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Research Report

O-Glycosylation of a male seminal fluid protein influences sperm binding and female postmating behavior

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Abstract

Glycoproteins are abundant within the human reproductive system and alterations in glycosylation lead to reproductive disorders, suggesting that glycans play an important role in reproductive function. In this study, we used the *Drosophila* reproductive system as a model to investigate the biological functions of O-glycosylation. We found that O-glycosylation in the male accessory glands, an organ responsible for secreting seminal fluid proteins, plays important roles in female postmating behavior. The loss of one O-glycosyltransferase, PGANT9, in the male reproductive system resulted in decreased egg production in mated females. We identified one substrate of PGANT9, lectin-46Ca (CG1656), which is known to affect female postmating responses. We further show that the loss of lectin-46Ca O-glycosylation affects its ability to associate with sperm tails, resulting in reduced transfer within the female reproductive system. Our results provide the first example that O-glycosylation of a seminal fluid protein affects its ability to associate with sperm in vivo. These studies may shed light on the biological function of O-glycans in mammalian reproduction.

Keywords: lectin, mucin, O-glycosylation, *Drosophila*, reproduction

Significance Statement

Glycosylated proteins are abundant within the reproductive systems of many species, including mammals, and are thought to play roles in reproductive fitness. However, the details of how glycosylation affects reproductive fitness remain largely unknown. In this study, we show that the glycosylation of one seminal fluid protein affects its ability to associate with sperm. This disruption in sperm association inhibits movement through the female reproductive tract, resulting in decreased egg laying. Understanding the factors that influence reproductive fitness may provide novel insights into the causes of infertility and potential strategies to address it.

Introduction

Glycosylation is an evolutionarily conserved protein modification that impacts all aspects of cell and organismal biology, from early stages of development to organ protection and function. The two primary forms of glycosylation present on membrane bound and secreted proteins are N-linked and mucin-type O-linked glycosylation. N-linked glycosylation occurs co-translationally when glycans are added en bloc to asparagine, and subsequently modified as the substrate protein moves through the secretory apparatus. O-linked glycosylation involves the posttranslational addition of a single *N*-acetylgalactosamine (GalNAc) onto serine and threonine residues through the action of a large evolutionarily conserved family of enzymes known as the UDP-GalNAc:polypeptide *N*-acetylgalactosaminyltransferases (GALNTs in humans and PGANTs in *Drosophila*) (1–4). This GalNAc can then be extended by the action of other glycosyltransferases in the secretory apparatus (3). Both forms of glycosylation are essential and play diverse roles in protein stability, folding, structure, and function.

Glycans are abundantly present throughout the male and female reproductive tracts, and their roles in mammalian reproduction have been extensively investigated (5, 6). The thick glycocalyx of sperm as well as glycoproteins within the seminal fluid are believed to influence sperm development and maturation; movement through the female reproductive tract; protection from the female immune response; and binding and fusion with eggs (7–13). The mammalian uterus is also enriched in glycoproteins, which are involved in interacting with sperm and protection against pathogens (5, 14). Glycoproteins within the zona pellucida, a glycan-rich layer surrounding the egg, have also been implicated in controlling sperm–egg binding (5). Furthermore, defects in glycosylation have been associated with infertility (12, 15, 16). However, the details of where glycans are present and how they affect specific reproductive processes remain poorly understood. *Drosophila melanogaster* has been used to dissect the details of glycosylation in many areas of development and organ function, given its extensive genetic tools, and conserved genes and cellular processes (3, 4, 17, 18). Indeed, the family of glycosyltransferases responsible for initiating O-glycosylation is highly conserved from mammals to flies, both at the sequence and enzymatic levels (3, 4). *Drosophila* has also

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been extensively used to study the complexities of reproductive biology and diseases of reproductive organs, including prostate and ovarian cancers (19, 20). The male accessory glands (MAGs) of *Drosophila* are active in producing and secreting seminal fluid proteins, similar to the function of the human prostate gland (20).

Here, we use *D. melanogaster* to investigate the role of O-linked glycosylation in reproductive biology. In our study, we find that O-linked glycans and members of the *pgant* gene family are abundantly expressed in the male reproductive system, particularly in the secretory cells of the MAG. Loss of one specific glycosyltransferase, *pgant9*, in the secretory cells of the MAG altered the O-glycosylation of a lectin (CG1656 or lectin-46Ca) known to influence female egg production after mating. We find that O-glycosylation of this lectin is required for its ability to bind sperm within the female uterus, thereby affecting its transfer to the female sperm storage organ. Loss of *pgant9* resulted in decreased glycosylation of lectin-46Ca, loss of sperm binding, loss of transfer to the female sperm storage organ, and decreased female egg production. Our studies provide important information about the roles of O-glycans and the associated glycosyltransferases in reproductive biology.

Results

pgant9 is the predominant glycosyltransferase family member expressed in the secondary cells of the MAG

The *Drosophila* adult male reproductive system is composed of one pair of testes, one pair of MAGs, an ejaculatory duct (ED) and an ejaculatory bulb (EB) (21) (Fig. 1A). Staining with the lectin *Helix pomatia* agglutinin (HPA), which detects GalNAc linked to the hydroxyl group of serine or threonine (Fig. 1B), reveals abundant staining within the secretory vesicles of the MAG and along the epithelium of the ED and EB (Fig. 1C and D). Thus, O-linked glycosylation is present in the MAG, ED, and EB of the male reproductive system.

