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# Rescue of *Drosophila Melanogaster l(2)35Aa* lethality is only mediated by polypeptide GalNAc-transferase *pgant35A*, but not by the evolutionary conserved human ortholog GalNAc-transferase-T11

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**Abstract** The *Drosophila l(2)35Aa* gene encodes a UDP-*N*-acetylgalactosamine: Polypeptide *N*-acetylgalactosaminyltransferase, essential for embryogenesis and development (*J. Biol. Chem.* 277, 22623–22638; *J. Biol. Chem.* 277, 22616–22). *l(2)35Aa*, also known as *pgant35A*, is a member of a large evolutionarily conserved family of genes encoding polypeptide GalNAc-transferases. Phylogenetic and functional analyses have proposed that subfamilies of orthologous GalNAc-transferase genes are conserved in species, suggesting that they serve distinct functions *in vivo*. Based on sequence alignments, *pgant35A* and human GALNT11 are thought to belong to a distinct subfamily. Recent *in vitro* studies have shown that *pgant35A* and *pgant7*, encoding enzymes from different subfamilies, prefer different acceptor substrates, whereas the orthologous *pgant35A* and human GALNT11 gene products possess, 1) conserved substrate preferences and 2) similar acceptor site preferences *in vitro*. In line with the *in vitro pgant7* studies, we show that *l(2)35Aa* lethality is not rescued by ectopic *pgant7* expression. Remarkably and in contrast to this observation, the human *pgant35A* ortholog, GALNT11, was shown not to support rescue of the *l(2)35Aa* lethality. By use of genetic "domain swapping" experiments we demonstrate, that lack of rescue was not caused by inappropriate sub-cellular targeting of functionally active GalNAc-T11. Collectively our results show, that fly embryogenesis specifically requires functional *pgant35A*, and that the presence of this gene product during fly embryogenesis is functionally distinct from other *Drosophila* GalNAc-transferase isoforms and from the proposed human ortholog GALNT11.

**Keywords** GalNAc-transferase · GALNT11 · *pgant35A* · Drosophila · Glycosyltransferase · Mucin-type *O*-glycosylation

**Abbreviations**

| | |
|---|---|
| GalNAc-transferase | UDP-*N*-acetyl-α-D-galactosamine:Polypeptide *N*-acetylgalactosaminyltransferase |

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| | |
|---|---|
| PCR | polymerase chain reaction |
| Mab | monoclonal antibody |
| fly ortholog of human GalNAc-T11 is CG7480 (=*pgant35A*) | CG6394 (=*pgant7*) the fly ortholog of human GalNAc-T7 |

## Introduction

Mucin-type O-glycosylation is one of the most abundant types of protein glycosylation conserved throughout evolution from *C.elegans*, *Drosophila* to man [1, 2]. Mucin-type O-glycans are widely found on most secreted and cell surface glycoproteins, and are involved in important biological functions, such as cell-cell interactions [3, 4], intracellular transport and sorting of glycoproteins [5–13], receptor ligand interactions [14], leukocyte trafficking [15, 16], and cancer metastasis [17]. The initial step in mucin-type O-glycosylation, involving transfer of GalNAc from UDP-GalNAc to serine or threonine residues in proteins, is controlled by a large homologous family of UDP-GalNAc: polypeptide *N*-acetylgalactosaminyltransferases (GalNAc-transferases) (EC 2.4.1.41). This gene family is found in all higher eukaryotic species and includes >10 members in *Drosophila*, 9 in *C.elegans* and 20 members in man [18]. As such, the GalNAc-transferase gene family constitutes by far the largest glycosyltransferase gene-family catalysing a single glycosidic linkage. It is currently not clear why such a large gene family has evolved. GalNAc-transferase isoforms have distinct peptide substrate specificities and expression patterns, however, there is considerable overlap in both specificities and expression patterns among isoforms [19–21]. In striking contrast to this enormous potential redundancy and genetic backup for control of the first initiation step in O-glycosylation, the second step being core 1 (Galβ1-3GalNAcα1-O-Ser/Thr) or core 3 (GlcNAcβ1-3GalNAcα1-O-Ser/Thr) O-glycosylation is covered only by single genes, and importantly the core 1 β3galactosyltransferase is essential for mouse embryogenesis [22].

Targeted disruption of GalNAc-transferase genes in mouse has not provided clear evidence for distinct phenotypes [2], although a recent report demonstrates that the GalNAc-T1 isoform is required for normal B-cell development and blood clotting factor levels [23]. A more conclusive role of individual GalNAc-transferase isoforms has emerged from studies of the autosomal recessive diseases familial tumoral calcinosis and hyperostosis-hyperphosphatemia syndrome [24], which may be caused by mutations in the GALNT3 gene as well as FGF23, a key regulator of phosphate homeostasis. The GalNAc-T3 isoform mediates O-glycosylation of a specific acceptor site in a furin protease inactivating site of FGF23, which was found to be required for normal function of this important serum factor [25]. This is the first example of molecular dissection of the importance of individual GalNAc-transferases. The finding that mutations in either GALNT3 or FGF23 results in presently indistinguishable clinical features, suggests that O-glycosylation is indeed governed by a high degree of genetic and functional specificity. It furthermore seems to predict that clinical characteristics associated with loss of function of individual GalNAc-transferase isoforms may be caused by very subtle changes in O-glycosylation of specific sites in a limited number of glycoproteins, which combined with the high degree of redundancy in O-glycosylation in general makes it very difficult to identify and decipher defects in GalNAc-transferase mediated O-glycosylation.

