reduced relative to wild type embryos and variable over the length of the tracheal tubes. In general, the severity of the tracheal phenotypes correlated with the severity of the mutant allele (Figs. 1 and 2). Additionally, the percentage of embryos with affected tracheal tubes was also greater in the pgant35A\textsuperscript{H10/3775} GLC crosses (86% of pgant35A\textsuperscript{H10/3775} embryos affected, n = 98) than in the pgant35A\textsuperscript{SF32/3775} GLC crosses (72% of pgant35A\textsuperscript{SF32/3775} embryos affected, n = 83).

RNA in situ hybridization showed pgant35A expression in the dorsal longitudinal tracheal trunks and tracheal transverse connectives at stages 15–17 (Fig. 2, J–M). In situ hybridization combined with immunostaining for the luminal 2A12 protein demonstrates that pgant35A expression is confined to the tracheal cells at stage 12 (30). The time of pgant35A expression (stage 15) corresponds to a period of intense apical secretion in the developing tracheal system and correlates well with the time of onset of the tracheal defects observed in pgant35A m/z mutants.

pgant35A Mutants Have Altered Tracheal Cell Morphology—To further investigate the stage at which tracheal defects are first detected, we stained stage 15 embryos for 2A12 and the apical determinant, Crbs. In pgant35A m/z homozygous mutants, decreased levels of 2A12 are seen in the tracheal lumen, with a corresponding increase in 2A12 staining within the cytoplasm of the tracheal cells relative to wild type (Fig. 3, A–A’). Additionally, apical Crbs staining in pgant35A mutants is also slightly reduced, with increased Crbs present in cytoplasmic puncta within the tracheal cells (Fig. 3, B–B’). These results suggest that trafficking of secreted and membrane-bound proteins, such as 2A12 and Crbs, is compromised in pgant35A mutants, with increased amounts of protein being retained in cytoplasmic vesicles.

By embryonic stage 17, when tracheal tube diameter expansion is normally occurring, the tracheal tubes of the pgant35A m/z mutants display the irregular diameter and morphology first detected with the 2A12 staining. Stage 17 embryos stained with the cytoskeletal protein, \(\alpha\)-spectrin (34), reveal irregular cell shape and size along the length of the tracheal tube; cells no longer display the evenly spaced, regular appearance but are either much larger or smaller than wild type and highly irregular in shape (Fig. 3, C–C’), thus contributing to the irregulari-
levels are reduced along the apical region of the cells relative to wild type (WT) (A). Similar results are seen with Crbs staining at stage 15 in WT (B), pgant35A SF32/3775 (B'), and pgant35A HG6/3775 (B'') m/z mutants. At stage 17, α-spectrin (α-spec) cytoskeletal staining is reduced apically and reveals altered cell shape in pgant35A m/z mutants (C' and C''). Relative to wild type trachea (C). In wild type, the dorsal longitudinal tracheal trunk shows the regular alignment of tracheal cells (arrowhead in C), but pgant35A mutants show abnormal tracheal cell shape and size (arrowheads and asterisk in C' and C''). Apical Crbs staining at stage 17 is greatly reduced in pgant35A m/z mutants (D' and D'') relative to wild type (D). Zona adherens formation in the embryonic trachea, as visualized with DE-cadherin (DE-cad), is incomplete in pgant35A m/z mutants (C' and C'') relative to wild type (C'). The septate junction protein, Sinuous (Sinu), is mislocalized in the pgant35A m/z mutant trachea (F' and F'') relative to wild type (F), since the mutants show reduced lateral localization and increased apical distribution (arrows in F--F''). The septate junction protein, Discs large (Dlg), also has reduced lateral staining in pgant35A m/z mutants relative to wild type (G'--G''). Panels E--G' are co-stained with the luminal marker 2A12. All tracheal sections are oriented similarly, with the lumen extending left to right across the center of each panel. Scale bar, 10 μm.