We next investigated the expression of the UDP-GalNAc:polypeptide *N*-acetyl galactosaminyltransferase (*pgant*) genes that encode the glycosyltransferases that add GalNAc to serine/threonine. qPCR revealed that *pgant4*, *pgant5*, and *pgant9* are the most abundantly expressed family members in the MAG, an organ responsible for secreting a variety of seminal fluid peptides and proteins that are transferred to the female upon mating, affecting fertility, and postmating behavior (22) (Fig. 1E). *pgant9* is known to have two splice variants (*pgant9A* and *pgant9B*) that are differentially expressed in certain tissues (23); both are expressed in the MAG, with *pgant9A* being higher than *pgant9B* (Fig. 1E). We next performed immunostaining to determine the cells in which each gene product is expressed. The MAG is composed of two types of secretory cells, main cells, and secondary cells (Fig. 1F). The spherical secondary cells located at the distal tip of the MAG are filled with large secretory granules containing seminal fluid peptides and proteins that stain abundantly for O-glycosylation (24) (Fig. 1D and F). As shown in Figs. 1G–I and S1, PGANT4 is expressed predominantly in the main cells, PGANT5 has limited expression in the secondary cells, and PGANT9 is abundantly expressed in both main and secondary cells.

Loss of PGANT9 in the male reproductive tract affects female reproductive fitness

To investigate the function of O-glycosylation in the male reproductive system, we performed RNA interference (RNAi) on the most abundant member of the *pgant* family, *pgant9* (both *pgant9A* and *pgant9B*; Fig. 1E), which is expressed within all cells of the MAG. RNAi was performed in all cells of the MAG using a Gal4 driver (MAG-Gal4) that is expressed in both the main and secondary cells, as shown by green fluorescent protein (GFP) expression (MAG-Gal4 > UAS-GFP; Fig. 2A) or by using a Gal4 driver that expresses only within the secondary cells of the MAG (SC-Gal4 > UAS-GFP), as shown in Fig. 2B. The MAG-Gal4 driver efficiently and specifically decreased expression of *pgant9* ($pgant9^{RNAi>MAG}$; Fig. 2C). To discern the effects of the loss of *pgant9* in male reproductive fitness, we mated *WT* females with *WT* males, $pgant9^{RNAi>MAG}$ males, or $pgant9^{RNAi>SC}$ males and counted the number of eggs laid postmating for 10 days (Fig. 2D). Interestingly, loss of *pgant9* throughout the MAG or only in the secondary cells of males resulted in a significant decrease in egg production by mated females relative to females mated with *WT* males (Fig. 2D). These results suggest that PGANT9 glycosylation within secondary cells of the MAG affects male reproductive fitness by influencing egg production in females.

PGANT9 glycosylates a lectin within the male reproductive system

To determine how the loss of *pgant9* affects female egg production, we next examined MAG extracts from *WT*, $pgant9^{RNAi>MAG}$, and $pgant9^{RNAi>SC}$ males to identify differentially glycosylated proteins. Western blots probed with lectins that specifically recognize O-linked GalNAc (HPA) showed reduced glycosylation of three bands in $pgant9^{RNAi>MAG}$ MAG extracts when compared with *WT* (Figs. 3A and S2A). RNAi to *pgant9* only in the secondary cells ($pgant9^{RNAi>SC}$) resulted in a reduction of two HPA bands that were similar in size to those seen upon RNAi to *pgant9* throughout the MAG (Figs. 3B and S2B). This suggests that two proteins (~55 and ~35 kDa) are glycosylated by PGANT9 in secondary cells.

Based on the size of one protein (~55 kDa), we hypothesized that it may be the lectin-46Ca (CG1656), a seminal fluid protein known to have a role in reproductive fitness (25–27). Indeed, westerns of $pgant9^{RNAi>MAG}$, $pgant9^{RNAi>SC}$, or *WT* MAG extracts probed with an antibody to lectin-46Ca show that it overlaps with the HPA band in *WT* and runs at a lower molecular weight with less HPA reactivity (suggesting loss of O-glycans) upon loss of *pgant9* (Fig. 3A and B). CG1656 or lectin-46Ca is produced in the secondary cells of the MAG and has been shown to bind sperm and affect long-term female postmating responses, including egg production (26, 27). To further explore whether other seminal fluid proteins are also O-glycosylated and investigate the identity of the ~35 kDa protein, we performed additional westerns using HPA and antibodies to other seminal fluid proteins (28) (Fig. S3). Only lectin-46Ca and CG14560 (~15 kDa protein) overlapped with HPA staining, suggesting that other known seminal fluid proteins are not modified with GalNAc (Fig. S3). Further attempts to determine the identity of the 35 kDa band were unsuccessful.

To test the ability of PGANT9 to glycosylate the lectin-46Ca, we next co-expressed it with *pgant9A* or *pgant9B* in *Drosophila* $S2R^+$ cells. As shown in Fig. 3C, expression of *lectin-46Ca* with either *pgant9A* or *pgant9B* resulted in lectin-46Ca becoming HPA reactive and increasing in molecular weight, suggesting that both PGANT9A and PGANT9B isoforms are capable of glycosylating it. Next, we purified the lectin-46Ca and performed mass spectrometry to verify glycosylation. Two O-glycosylated residues were identified at Thr264 and Thr295 in lectin-46Ca when PGANT9A and PGANT9B were co-expressed (Figs. 3D, E, S2C, and S4).