In this respect the fruit fly, *Drosophila melanogaster*, offers a simpler model system with availability of well defined genetic tools, and we and others have previously demonstrated that a single GalNAc-transferase isoform, *pgant35A*, is an essential gene for development [26, 27]. More recent studies have shown that the *pgant35A* gene has an important role for epithelial tube formation [28], but our understanding of the role of GalNAc-transferase isoforms and O-glycosylation in fly development is still limited.

In the present study we have used the *Drosophila pgant35A* model system to shed light on the importance of distinct GalNAc-transferase isoforms, their functions and evolutionary conservation. With a transgene-based assay, we found that *l(2)35Aa* lethality can be fully rescued by a minimal transgene containing essentially only the coding region of *pgant35A*. Using this construct we found that the catalytic activity of *pgant35A* was required for rescue. We also demonstrated that another *Drosophila* GalNAc-transferase isoform, *pgant7*, with different *in vitro* substrate specificity did not rescue. Finally, we tested our previous hypothesis of evolutionary conserved subfamilies of GalNAc-transferases, and show that the predicted human ortholog GALNT11 surprisingly does not substitute for *pgant35A in vivo*. Despite the finding that the catalytic functions evaluated by *in vitro* assays apparently are conserved, the human enzyme cannot replace the fly enzyme *in vivo.* Possible explanations for this are discussed.

## Materials and methods

### Genomic *pgant35A* and *pgant35A (D243N)* complementation constructs

To eliminate the potential influence of flanking *Rab14* and *Spel1* sequences from the original 5.2 kbp genomic *pgant35A* complementation clone *pCaGa1* [29], the DNA was sub-cloned by PCR to yield a 3.8 Kbp genomic clone

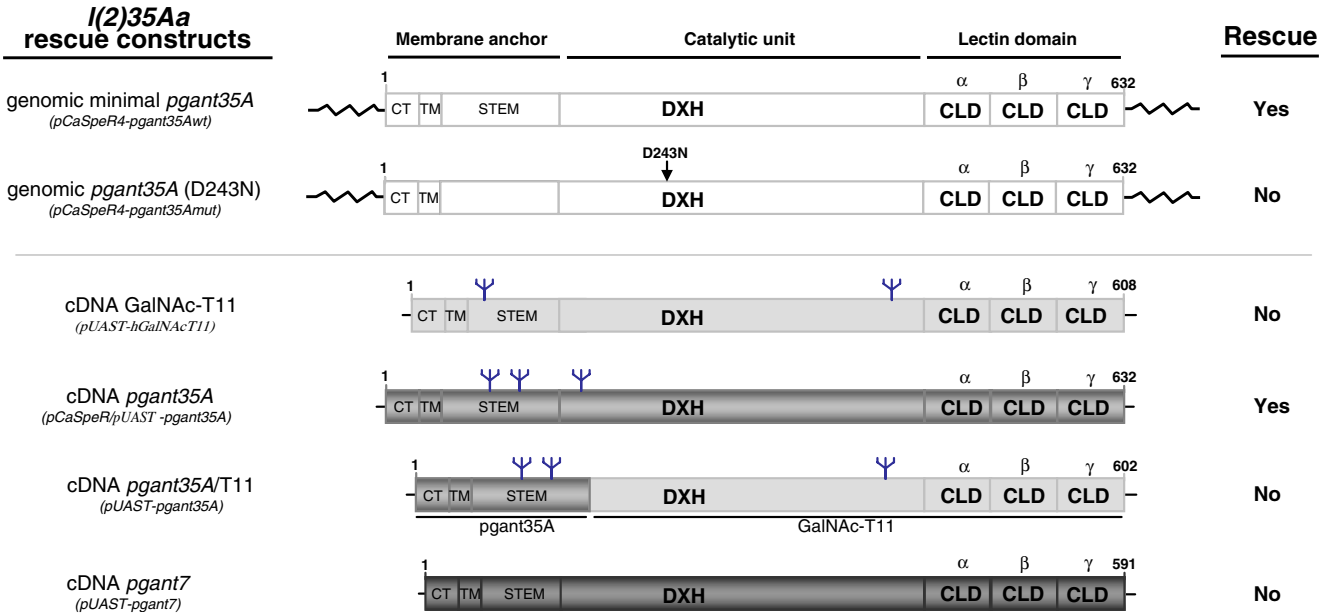


**Fig. 1** Schematic representation of all constructs generated and outcome of *pgant35A*$^{3}$ (described as *l(2)35Aa*$^{SF12}$ [29]) rescue attempts. Genomic *pgant35A* rescue constructs are shown in white, human GalNAc-T11 cDNA construct in patterned light grey, *Drosophila pgant35A* cDNA constructs in grey and *pgant7* cDNA construct in dark grey. Approximate positions of the conserved GalNAc-transferase catalytic DXH motif, inactivating *pgant35A* D243N mutation and conserved lectin CLD motifs are indicated. For *pgant35A*/T11 the size of the N-terminal *pgant35A* (amino acids 1–110) and C-terminal GalNAc-T11 (amino acids 117–608) are underlined. Figures are based on assignments from GenBank accession numbers given in Section 2. Potential N-glycan sites (Ψ) are shown for GalNAc-T11, *pgant35A* and *pgant35A*/T11. Designated names of the constructs used in this study, as described in the Materials and methods section, are shown in parenthesis