FIGURE 3. pgant35A mutations affect cell shape, polarity, and septate junction localization. Tracheal staining with the luminal marker 2A12 (green) at stage 15 in pgant35A SF32/3775 (A') and pgant35A HG6/3775 (A'') m/z mutants reveals decreased luminal staining and increased cytoplasmic staining relative to wild type (WT) (A). Similar results are seen with Crbs staining at stage 15 in WT (B), pgant35A SF32/3775 (B'), and pgant35A HG6/3775 (B'') m/z mutants. At stage 17, α-spectrin (α-spec) cytoskeletal staining is reduced apically and reveals altered cell shape in pgant35A m/z mutants (C' and C'') relative to wild type trachea (C). In wild type, the dorsal longitudinal tracheal trunk shows the regular alignment of tracheal cells (arrowhead in C), but pgant35A mutants show abnormal tracheal cell shape and size (arrowheads and asterisk in C' and C''). Apical Crbs staining at stage 17 is greatly reduced in pgant35A m/z mutants (D' and D'') relative to wild type (D). Zona adherens formation in the embryonic trachea, as visualized with DE-cadherin (DE-cad), is incomplete in pgant35A m/z mutants (C' and C'') relative to wild type (C'). The septate junctions protein, Sinuous (Sinu), is mislocalized in the pgant35A m/z mutant trachea (F' and F'') relative to wild type (F), since the mutants show reduced lateral localization and increased apical distribution (arrows in F--F''). The septate junction protein, Discs large (Dlg), also has reduced lateral staining in pgant35A m/z mutants relative to wild type (G'--G''). Panels E--G' are co-stained with the luminal marker 2A12. All tracheal sections are oriented similarly, with the lumen extending left to right across the center of each panel. Scale bar, 10 μm.

mutants across the entire length of the dorsal tracheal trunk in stage 16–17 embryos (Fig. 3, F–F''), suggesting alterations in the polarity of these cells at this stage. Because Crbs is known to regulate adherens junction formation (9, 11), we next examined the adherens junction protein, DE-cadherin in pgant35A m/z mutants. DE-cadherin is normally found in an even band around wild type tracheal cells in the sub-apical region (Fig. 3E). In pgant35A mutants, DE-cadherin is present but does not form a continuous band around the tracheal cells, indicating a discontinuous ZA in the mutant tracheal cells (Fig. 3, E' and E''). Similar results were obtained with another ZA marker, Armadillo (supplemental data). Taken together, these results suggest that loss of pgant35A results in reduction of apical and luminal components and subsequent alterations in cell shape and ZA formation at stages 16–17. These changes are likely the result of altered intracellular trafficking observed at stage 15, where mutants had increased cytoplasmic retention of apical and luminal proteins.