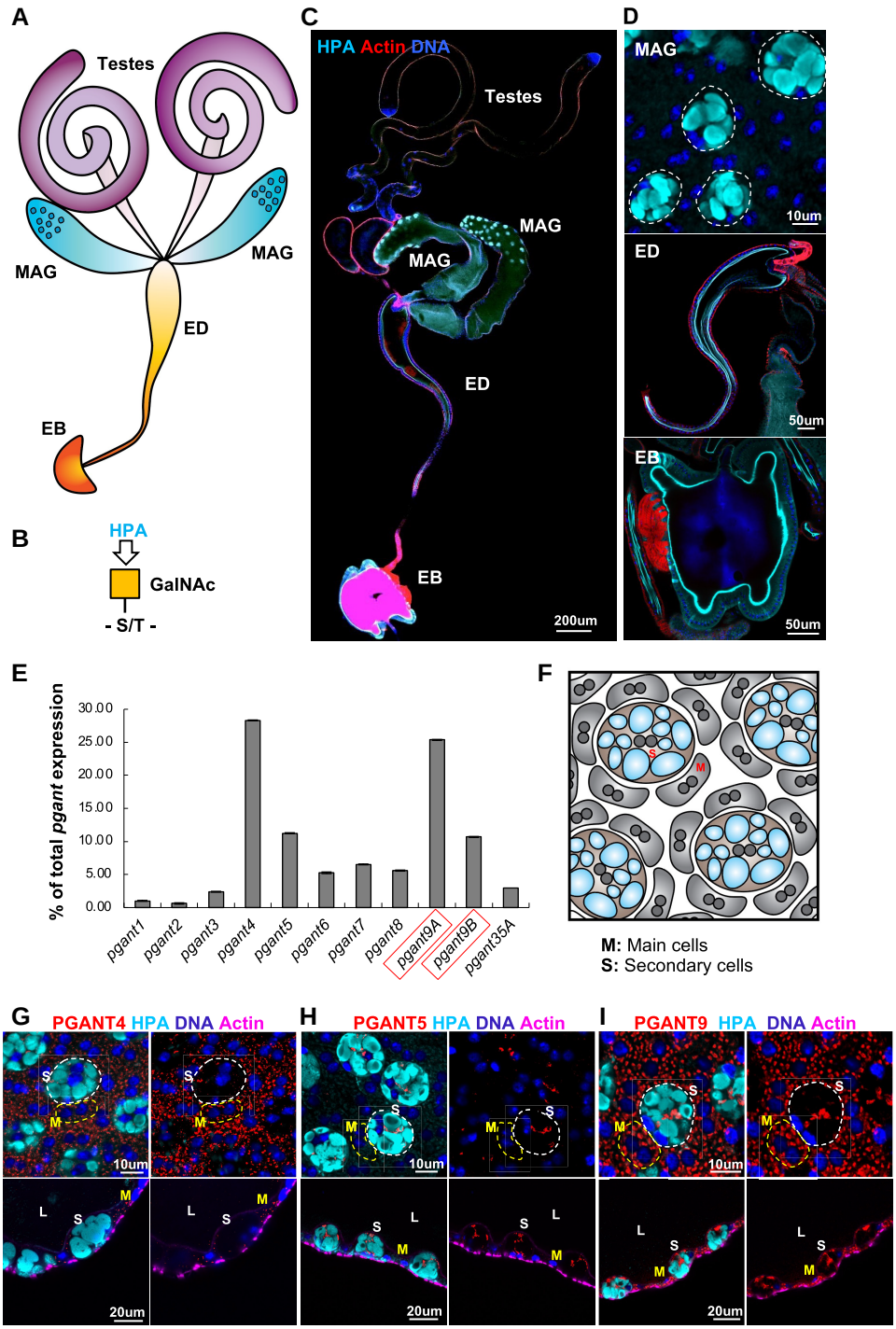


Fig. 1. O-glycans and *pgant*s are abundantly expressed in the *Drosophila* adult male reproductive system. A) Schematic of the male reproductive system, including testes, MAGs, ED, and EB. B) The lectin HPA specifically binds to GalNAc linked to serine or threonine residues. C) HPA staining (cyan) in the whole male reproductive system. Scale bar, 200 µm. The actin (red) and nuclear counterstaining (dark blue) are shown. Strong HPA staining is detected in the MAG, ED, and EB, while weak HPA staining is present in the testes. D) Magnified images of HPA staining in the MAG, ED, and EB are shown. Abundant HPA staining is present in the secretory vesicles in the secondary cells of MAG (top panel). Scale bar, 10 µm. Continuous HPA-reactive membranes along the epithelia in the ED and EB are present (middle and bottom panels). Scale bars, 50 µm. E) Real-time qPCR data reveal that *pgant*9A, *pgant*9B, *pgant*4, and *pgant*5 are highly expressed in the MAG. The percentage of total *pgant* expression is shown for each *pgant* family member. F) Two types of secretory cells exist in the MAG: main cells (M) and secondary cells (S). Secondary cells are shown with large HPA-reactive secretory vesicles (light blue). Immunostaining with PGANT4 (G), PGANT5 (H), and PGANT9 (I) antibodies (red) in the MAG. HPA staining of secondary cells is shown in cyan. Actin staining (pink) and DNA (dark blue) are also shown. M, main cells; L, lumen; S, secondary cells. Scale bars, 10 µm (top panels) or 20 µm (bottom panels).