of *pgant35A*, hereafter designated *pCaSper4-pgant35Awt*. In brief, primers TSHC269 (5′-CGA**GGATCC**AACAACAGCGACAGCAAC) and TSHC270 (5′-CGA**GGATCC**TGCCTGCTGTCGGTTGC) were used to amplify base pairs 722 to 4568 of *pCaGa1* (BamHI restriction sites are shown in bold) covering *pgant35A*. The generated product was inserted into the BamHI site of pBluescript KS+ (Stratagene) and fully sequenced. The *pgant35A* insert was cloned into the BamHI site of the *Drosophila* expression vector *pCaSpeR4* (GenBank accession number U60731) to yield *pCaSpeR4-pgant35A*wt. The conserved GalNAc-transferase GT1 DXH motif of *pgant35A* was mutated to NSH by assembly-PCR using TSHC269 and TSHC272 (5′-ATGCGAGTTGAGGAAGACGAG) for the 5′ part and TSHC271 (5’-CGTCTTCCTCAACTCGCATATC) and TSHC270 for the 3′ portion (mutated position 2505 underlined). Amplification of the assembled fragments using TSHC269 and TSHC 270 yielded a genomic *pgant35A* clone with position 2505G exchanged to A. The clone was fully sequenced in pBluescript KS+ and inserted into pCaSpeR4 as described above and designated *pCaSper4-pgant35Amut*.

Constructs for human *GalNAc-T11*, *pgant35A*, chimeric *pgant35A/T11* and *pgant7*

Full length human GalNAc-T11 (GenBank accession number Y12434) encoding amino acids 1-609 was amplified using primers EBHC615 (5′-GCGAATTCACCATGGGAAGTGT

**Table 1** Rescue attempts of the *l(2)35Aa* recessive lethality by genomic transgenes, *pCaSpeR4-pgant35A wt* (wild type *pgant35A* gene) or *pCaSpeR4-pgant35A mut* (D243N inactivated *pgant35A* gene)

| Crosses | Progeny with genomic transgene | |
|---|---|---|
| | $Cy^{+}$ [a] (trans-heterozygous mutant) | $Cy^{-}$ [a] (heterozygous mutant) |
| ♂ *w- / Y ; Df(2L)b84h1/ CyO P{w+GFP}* X ♀ *w- pCaSpeR4- pgant35Amut / w-; b pgant35A*$^{3}$ */ CyO P{w+GFP}* | 0 | 692 |
| ♂ *w- / Y ; Df(2L)b84h1/ CyO P{w+GFP}* X ♀ *w- pCaSpeR4- pgant35Awt/ w-; b pgant35A*$^{3}$ */ CyO P{w+GFP}* | 313 | 1061 |

[a] $Cy^{-}$ indicates flies carrying curly wings; $Cy^{+}$ indicates non-curly flies

**Table 2** Rescue attempts of the *l(2)35Aa* recessive lethality by *pgant35A* cDNA transgene *pCaSpeR-pgant35A* under temperature dependent *Hsp70* promoter control

| Crosses | $Cy^+$ progeny | | | |
|---|---|---|---|---|
| | $b^+$ [a] (heterozygous mutant) | | $b^{-a}$ (trans-heterozygous mutant) | |
| | $w^+$ [b] (with transgene) | $w^{-b}$ (without transgene) | $w^+$ (with transgene) | *w*-(without transgene) |
| ♂ *w- / Y ; b pgant35A*$^3$ */ CyO P{w+GFP}* X ♀ *w- pCaSpeR- pgant35A / w-; Df(2L)b84h1/ +* | 308 | 258 | 293 | 0 |

[a] Deficiency chromosome *Df(2L)b84h1* carries a recessive marker, *black*. $b^-$ indicates flies carrying black body color; $b^+$ indicates non-black flies. [b] $w^-$ indicates flies with white eye color; $w^+$ indicates flies with red eye color which is the marker of transgenes. * The cross was performed at 25 degree

CACAGTTCGG)/EBHC631 (5′-GCGAATTCCACCTTA ACCTTCCAAATGC), human kidney total RNA (Clontech, Palo Alto, USA) SuperscriptII reverse transcriptase (Invitrogen, Carlsbad, USA) and PfU Ultra DNA polymerase (Stratagene, La Jolla, USA) as recommended by the supplier. EcoRI restriction enzyme overhangs are underlined. Generated product was inserted into the EcoRI site of pBluescript/PBKS (Stratagene, La Jolla, USA) and insert fully sequenced using BigDye terminator chemistry (Applied Biosystems, Foster City, USA) and ABI377 Prism sequenator (Applied Biosystems, Foster City, USA), generating GalNAc-T11/PBKS. Human GalNAc-T11 insert was sub-cloned into the EcoRI site of hsp70 promoter driven *Drosophila* expression vector *pCaSpeR-hs-act* (GenBank accession number U60735), or the Gal4 driven vector pUAST [30], generating *pCaSpeR*-GalNAc-T11 and *pUAST*-GalNAc-T11, respectively.