The tracheal phenotypes seen for the pgant35A mutants are highly reminiscent of the diameter and length irregularities seen in some septate junction mutants and thus prompted us to examine the expression and localization of two septate junction components, Sinuous (19) and Discs large (Dlg) (16). Septate junctions are unique paracellular structures that maintain a permeability barrier in epithelial tubes and represent the functional equivalent of mammalian tight junctions (2, 4). In wild type embryos, the septate junction proteins are normally localized to the lateral membrane of polarized epithelial cells that comprise the trachea (Fig. 3, F and G). In the pgant35A mutants, however, Sinu and Dlg are mislocalized, being reduced in the lateral regions and increased along the apical surface (Fig. 3, F--F'' and G'--G''). Again, as expected, the mislocalization of septate junction components appears more severe in the pgant35A HG6 GLC cross than in the pgant35A SF32 GLC cross. These data demonstrate that mutations in pgant35A result in alterations in cell shape and polarity as well as mislocalization of septate junction proteins in the embryonic tracheal system.
Diminished O-Glycosylation within the Trachea of pgant35A Mutants—To examine the effects of the pgant35A mutation on the glycans composition in the developing tracheal system, we used a variety of lectins to stain wild type and homozygous pgant35A m/z mutants. The lectins, Jacalin (which detects GlcNAcβ1-3GalNAcα-S/T or Galβ1-3GalNAcα-S/T) (35), peanut agglutinin (which detects Galβ1-3GalNAcα-S/T), soybean agglutinin (which detects GalNAcα-S/T), and V. villosa agglutinin (which detects GalNAcα-S/T) were used to detect a number of different core structures present on mucin-type O-glycans. Additionally, the anti-Tn-antigen antibody (Tn Ab) was also used to detect unmodified Tn antigen (GalNAcα-S/T). Intense staining along the apical surface and lumen of wild type tracheal tubes was detected with soybean agglutinin, V. villosa agglutinin, and the Tn Ab (Fig. 4, A–D) (data not shown), suggesting that the primary O-glycans present in the tracheal system are GalNAcα-S/T in structure and concentrated along the apical region and lumen of the tracheal tubes. Indeed, Tn Ab staining co-localized with Crbs along the apical surface of the tracheal tube (Fig. 4, A” and B”). During stage 15, co-localization of Tn Ab and Crbs was seen along the apical cell surface and in portions of the lumen (Fig. 4, A–A”). At stage 17, co-localization was primarily confined to the apical surface; additional Tn Ab staining that did not co-localize with Crbs was seen in the lumen (Fig. 4, B–B”). No tracheal staining was seen using peanut agglutinin, and only very weak staining was detected with Jacalin, suggesting that core 1, core 3 and sialyl-T Ag are not major components of the embryonic tracheal system. Tn Ab apical and luminal staining was present but slightly reduced in pgant35A mutants at stage 15 (Fig. 4, C–C”) and substantially reduced by stage 17 relative to wild type (Fig. 4, D–D”). Tn Ab staining seen at stage 15 is probably due to the activity of the other family member (pgant2) that is expressed during tracheal development (30). We also examined Tn Ab staining in other tissues of ectodermal origin (hindgut and salivary glands) in pgant35A mutants; apical and luminal staining was reduced in these tissues as well but to a lesser extent than that seen in the tracheal system (Fig. 4, E–F’). Additionally, these tissues (hindgut and salivary glands) maintained normal morphology and staining patterns.
O-Glycosylation in Epithelial Tube Formation

with the markers mentioned previously (Crbs, DE-cadherin, α-spectrin, Sinu, and Dlg) (Fig. 4, E–F’ and supplemental data). Therefore, lectin and antibody staining reveals specific decreases in the amount of GalNAcα-S/T glycans on the apical surface of the tracheal cells of pgant35A mutants concomitant with decreases in apical protein localization, alterations in cell shape, and mislocalization of septate junction proteins. No differences were detected with the chitin-binding protein (which detects the filamentous GlcNAc polymeric chitin cable present in the tracheal lumen) or wheat germ agglutinin (which detects both N-glycans and chitin) (Fig. 4, G–H’), indicating that the tracheal phenotypes are not associated with alterations in other glycans but are specific to mucin-type O-glycans of the GalNAcα-S/T structure.

Paracellular Barrier Formation Is Disrupted in pgant35A Mutants—To assess the effect of pgant35A mutations on the epithelial diffusion barrier function of the tracheal tubes, mutant embryos were injected with 10-kDa dextran dye. If the cells of the tracheal system maintain a proper diffusion barrier, no dye will be found within the tracheal tubes (Fig. 5A, wild type (WT)). However, in the majority of homozygous pgant35A mutants, dye was found within the dorsal tracheal tubes within 30 min, indicating that septate junction function and paracellular diffusion barrier formation is disrupted (Fig. 5, B and C). Additionally, epithelial barrier disruption is specific to the tracheal system, since dye did not readily diffuse into the salivary glands (data not shown).

Tracheal Expression of pgant35A Results in Partial Rescue—To assess the importance of pgant35A expression in the trachea and its contribution to the lethality observed in homozygous mutants, we also expressed the UAS-pgant35A wild type cDNA under the control of the trachea-specific breathless-Gal4 driver (btl-Gal4). Homozygous mutants from heterozygous crosses also display convoluted tracheal trunks, although the phenotype is less severe than that seen in the GLC progeny. In heterozygous crosses containing the btl-Gal4 driver and the UAS-pgant35A transgene, we saw a reduction in the percentage of total progeny displaying curved, irregular shaped dorsal longitudinal tracheae relative to progeny from heterozygous crosses without the UAS-pgant35A transgene present (Table 3). Additionally, we were able to rescue a limited percentage of homozygous mutant animals to adulthood (7%) with btl-driven expression of the wild type pgant35A transgene. However, all homozygous mutant adults died during or shortly after eclosion, indicating that although pgant35A expression in the tracheal system will allow some animals to proceed to adulthood, additional expression elsewhere may be necessary to restore complete viability. The low rescue frequency may also result from differences in the temporal and spatial expression of the btl-driver and endogenous pgant35A in the developing tracheal system (30, 36).