Additionally, another glycosylation site at Thr312 was identified in the absence of PGANT9A or PGANT9B expression, suggesting this site is glycosylated by another PGANT family member. These data may explain why O-glycosylation of lectin-46Ca is greatly decreased but not completely lost upon RNAi to *pgant*9 (Fig. 3A and B).

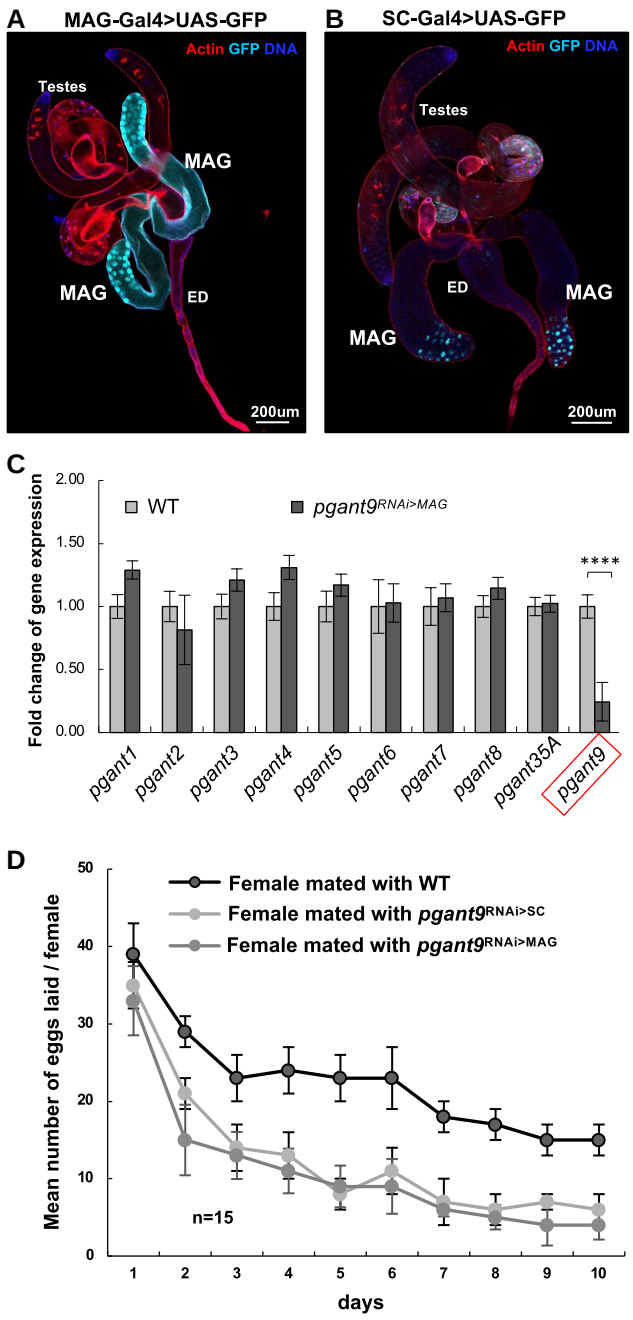


Fig. 2. RNAi to *pgant9* in the male reproductive system affects reproductive fitness. Expression patterns of Gal4 driver lines used to perform RNAi to *pgant9* in the male reproductive system were verified using a UAS-GFP reporter. A) MAG-Gal4 drives UAS-GFP expression (cyan) in both main and secondary cells of MAG. B) SC-Gal4 drives UAS-GFP expression in secondary cells only. Actin staining (red) and DNA (dark blue) are also shown. Scale bar, 200 µm. C) qPCR showed a specific reduction in *pgant9* expression in the MAG of $pgant9^{RNAi>MAG}$ relative to *WT*. ****$P < 0.0001$. D) The egg production of 15 *WT* females ($n = 15$) mated with $pgant9^{RNAi>MAG}$ or $pgant9^{RNAi>SC}$ males was decreased compared with *WT* females mated with *WT* males. Egg production from each female was quantitated every 24 h for 10 days after mating. Significance on day 1, $P < 0.05$; significance on days 2–10, $P < 0.0001$).

O-glycosylation influences binding to sperm and lectin transfer to the female seminal receptacle