Full length *pgant35A* (GenBank accession number AF158747) cDNA was generated by PCR using TSHC1 and TSHC3 [27] and EST clone LD24449 as template. The product was inserted into pBluescript KS+, fully sequenced, and cloned into the BamHI site of *pCaSpeR-hs-act* allowing for Hsp70 heat inducible gene induction [31], generating *pCaSpeR-pgant35A*. To allow for expression in the *Gal4* driver lines, full-length *pgant35A* cDNA was inserted into the BglII-site of pUAST, generating *pUAST-pgant35A*. Full length cDNA of *pgant7* (CG6394) (GenBank accession number AF493067) was cloned as described, fully sequenced, and inserted into the EcoRI site of pCaSpeR-hs-act, yielding *pCaSpeR-pgant7*, and the EcoRI site of pUAST, generating *pUAST- pgant7*, respectively. The *pgant35A*/GalNAc-T11 chimera was made by fusion of the *pgant35A* N-terminal part encoding amino acids 1–110, with the C-terminal part of human GalNAc-T11 encoding amino acids 117–609. A DNA fragment encoding the N-terminal part of *pgant35A* (1–110) was amplified using *pBKS-pgant35A* as template, vector T7primer/DT1STEM (5′-CGAGCTCCTG AGGCAAGCTTGTAGCCAATGTCGCGTATG) and PfU Ultra DNA-polymerase. A DNA fragment encoding the c-terminal part of human GalNAc-T11 (117–609) was amplified using *pBKS*-GalNAc-T11 as template, vector T3primer/T11CAT (AGCGAAGCTTCATGCTCTTAATATGCTTAT CAGTGAC) and PfU Ultra DNA-polymerase. In frame HindIII primer overhangs have been underlined. N-terminal *pgant35A* NotI/HindIII fragment and C-terminal human GalNAc-T11 HindIII/KpnI fragments were ligated into the NotI/KpnI site of pUAST, generating the chimeric construct *pUAST-pgant35A*/GalNAc-T11- (*pUAST-pgant35A*/T11).

A secreted N-terminally HIS-tagged *pgant35A*/T11 insect cell expression construct was made. In brief, *pUAST-pgant35A*/T11 was used as template for amplification of a

**Table 3** Rescue attempts of the *l(2)35Aa* recessive lethality by *UAS* transgenes of *pgant35A* cDNA (*pUAST-pgant35A*), another *Drosophila* isoform *pgant7* cDNA (*pUAST-pgant7*), or human ortholog *GalNAcT11* cDNA (*pUAST-hGalNAcT11*). Transgenes were driven under ubiquitous *armadillo-Gal4* using *UAS/Gal4* system

| Crosses | $Tb^+$ [a] (progeny with transgene) | |
|---|---|---|
| | $Cy^+$ (trans-heterozygous mutant) | *Cy* (heterozygous mutant) |
| ♂ *Df(2L)b84h1/ CyO; pUAST- pgant35A / TM6B* X ♀ *pgant35A*$^3$*, arm-Gal4/ CyO* | 70 | 160 |
| ♂ *Df(2L)b84h1/ CyO; pUAST-pgant7/ TM6B* X ♀ *pgant35A*$^3$*, arm-Gal4/ CyO* | 0 | 338 |
| ♂ *Df(2L)b84h1/ CyO; pUAST-hGalNAcT11/ TM6B* X ♀ *pgant35A*$^3$*, arm-Gal4/ CyO* | 0 | 200 |

[a] $Tb^+$ indicates flies without dominant marker *Tubby*, a marker on *TM6B* balancer

secreted construct of the chimera lacking amino acids 1–30 (of the *pgant35A*) using primers dT1SOL (5′-GCG GAATTCTCTCGCACAGCCTGCGCAGCTCCATC)/ EBHC631 (EcoRI overhang shown underlined). PCR product was inserted into the EcoRI/NotI sites, in-frame and downstream of the previously described 6xHis-T7-tagged pAcGP67 vector [32], creating pAcGP67-HIS-*pgant35A*/T11.

## Polypeptide *GalNAc*-transferase assays

Human GalNAc-T11 and *pgant35A*/T11 were expressed as soluble secreted N-terminally truncated proteins in insect cells and semi-purified as previously described [32]. Screening assays for GalNAc-transferases with peptides were performed with UDP-[$^{14}$C]-GalNAc essentially as previously described using 10 ug of acceptor peptides, and 1 μg of purified recombinant *pgant35A*/T11 or GalNAc-T11. Peptides were custom synthesized by Neosystems (France) or Schafer-N (Denmark).

## Expression of human *GalNAc-T11* and chimeric *pgant35A/T11* in *S2* cells

*Drosophila* Schneider's cells line 2 (S2 cells) were cultured in insect cell SFM (Serum-free medium, Invitrogen) and transiently co-transfected *pUAST*-GalNAc-T11 or *pUAST-pgant35A*/T11 and pRmHa3-Gal4, and pRmHa3-Notum-GT [33] using Cellfectin (Invitrogen). Expression was induced by addition of 0.7 mM $CuSO_4$ for 2 days.