DISCUSSION

Here we present a new class of genes involved in proper epithelial tube formation and function. In this study, we demonstrate a crucial role for mucin-type protein O-glycosylation in regulating epithelial cell...
O-Glycosylation in Epithelial Tube Formation

shape, polarity, and transepithelial barrier formation using the *Drosophila* embryonic tracheal system as a model. Although different roles for *O*-glycans on proteins have been proposed, unambiguous confirmation of their biological import has proven to be very difficult; specific inhibitors of the many unique members (≥14 in mammals and ≥9 in flies) of this enzyme family do not exist, and deletion of individual isoforms in mice has proven uninformative, due to functional redundancy within this multigene family. Additionally, identification and confirmation of *O*-glycosylated substrates remains a continuing challenge, since there is no reliable way to predict or detect the wide diversity of *O*-glycans synthesized *in vivo*. In contrast to N-linked glycosylation, there is no known consensus sequence for *O*-GalNAc addition, no single reagent that will specifically detect all classes of mucin-type *O*-glycans, and no endoglycosidase that will remove all O-linked chains regardless of their length or structure. These experimental limitations have hampered efforts to discern the biological role of *O*-glycosylation. Using a model organism and tissue with less enzymatic redundancy, the work presented here is the first example of the requirement for mucin-type *O*-glycans in a specific developmental process. This study represents a substantial advance in an experimentally difficult area of glycobiology.

Although previous work demonstrated that *pgant35A* is required for viability in *Drosophila*, its specific developmental roles were unknown. The results presented here suggest that *pgant35A* is required for efficient trafficking of apical and luminal proteins during the later stages (stages 15–17) of embryonic tracheal development. *pgant35A* mutants showed reduced apical Crbs and luminal 2A12 staining with increased punctate staining seen in the cytoplasm of the tracheal cells, suggesting slower/less efficient trafficking of proteins out of the secretory apparatus at stage 15. By stage 17, apical Crbs levels were greatly reduced relative to wild type, concomitant with a reduction in the apical *O*-glycans normally present in the developing trachea. Our results demonstrate that loss of *pgant35A* results in a severe reduction in apical and luminal proteins, such as Crbs and 2A12. Alterations in the apical composition of the tracheal tube likely contribute to the subsequent changes observed in cell size, shape, and ZA formation, since Crbs is known to interact with the apical cytoskeleton (13) as well as establish epithelial polarity (7–10). Additionally, Crbs and other apical proteins establish an apical boundary that limits the position of lateral components, such as the septate junction proteins. Without the proper apical composition, the septate junction proteins may now be able to move to more apical positions, thereby compromising the diffusion barrier normally formed along the lateral surface (Fig. 5D).

The tracheal phenotypes seen here are reminiscent of those seen with the loss of certain septate junction components. Like mutations in septate junction proteins, *pgant35A* mutations resulted in a defective diffusion barrier across the tracheal tube. Furthermore, the tracheal tube shape is altered in much the same way as is seen for mutations in the claudin transmembrane proteins, Megatrachea and Sinuous (18, 19), as well as the cell adhesion protein, Lachesin (21). Given the phenotypic similarities, it is possible that these membrane-bound proteins may be substrates of PGANT35A, requiring *O*-glycans for proper function. However, we did not detect significant *O*-glycan staining along the lateral surfaces of wild type tracheal cells, supporting the possibility that septate junction formation and function may be regulated indirectly, through the *O*-glycosylation of apical and luminal proteins that influence lateral cell boundaries. One obvious candidate substrate is the Crbs protein itself, since it displayed considerable co-localization with Tn Ab staining along the apical surface. Crbs is a transmembrane protein containing EGF (epidermal growth factor) repeats with many potential sites of *O*-glycosylation. We are currently investigating whether septate junction proteins, Crbs, or proteins with which Crbs interacts are *O*-glycosylated.