As mentioned earlier, lectin-46Ca is a seminal fluid protein that is known to affect female long-term postmating responses, including egg production (25–27). Upon transfer to the female uterus during mating, lectin-46Ca associates with the sperm tail and is then transferred with the sperm to the seminal receptacle, the organ responsible for sperm storage in the female after mating (26, 27, 29). Lectin-46Ca is required for the transfer of sex peptide to the seminal receptacle, where sex peptide influences female postmating behavior (25, 27). Loss of lectin-46Ca results in loss of sex peptide in the seminal receptacle, resulting in a decrease in egg production by the mated female (25, 27). Given that the loss of *pgant9* results in decreased egg production, this suggests that O-glycosylation affects some aspects of lectin-46Ca stability, transfer, and/or function. To investigate this, we crossed *WT* or $pgant9^{RNAi>SC}$ males with *WT* females and assessed whether lectin-46Ca is transferred to each portion of the female reproductive tract (Fig. 4A). Protein extracts from female uteri or seminal receptacles (from females mated with *WT* males, $pgant9^{RNAi>SC}$ males, or not mated) were western blotted and probed with antibodies to lectin-46Ca. As shown in Fig. 4B, lectin-46Ca is present in the uteri of females mated to both *WT* or $pgant9^{RNAi>SC}$ males, indicating that the loss of O-glycosylation does not result in its degradation or prohibit its initial transfer to females. Interestingly, females mated to $pgant9^{RNAi>SC}$ males had more lectin-46Ca present in the uterus when compared with females mated to *WT* males (Fig. 4B). However, the opposite was observed when examining the female seminal receptacle—females mated to $pgant9^{RNAi>SC}$ males had less lectin-46Ca present when compared with females mated to *WT* males (Fig. 4C). These results suggest that the loss of O-glycosylation of lectin-46Ca does not result in its degradation but rather affects its ability to progress through the female reproductive tract.

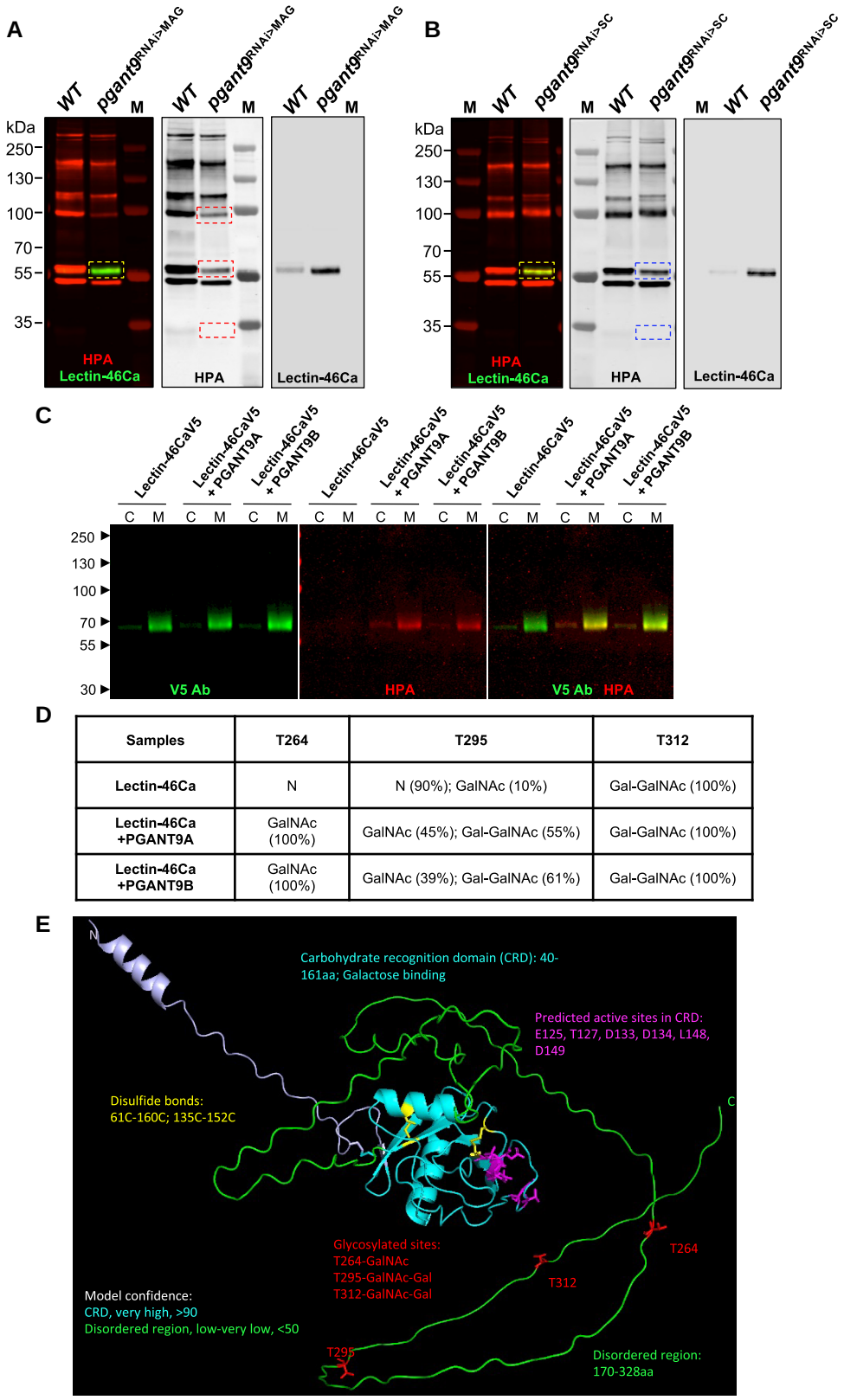
To examine how lectin-46Ca may be inhibited from progressing through the female reproductive tract, we next examined its ability to associate with sperm. Matings were performed as described above, and sperm from the seminal receptacles and uteri were collected and stained for lectin-46Ca. As shown in Fig. 4D and E, lectin-46Ca is abundantly present on the tails of sperm from *WT* males. However, lectin-46Ca from $pgant9^{RNAi>SC}$ males is no longer able to bind sperm (Fig. 4D and E). These results indicate that the loss of O-glycosylation decreases the ability of lectin-46Ca to bind sperm in the uterus, leading to decreased transfer to the seminal receptacle, where it normally influences egg production (Fig. 4F).