*Drosophila* S2 cells grown in chamber slides (Nunc) were fixed in 4% Formaldehyde diluted in PBS for 10 min followed by two 10-minute washes with PBT (0.2% Triton X-100 in PBS) and 30 min blocking in BBT (0.1% BSA in PBT). The samples were incubated with primary and secondary antibodies for 1 h each at room temperature, with three 15-minute washes with BBT in between.

Antibodies and reagents used for staining were as follows: Murine anti-human GalNAc-T11 monoclonal antibody UH8, without dilution [27], guinea pig anti-Notum 1:100 [33], fluorophore-conjugated secondary antibodies (Jackson ImmunoResearch), and DAPI (Sigma) for nuclei staining.

## Drosophila strains and genetics

*Armadillo-Gal4* (II), and *tubulin-Gal4* (III) are described in FlyBase (http://flybase.bio.indiana.edu/). *Pgant35A*$^{3}$, *Df (2L)b84h1/CyO* (34D4; 35A4) are described in Flybase and [27]. In brief, *Pgant35A*$^{3}$ carries a nonsense mutation introducing a stop codon at position 195 of *pgant35A* [29]. Stocks carrying *Pgant35A*$^{3}$ with a ubiquitous driver (*armadillo-Gal4* or *tubulin-Gal4*) and stocks carrying *Df (2L)b84h1/CyO* with a UAS line were established. Two to three independent UAS insertions were tested for each construct. The transgenic flies were obtained by co-injecting the construct with helper DNA. Their ability to rescue the lethality of trans-heterozygous mutant *Pgant35A*$^{3}$/*Df(2L)b84h1* was assayed by counting the number of mutants that survived with and without the transgenes.

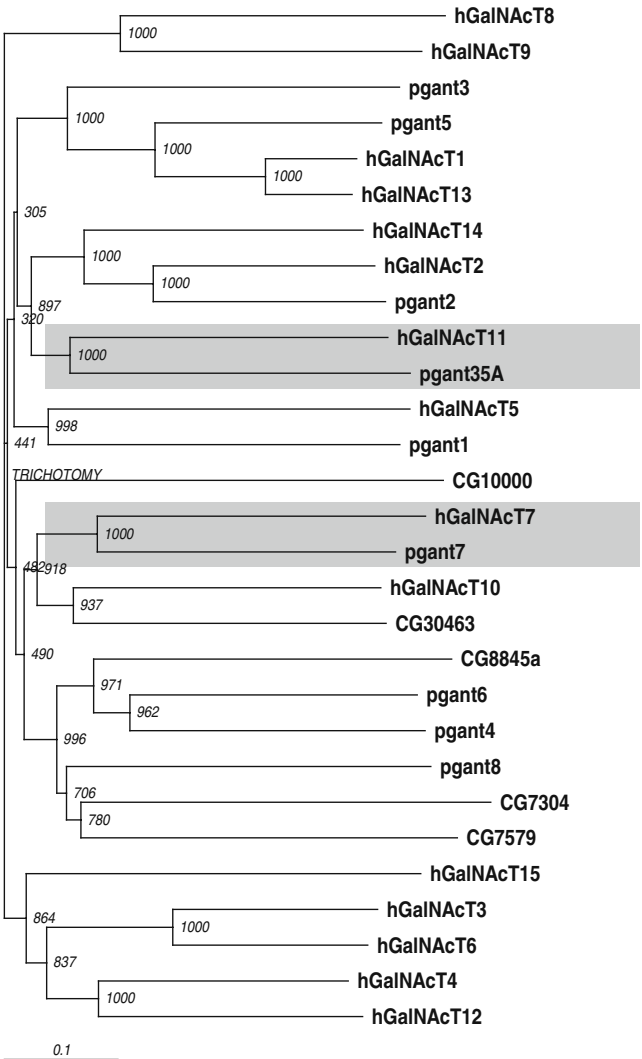


**Fig. 2** Phylogenetic analysis of published human and fly [27, 54] GalNAc-transferase genes. Human GenBank accession numbers used for the analysis were; GalNAcT1 X85018, GalNAcT2 X85019, GalNAcT3 X92689, GalNAcT4 Y08564, GalNAcT5 AJ245539, GalNAcT6 Y08565, GalNAcT7 AJ002744, GalNAcT8 AJ271385, GalNAcT9 AB040672, GalNAcT10 AJ505950, GalNAcT11 Y12434, GalNAcT12 AJ132365, GalNAcT13 AJ505991 , GalNAcT14 Y09324, GalNAcT15 NM_054110. Phylogenetic tree was based on Clustal W alignments [55] and trees were drawn using TreeView (Win32) software (http://taxonomy.zoology.gla.ac.uk/rod/rod.html). The two distinct clades containing hGalNAc-T11/*pgant35A* and hGalNAcT7/*pgantt7* have been highlighted in shaded boxes

## Results

### The coding region and catalytic function of *pgant35A* is required for rescue of *l(2)35Aa*

We initially demonstrate that the coding region of *pgant35A* is essential for development by rescue of the *l(2)35Aa* mutant with a 3.8 kb genomic construct containing the minimal *pgant35A* locus devoid of all other flanking sequences *pCaSpeR4-pgant35Awt* (Fig. 1 and Table 1) or cDNA containing only the full coding region of *pgant35*, *pCaSpeR-pgant35A* (Fig. 1 and Table 2). In both cases flies carrying one copy of *pgant35A* were found to rescue the lethality of *l(2)35Aa*. These results are in agreement with a recent report by E. Tian *et al.* [28], where it was shown that *pgant35A* cDNA under control of the ubiquitous tubulin-Gal4 driver, rescued the lethality and gave rise to viable and fertile progeny. We further confirmed that the catalytic functions of *pgant35A* is required by demonstrating that a mutant construct with a single inactivating substitution in the DxH motif, D243N, did not rescue *l(2)35Aa* (Fig. 1 and Table 1).