*O*-Glycans were also detected in the lumen of the trachea and were lost in the *pgant35A* m/z mutants. It remains possible that *O*-glycoproteins in the lumen may also influence trachea formation by contributing to the luminal matrix. Recent work has demonstrated the role of chitin and chitin-modifying enzymes in formation of the luminal matrix that coordinates proper tracheal tube expansion (37–40). However, while chitin matrix mutants display irregularly shaped tracheal tubes, they still maintain intact transepithelial barriers, in contrast to the *pgant35A* mutants. This indicates that although *pgant35A* may contribute to the luminal matrix, it plays an additional role specific to paracellular barrier formation.

The epithelial tube shape, diameter, and cell polarity changes seen in the *pgant35A* mutants were limited to the tracheal system. When we examined the hindgut and the salivary glands, only slightly reduced glycosylation was seen, and Crbs, DE-cadherin, α-spectrin, Sinu, and Dilg staining were normal. This result is probably due to the functional redundancy of the 10 and six isoforms expressed in the hindgut and salivary glands, respectively (30). In contrast, only two isoforms (*pgant35A* and *pgant2*) are expressed in the trachea. Whether the role of *O*-glycosylation in hindgut and salivary gland tissues is similar to that seen in the tracheal system remains to be determined. These experiments will require ablation of multiple isoforms to overcome the compensatory activity of the family members expressed there.

Previous studies have suggested a role for *O*-glycans in intracellular trafficking of apical proteins and receptors (41–45). One screen identified mutations in two mucin genes (encoding proteins that are typically heavily *O*-glycosylated) that result in a significant decrease in glutamate receptor expression/localization (41). Work on another mucin, Muc4, has demonstrated its role in ErbB2 and ErbB3 receptor trafficking to the cell surface (45). In other studies, regions crucial for apical sorting of certain proteins have been shown to contain domains rich in *O*-glycosylated serines and threonines (41). Treatment of cells with a competitive inhibitor of *O*-glycan elongation resulted in reduced apical trafficking of proteins, concomitant with increased accumulation within cytoplasmic vesicles (42). However, the specific role of *O*-glycans in apical sorting was not definitive, since the compound used also interferes with *N*-glycan and glycolipid synthesis. The studies presented here, examining a specific *O*-glycosyltransferase mutant, provide further support for the role of *O*-glycans in intracellular trafficking of apical proteins. Current efforts are focused on identifying the
complement of embryonically expressed proteins containing O-linked GalNAc.

Altogether, our studies demonstrate a novel role for a member of the pgant family in the proper formation of the embryonic tracheal system in Drosophila. Our results have notable implications for the role of O-glycosylation in various aspects of chordate development that involve cell polarity and epithelial tube formation (kidney, lung, salivary gland, and vasculature development), since this gene family has been conserved throughout evolution (22, 25, 46, 47). Additionally, the mammalian ortholog of pgant35A (pGalNTase-T11) is most highly expressed in the mouse lung and kidney, two organs whose function is dependent on intact epithelial tubes (48). Indeed, our results are reminiscent of the circuitous, irregular microvasculature seen in the B1,3-galactosyltransferase-deficient mice (49). This enzyme modifies the O-linked GalNAc on glycoproteins through the addition of galactose in a B1,3 linkage, forming the core 1 structure. These mice died during embryogenesis from brain hemorrhages and exhibited distorted endothelial cell shape and irregular capillary lumens. In light of our studies, these phenotypes may be the result of aberrations in apicobasal polarity and tight junction formation within capillaries that fail to express appropriate O-glycoproteins necessary for the transport of apical proteins. These results, taken in combination with ours, suggest a previously unrecognized role for O-glycans in epithelial tube formation in diverse organs and species. Future studies will be aimed at identifying the substrates of this enzyme in Drosophila as well as characterizing the role of its ortholog in mammalian development.

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