Examination of the sites of PGANT9 glycosylation reveals that they lie in a disordered region of the protein away from the predicted carbohydrate recognition domain (CRD; Fig. 3E). Given that the loss of *pgant9* does not appear to affect the stability of lectin-46Ca, we propose that the glycans may be directly recognized by other proteins or they may alter the structure of this region to allow recognition of regions vicinal to the sites of glycosylation. As associations of the seminal fluid proteins are transient and specific to the environment of the female reproductive tract, elucidating the exact binding interactions mediated by these glycans will be a future area of investigation.

Taken together, we propose a model where O-glycosylation of a seminal fluid protein enables sperm binding, which then mediates transfer throughout the female reproductive tract to influence female postmating behavior. This is the first example of O-glycosylation influencing seminal fluid peptide function in postmating behavior.

Discussion

Seminal fluid proteins exist across many species, including mammals, and are known to play roles in sperm viability, function,



Samples	T264	T295	T312
Lectin-46Ca	N	N (90%); GalNAc (10%)	Gal-GalNAc (100%)
Lectin-46Ca +PGANT9A	GalNAc (100%)	GalNAc (45%); Gal-GalNAc (55%)	Gal-GalNAc (100%)
Lectin-46Ca +PGANT9B	GalNAc (100%)	GalNAc (39%); Gal-GalNAc (61%)	Gal-GalNAc (100%)

Fig. 3. Lectin-46Ca (CG1656) is O-glycosylated by PGANT9A and 9B. Western blots of MAG extracts from *pgant9*$^{RNAi>MAG}$ (A) or *pgant9*$^{RNAi>SC}$ (B) probed with the HPA lectin (red) and the antibody to lectin-46Ca (CG1656) (green). Compared with WT, the O-glycosylation of some proteins highlighted in red boxes in A or blue boxes in B is altered in RNAi glands. The lectin-46Ca antibody-reactive band (yellow box in A and B) overlaps with one HPA-reactive band, and migrates lower with a decreased HPA-reactive signal (indicative of reduced O-glycosylation) in *pgant9* RNAi glands. M, marker lane. Protein size markers are shown to the left of each blot. C) Western blots of proteins from cells (C) and media (M) expressing lectin-46CaV5 alone or lectin-46CaV5 with PGANT9A or PGANT9B probed with HPA lectin (red) and the antibody to V5 (green). The O-glycosylation of lectin-46Ca is dramatically increased when co-expressing with PGANT9A and PGANT9B. D) O-glycosylation modifications (as determined by mass spectrometry) at T264, T295, and T312 in lectin-46Ca purified from the media are summarized. *N*, no modification found. E) The AlphaFold2 predicted structure of lectin-46Ca is shown. The CRD (cyan) and identified sites glycosylated by PGANT9A and B (red) within the disordered region (green) are shown.