Rescue was also obtained when *pgant35A* cDNA was put under control of the ubiquitously expressed *armadillo-GAL4* driver [30] to direct expression of *pUAS-pgant35A* (Table 3), which indicates that ectopic expression of *pgant35A* does not affect development. Comparable rescue was obtained using *actin-GAL4* (data not shown) and using a *Hsp70* promoter to direct *pgant35A* cDNA expression (Table 2). Since ubiquitous *pgant35A* clearly supported rescue, an attempt was made to determine at what embryonic stage *pgant35A* expression might be critical for development. This was done by putting ectopic *pgant35A* expression under inducible Hsp70 promoter control, allowing for heat inducible expression of *pgant35A*. Surprisingly, transgenic flies possessing an ectopic *Hsp70* driven *pCaSpeR- pgant35A* copy were found to rescue *l(2)35Aa* lethality even when grown under non-permissive conditions, see Table 2. This latter finding is most likely due to a low basal level of "leaky" *Hsp70* promotor activity even under non-permissive conditions [31].

### Rescue of *l(2)35Aa* is isoform specific

GalNAc-transferase isoforms have different *in vitro* substrate specificities, although a degree of overlap exists. We previously demonstrated that *pgant35A* and *pgant7* cluster into two distinct evolutionary conserved subfamilies (Fig. 2) and that they have markedly different *in vitro* substrate specificities using a panel of peptides and GalNAc-glycopeptides derived from human glycoproteins [27]. We therefore tested if *pgant7* could rescue *l(2)35Aa* mutants. Transgenic flies expressing *pgant7* under the control of arm-GAL4 did not rescue *l(2)35Aa* (Table 3). Wild type flies with the armadillo-GAL4 and UAS-*pgant7* were viable and fertile suggesting that ectopic expression of this isoform does not affect normal development. Several independent transgenic lines of UAS-*pgant7* were tested with armadillo-GAL4 and with actin-GAL4 or under control of the Hsp70 promoter and all failed to rescue the *l(2)35Aa* mutant (data not shown). This result provides additional support for the specific requirement of distinct functions of GalNAc-transferase isoforms in whole organisms.

### The predicted human ortholog of Drosophila *pgant35A*, GALNT11, does not rescue *(1(2)35Aa*

Previous *in vitro* analyses of *pgant35A* and human GalNAc-T11 iso-forms have suggested that these enzymes were putative orthologs with almost identical substrate specificities and site preference [27, 34]. Phylogenetic

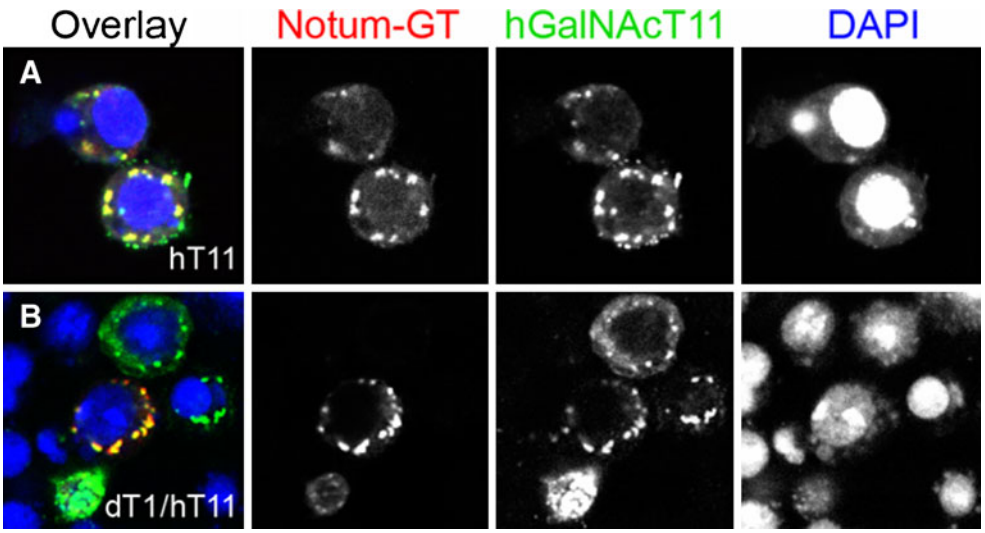


**Fig. 3** Cytolocalization of human GalNAc-T11 (hT11) and chimeric *pgant35A*/T11 (dT1/hT11) in S2 cells. Human GalNAc-T11 **(panel A)** or chimeric *pgant35A*/T11 **(panel B)** expressed and co-localized with Golgi Notum marker (Notum-GT). Immunohisto-chemical stainings show Golgi marker and GalNAcT11 or *pgant35A* /T11 co-localized in Golgi, overlay in yellow. Nuclei were stained with DAPI in blue. **Panels A and B from left to right**; merged image, split channel of Golgi marker, human GalNAcT11 or *pgant35A* /T11 and DAPI