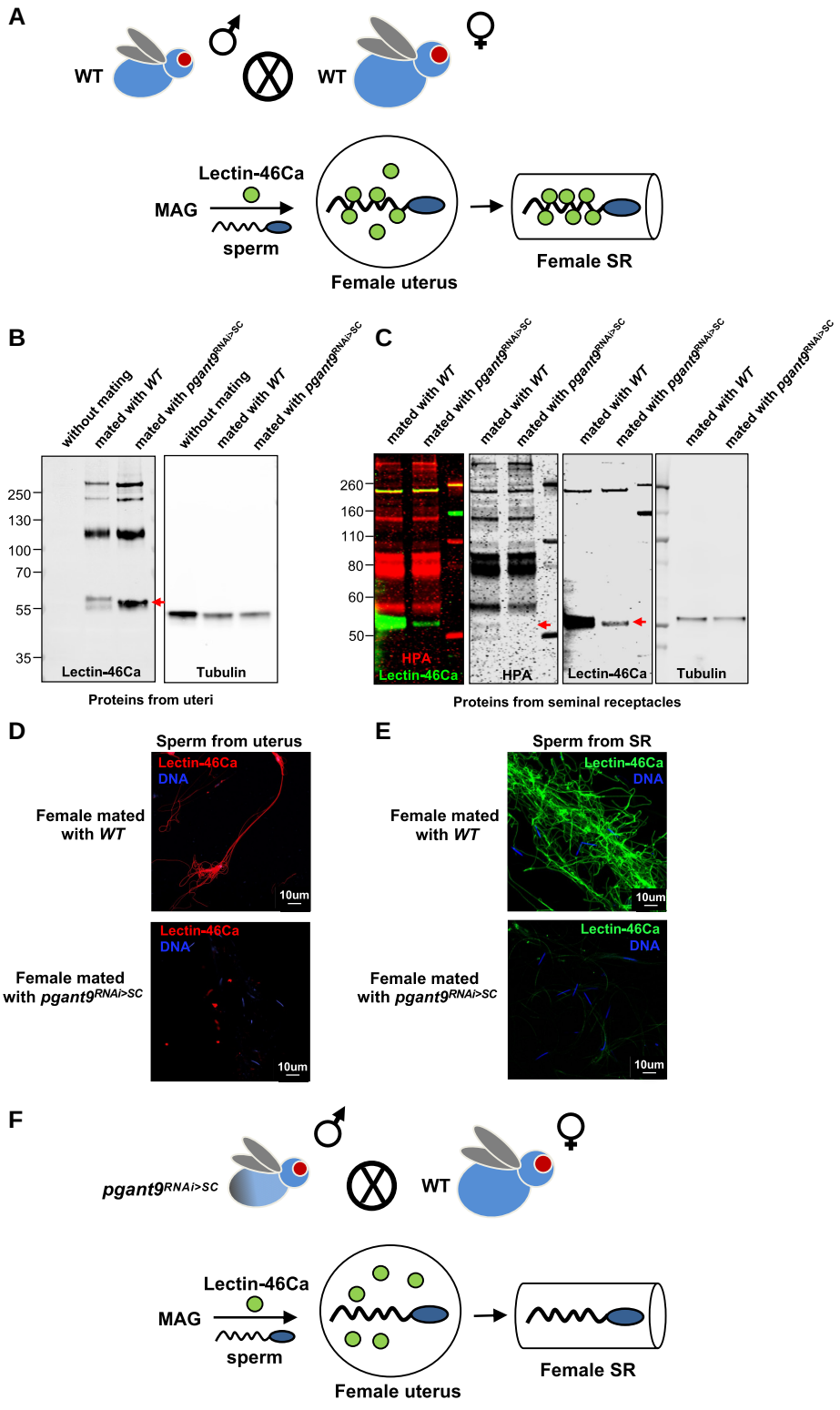


Fig. 4. Loss of lectin-46Ca O-glycosylation leads to decreased sperm binding and reduced transfer to the seminal receptacle (SR) of females. A) Schematic diagram of the transfer of lectin-46Ca from the MAG to the female uterus to the female SR (the sperm storage organ). B) Western blots of extracts from female uteri 30 min after mating probed with the lectin-46Ca antibody and a duplicate blot probed with the tubulin antibody to control for loading. The red arrow indicates the lectin-46Ca protein. C) Western blots of extracts from female SRs 1 h after mating probed with HPA and the lectin-46Ca antibody. A duplicate blot was probed with the tubulin antibody to control for loading. Red arrows indicate the lectin-46Ca protein. D) Immunostaining using the lectin-46Ca antibody on sperm dissected from uteri of females. There is abundant anti-lectin-46Ca staining (red) on sperm tails in the uteri of females mated with *WT* males but no staining in the females mated with *pgant9*$^{RNAi>SC}$ males. DNA staining is shown in dark blue. E) Immunostaining using the lectin-46Ca antibody on sperm dissected from the SRs. There is abundant anti-lectin-46Ca staining (green) on sperm tails in the SRs of females mated with *WT* males but no staining in females mated with *pgant9*$^{RNAi>SC}$ males. DNA staining is shown in dark blue. F) Summary of the role of O-glycosylation on lectin-46Ca function. Loss of glycosylation of lectin-46Ca in *pgant9*$^{RNAi>SC}$ males resulted in decreased sperm tail binding in the uteri of females, leading to the loss of transfer of lectin-46Ca to the SRs of females.

transfer to the female reproductive tract, and fusion with the egg (7, 8, 11, 13, 24). Seminal fluid proteins also aid in sperm storage within the female reproductive tract, a mechanism used by animals that undergo internal fertilization to enhance fitness. Defects in seminal fluid protein production and sperm storage can dramatically alter reproductive fitness and fertility (22, 24–27, 29). In this study, we present the first example of how O-glycosylation of a critical seminal fluid protein plays an active role in reproductive fitness. We show that O-glycosylation of the lectin-46Ca, a critical seminal fluid protein, influences its association with sperm, thereby affecting its ability to move throughout the female reproductive tract. Lectin-46Ca was previously shown to influence postmating behavior when transferred to females (25) and is one of a group of seminal fluid proteins that are required for the localization and long-term persistence of sex peptide in the female reproductive tract. Sex peptide is the protein that directly controls female postmating behavior, as it is slowly released from sperm in the female sperm storage organ and signals through the G-protein coupled receptors of neurons to influence female egg laying and receptivity (30–32). Thus, the O-glycosylation of lectin-46Ca is another aspect of this conserved signaling cascade designed to enhance reproductive fitness. In the absence of glycosylation by PGANT9, this lectin fails to bind to sperm and fails to move to the female sperm storage organ, where it would normally influence female postmating behavior.

The details of how the O-glycans are influencing the association of lectin-46Ca with sperm tails are both interesting and complex. According to our data, O-glycosylation does not appear to affect the initial transfer of lectin-46Ca to females upon mating. Nor do O-glycans affect the stability of lectin-46Ca, as it is present at levels comparable with WT. However, the loss of O-glycans dramatically altered the association of lectin-46Ca with the sperm tail within the female uterus, thus influencing its ability to be transferred throughout the female reproductive tract. The association of lectin-46Ca (and other seminal fluid proteins) with sperm is transient and dependent on the female reproductive environment, and thus not easily dissected ex vivo (26, 27). Previous work has highlighted the interdependent interacting networks among seminal fluid proteins, most of which play roles in the transfer, localization, and slow release of sex peptide (25–27). Certain proteins (CG9997) are required for the transfer of lectin-46Ca and other seminal fluid proteins to the female (26). Once transferred, lectin-46Ca is required for the stability of CG9997 (26). CG17575 is required for the association of lectin-46Ca with sperm, although CG17575 itself does not associate with sperm, thus obscuring the nature (direct or indirect) of these interactions. Lectin-46Ca (and other seminal fluid proteins) are required for long-term association of sex peptide with sperm, although lectin-46Ca is only transiently associated, being lost within 4 h of transfer to females (26, 27, 33). Whether many of these interactions are direct or indirect remains unknown. Additionally, all of these interactions are transient and dependent on the specific environments of the female uterus and sperm storage organ (29), making in vitro or ex vivo assays uninformative. While the transient and environment-specific nature of these associations creates many experimental hurdles, it nonetheless highlights the intricate mechanisms that have evolved to optimize reproductive fitness.