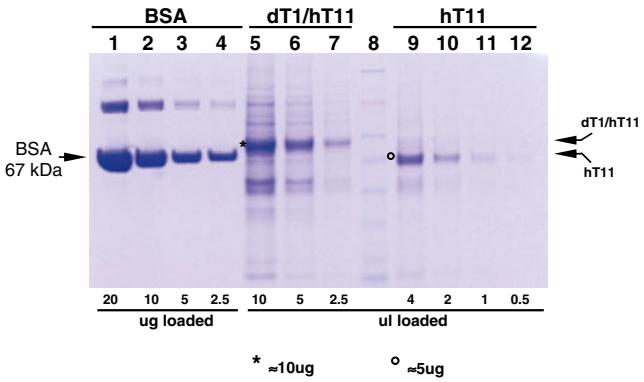


**Fig. 4** SDS PAGE Coomassie stain of recombinant secreted forms of chimeric *pgant35A*/T11 (dT1/hT11) and human GalNAc-T11 (hT11), semi-purified from Sf9-cells. Known amounts of BSA was loaded together with various volumes of semi purified dT1/T11 or human hT11. Estimated protein sizes for dT1/T11 and human hT11 are app. 68 KDa, size difference observed is attributed to the presence of potentially 1 additional N-glycan in dT1/T11, see Fig. 1. * marked dT1/hT11 band, amount estimated to 10 ug/10 ul loaded =1 mg/ml protein concentration. ° marked hT11 band, amount estimated to 5 ug/4 ul loaded =1.25 mg/ml protein concentration

analyses clearly support that the two genes are potential orthologs and as shown in Fig. 2 the two genes group in the same clade. Thus, the hypothesis that the two genes have been functionally conserved during evolution was tested by expressing *pUAS-GalNAc-T11* in trans-heterozygous mutant *l(2)35Aa* flies (Table 3). Rescue experiments were performed on two independent transgenic lines in combination with *armadillo-GAL4* or *actin-GAL4* (data not shown). Over expression of human GalNAc-T11 did not lead to lethality, and immunocytology studies indicated that expression was properly co-localized with a known Golgi-marker [33] in *Drosophila* cells (Fig. 3). Nevertheless, GalNAc-T11 did not appear to rescue the *l(2)35Aa* mutant. We further tested if a chimeric construct, *pUAS-pgant35A/T11*, comprised of the cytoplasmic, transmembrane and part of the *pgant35A* stalk fused to the catalytic and lectin domains of human GalNAc-T11 complemented the mutation (Fig. 1). The chimeric protein was expressed and localized to the Golgi compartment in *Drosophila* S2 cells (Fig. 3) and purified soluble secreted chimeric protein expressed in insect cells was shown to have the predicted catalytic function of human GalNAc-T11 (Table 4). Several independent transgenic lines expressing *pUAS- pgant35A*/T11-chimera under control of *tubulin-GAL4* or *actin-GAL4* also failed to rescue the *l(2)35Aa* mutants (Table 5). The reason for lack of complementation with human GalNAc-T11 is unknown. Cross-species transgene expression of human gene homologs in *Drosophila* has been successful in the past [35, 36], and we have found that a human β4-galactosyltransferase, β4Gal-T6, rescues the *Drosophila egghead* β4-mannosyltransferase mutant despite the fact that the two enzymes produce different products [37].

## Discussion

The results of this study provide evidence that the catalytic function of the *pgant35A* GalNAc-transferase isoform is required for *Drosophila* development. The study furthermore demonstrates that the essential catalytic function of this enzyme cannot be substituted by another GalNAc-transferase isoform or even the putative human ortholog. The results stress that polypeptide GalNAc-transferases in animal cells serve unique and non-redundant functions directed at least in part by the substrate specificity of the catalytic unit.

GalNAc-transferases are unique among mammalian glycosyltransferases in having a C-terminal distinct lectin domain [38]. The lectin domain is a separate fold linked through a short flexible linker region [39]. Studies of some isoforms show that the lectin domains bind GalNAc-glycopeptides [32] and modulate the substrate specificity and kinetic properties of GalNAc-transferases [40–43], and it is expected that most if not all have functional lectin

**Table 4** Acceptor substrate specificities of GalNAc-transferase chimera *pgant35A*/T11 and human GalNAc-T11

| Acceptor peptide | Amino acid sequence | *pgant35A*/T11 | | human GalNAc-T11 | |
|---|---|---|---|---|---|
| | | Substrate specificity[a] *mU/ml* | Specific activity *U/mg* | Substrate specificity[a] *mU/ml* | Specific activity *U/mg* |
| IgA hinge | VPSTPPTPSPSTPPTPSPSK | 34.74 | 0.87 | 53.26 | 1.33 |
| OSM | LSESTTQLPGGGPGCA | 4.17 | 0.10 | 4.25 | 0.11 |
| MUC1 | AHGVTSAPDTR | 7.59 | 0.19 | 35.65 | 0.89 |
| MUC2-1 | PTTTPITTTTTVTPTPTPTGTQTPTTTPISTTC | 10.06 | 0.25 | 22.79 | 0.67 |

[a] Activity was determined in 25 ul reactions containing 10 ug peptide substrate and 0.04 mg/ml semi purified *pgant35A*/T11 or human GalNAc-T11, 0.2 mM UDP-GalNAc (3153 cpm/nmol), 1× cacodylate buffer in a 30 min. 37C reaction. Enzyme amount was estimated from coomassie gels depicted in Fig. 4. The acceptor peptides were derived from sequences from the tandem repeats of ovine submaxillary mucin (OSM), human MUC1or MUC2 or the mucin like domain found in the hinge region of human IgA

**Table 5** Rescue attempts of the *l(2)35Aa* recessive lethality by *Drosophila*/human chimeric *pgant35A/T11* (*UAS-pgant35A/ T11*) transgene, under the control of *tubulin-Gal4*

| Crosses | $Ser^{+a}$ | | $Ser^{-a}$ | |
|---|---|---|---|---|
| | $Cy^{+}$ | $Cy^{-}$ | $Cy^{+}$ | $Cy^{-}$ |
| ♂ *Df(2L)b84h1/CyO; pUAST-pgant35A/T11/ TM3* X ♀ *pgant35A*$^{3}$*/ CyO; tub-Gal4/ TM3* | 0 | 77 | 0 | 120 |

[a] $Ser^{-}$ indicates flies carrying notched wings due to the dominant mutations Serrate; $Ser^{+}$ indicates flies without the dominant marker *Serrate*

domains with similar functions. The catalytic unit of GalNAc-transferases are distinct folds and active enzymes missing the entire lectin domain or with mutated lectin domains have been expressed and shown to retain intrinsic GalNAc-transferase enzymatic activity [40, 43].

We were surprised to find that human GalNAc-T11, which we previously showed to have essentially the same *in vitro* substrate specificity as Drosophila *pgant35A* [27] did not rescue *l(2)35Aa* mutants. The substrate specificities were studied using a large panel of peptide substrates from different human O-glycoproteins and both enzymes were distinctly different from many other human GalNAc-transferase isoforms tested. It is possible that substrates derived from *Drosophila* glycoproteins would show differences that can explain the lack of complementation, however, the identity of *Drosophila* O-glycoproteins are only now becoming available [44].

Another possibility we tested was whether the cytoplasmic, transmembrane and stem regions of *pgant35A* were important for rescue with the catalytic domain of the human GalNAc-T11 enzyme. A number of studies have demonstrated the importance of the transmembrane regions of glycosyltransferases for Golgi localization [45, 46]. Furthermore, in this study the chimeric construct included all amino-terminal non-catalytic regions of *pgant35A*, which should support correct steady state Golgi-localization through dynamic interactions with for example peripheral Golgi membrane proteins [47–49]. Interestingly, the consensus signal retention motif (-F/L-L/V-S/T-) identified in the cytoplasmic region of yeast glycosyltransferases [47] is not found in most *Drosophila* and mammalian glycosyltransferases, but the cytoplasmic regions of *pgant35A* (-L-G-T-) and GalNAc-T11 (-S-V-T-) appear to contain a motif similar to the suggested consensus motif. This motif has been shown to interact with the peripheral Golgi protein Vps74p and COPI in yeast, and found to play a decisive role in retrograde transport of Golgi resident glycosyltransferases. Vps74p is highly homologous to the human GMx33 Golgi matrix proteins, but further studies are required to address the existence and function of similar mechanisms in mammalian cells in general and in relation to the enzymes discussed in this study. In spite of these considerations, complementation with the functionally active Golgi targeted chimera failed to support rescue of the lethal *l(2)35Aa* fly phenotype. This result could be explained by the finding that *Drosophila* Golgi units are dispersed throughout the cell, and that these stacked units in *Drosophila* imaginal discs cells have been shown to be functionally diverse [50]. This could suggest that *Drosophila* Golgi resident proteins localize in distinct units. In contrast the Golgi complex in mammalian cells consists of a network of tubular membranes interconnected through intercisternal connections [51]. Thus, sub-cellular localization of glycosyltransferases in mammalian cells may depend on specific oligomeric interactions within sub-Golgi compartments in order for them to act correctly. This has been shown for enzymes involved in glycolipid biosynthesis where early acting glycosyltransferases physically associate in heteromeric Golgi complexes [52]. Likewise, the regulated biosynthesis of mucin-type O-glycans could be controlled by the restricted organization of glycosyltransferases with shared functions [53] and/or oligomerization of individual components of the "O-glycosylation machinery" in sub-Golgi compartments. Thus, the orchestration of individual glycosyltransferases in dispersed *Drosophila* Golgi units and in mammalian tubular Golgi membrane complexes may differ significantly, which could explain the lack of complementation obtained using the orthologous *pgant35A* and GalNAc-T11 genes in this study. Taken together, our results using human GalNAc-T11 and the chimeric rescue constructs, clearly suggest, that pgant35A and human GalNAc-T11 serve unique species specific cellular functions.

In summary, the *l(2)35Aa* model has allowed us to begin addressing functions of the functional domains of GalNAc-transferases as well as the functions of individual GalNAc-transferase isoforms *in vivo*. Further studies are needed to decipher the essential molecular O-glycosylation reaction(s) mediated by *pgant35A*.

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