Whether there are other substrates of PGANT9 in the reproductive system contributing to the phenotypes observed remains to be determined, particularly given the interacting networks among the seminal fluid proteins. It remains possible that other as yet to be identified substrates of PGANT9 may also be playing a role, such as the ~35 kDa protein with diminished HPA-reactivity upon loss of *pgant9* (Fig. 3B). Additionally, our westerns provide evidence that another seminal fluid protein (CG14560) is also O-glycosylated, although its glycosylation does not appear to be affected by PGANT9. Recently developed comprehensive mass spectrometry protocols have successfully cataloged the in vivo O-glycoproteome for Galnt2 in mouse tissues (34). However, the minute amounts of material obtained from fly tissues preclude this type of analysis currently. Nonetheless, given that lectin-46Ca is required for the transfer of sex peptide to the seminal receptable where it influences egg production (25, 27) and given that the loss of lectin-46Ca glycosylation influences its ability to move to the seminal receptacle, we hypothesize that the egg laying effects seen upon the loss of *pgant9* are, at least in part, due to the effects of O-glycosylation on lectin-46Ca.

Our mass spectrometry analysis indicated that both PGANT9A and PGANT9B glycosylate lectin-46Ca at Thr264 and Thr295. Lectin-46Ca is a C-type lectin, containing a CRD (40–161aa), which is proposed to bind galactose (35) (Fig. 3E). The three O-glycosylation sites identified are located within the C-terminal disordered region. It is possible that the CRD domain may bind to these O-glycans and form a stabilized structure, which may be important for association with the sperm tail. Another possibility is that these O-glycans may directly bind to other lectins on the sperm tail. A recent study investigating the binding between human lectins and the sperm surface found that three lectins (galectin-1, -7, and -8) can bind to human sperm (36). Additionally, galectin-8 promoted the acrosome reaction in vitro (36), suggesting that lectins play important roles in sperm maturation and fertility. In the future, generating mutations of each glycosylation site within lectin-46Ca may help to further define the function of O-glycosylation at each position.

O-linked glycoproteins are present throughout the mammalian male reproductive tract and form a protective mucus layer along the epithelia (37, 38). Additionally, some mucin proteins (MUC1, MUC5B, and MUC6) are expressed within the male urogenital tract epithelia (38). Likewise, we found a heavily O-glycosylated layer within the *Drosophila* male reproductive system, including along the epithelia of ED and the EB, suggesting the existence of a similar protective layer. Within the MAG, the large secretory vesicles of the secondary cells are filled with O-glycosylated proteins that are transferred to the ED and EB to mix with the sperm before being transferred to the female. In *Drosophila*, around 200 seminal fluid peptides and proteins have been identified and implicated in antimicrobial activity, sperm viability, and female postmating behavior (39–42). Using the NetOGlyc Server, we found that many seminal fluid proteins have predicted O-glycosylation sites, suggesting that O-glycosylation of these proteins may influence some aspect of their function. Similarly, many human seminal plasma proteins have been identified as O-glycosylated molecules (9, 43), suggesting that O-glycans may be involved in the function of seminal fluid proteins across diverse species.

The unique cell-specific expression patterns noted for the *pgant* family members within the reproductive system suggest that each member may have unique substrates and functions in each reproductive cell type. In mammalian systems, two Galnts have been associated with infertility. *Galnt3-deficient* mice have reduced O-glycosylation in the acrosomal regions of spermatids, leading to defects in acrosome formation and male infertility (15, 16). One protein, equatorin, which is involved in sperm–egg interactions, was identified as a potential substrate of *Galnt3* (15). Another family number, *Galntl5* (*Galnt15*), is also involved in acrosome formation. In mice, loss of *Galntl5* (*Galnt15*) reduces sperm

motility and results in male infertility (44, 45). All these studies suggest that O-glycans play important roles in successful fertilization across species. Here, our results reveal that O-glycosylation in the male reproductive system affects a key seminal fluid protein and influences female postmating behavior, suggesting that *Drosophila* is a valuable model to study the biological function of O-glycans in the reproductive system, shedding light on their roles in mammalian reproduction.

Materials and methods

Full details on the materials and methods used are described in the Supplementary Material, which includes fly strains and genetics; gene cloning and constructs used; antibody preparation; staining *Drosophila* tissues and cells; real-time PCR; western blotting; mass spectrometry; and immunoprecipitation.

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Supplementary Material

Supplementary material is available at *PNAS Nexus* online.

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Author Contributions

L.Z. and K.G.T.H. designed and planned the research, analyzed and discussed the data, and wrote the paper. L.Z. performed the experiments.

Data Availability

Data supporting the findings of this manuscript are available in the main text and in the Supplementary Material.

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