Characterization of interaction between GABPI and the β1,4GalNAcTransferaseB from *Drosophila melanogaster*

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Seht Ihr den Mond dort stehen?
Er ist nur halb zu sehen
und ist doch rund und schön.
So sind wohl manche Sachen
die wir getrost belachen
weil unsere Augen sie nicht seh'n.

(Abendlied, Mathias Claudius, 1740-1815)

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Zusammenfassung

Im Rahmen dieser Arbeit konnte der zuvor identifizierte Hilfsfaktor für die Aktivierung der glykolipidspezifischen β1,4Acetylgalactosaminyltransferase B aus *Drosophila melanogaster* β4GalNAcTB pilot (GABPI) näher charakterisiert, sowie die für die Interaktion mit der Glykosyltransferase verantwortlichen Domänen beider Enzyme identifiziert und analysiert werden. Erstmals konnte hiermit ein für die Herstellung der Akzeptorspezifität einer glykolipidspezifischen Glykosyltransferase in Frage kommender Mechanismus untersucht werden. GABPI, ein Mitglied der Familie der DHHC Proteine, welches sowohl für die Aktivierung der β4GalNAcTB als auch für deren Golgilokalisation verantwortlich ist, kann im Gegensatz zu anderen DHHC Proteinen keine Palmitoyltransferase-aktivität übernehmen. Diese Funktion konnte über Deletion des für diese Funktion vorausgesetzten DHHC-Motivs als auch aufgrund des, von der familientypischen Sequenz abweichenden, DHHS-Motivs ausgeschlossen werden. Topologievorhersagen, basierend auf der Aminosäuresequenz, ließen für GABPI ein Sechs-Transmembrandomänen Modell erwarten. Dieses konnte anhand der Untersuchung von verschiedenen Verkürzungs- und Insertionsmutanten in der Immunfluoreszenz nach spezifischer Detergenzbehandlung bestätigt werden. Die Topologie GABPIs ist, analog dem bereits charakterisierten Akr1, die eines Sechs-Transmembrandomänen-Proteins, dessen Amino- und Carboxyterminus, wie auch dessen DHHS-motiv im Cytosol gelegen sind. Aminoterminale Verkürzungen GABPIs zeigten die Fähigkeit zur Aktivierung der β4GalNAcTB, soweit sie die letzten vier Transmembrandomänen enthielten. Die zwei Aminoterminalen Transmembrandomänen sind, obwohl sie eine auch im humanen Homolog konservierte, cysteinreiche Domäne enthalten, für die Aktivierung der β4GalNAcTB nicht erforderlich. Anhand der in diesem Zuge generierten Mutanten konnten außerdem die beiden hinteren, luminal orientierten Loops 3-4 und 5-6 als für den Einfluss auf die β4GalNAcTB verantwortlich identifiziert werden. Alignments von GABPI, dem orthologen humanen zDHHC23 und weiterer Insekten homologer zeigten im Bereich dieser Regionen eine auffällig vom humanen zDHHC23 abweichende, insektenspezifische Konservierung. Die daraufhin durchgeführte, genauere Untersuchung dieser Bereiche über Generierung von Punktmutanten und deren Untersuchung hinsichtlich ihrer Funktion bestätigte die Glutaminsäuren 177 und 178, Histidin 335 und Prolin 346 ebenso wie eine Doppelmutation der Leucine 348/349 als essentiell. Analog wurde die β4GalNAcTB anhand von Hybridkonstrukten mit der GABPI-unabhängigen

β4GalNAcTA und eingebrachten Punktmutationen untersucht. Frühere Hybridmutanten ließen bereits auf die Wichtigkeit der Stammregion des Proteins schließen. Anhand der Untersuchung weiterer Hybridkonstrukte konnte der für die Wechselwirkung verantwortliche Bereich auf die ersten neun transmembrandomänennahen Aminosäuren eingegrenzt werden. Alignments mit weiteren Insektenhomologen GalNAc-Transferasen zeigten wiederum eine auffällige, insektenspezifische Konservierung von hydrophoben Aminosäuren in dieser Region. Demzufolge wurden vier deutlich konservierte Aminosäuren für Punktmutationsexperimente ausgewählt. Es konnte gezeigt werden, dass Phenylalanin 34 und Isoleucin 41 sowie das Zusammenwirken der beiden Tyrosine 38 und 40 in diesem Bereich für die Wechselwirkung zu GABPI unabdingbar sind. Hiermit wurde erstmals eine für die Funktion einer Glykosyltransferase unmittelbar erforderliche Domäne abseits der katalytischen Domäne identifiziert.

Abstract

In this work, we were able to further characterize the recently identified helping factor for activation of the glycolipid specific β4-N-acetylglactosaminyltransferaseB (β4GalNAcTB) from *Drosophila melanogaster* - GABPI, for β4GalNAcTB pilot. The responsible domains for the interaction in both enzymes were identified, analyzed and characterized. For the first time we were thereby able to describe a possible mechanism for establishing the acceptor specificity of a glycolipid specific glycosyltransferase, acting on the initial saccharides of glycolipids. GABPI, a member of the multitransmembrane DHHC protein family, which is needed for the activation as well as for correct translocation to the Golgi apparatus, in contrast to other described DHHC proteins is not a palmitoyltransferase. This activity could be excluded by complete deletion of the eponymous amino acid sequence motif. It actually already lacked the essential cysteine for the palmitoylation reaction, as it contained a natural alteration to DHHS. Topology predictions, based on the primary sequence, made us expect a topology of six transmembrane domains (TMDs). This model was confirmed by generation of various deletion and insertion mutants which were analyzed by immunofluorescence after different detergent treatments. GABPI's topology is analogous to the characterized DHHC protein Akr1. It displays six transmembrane domains, cytosolic amino- and carboxy-termini as well as a cytosolic DHHS motif. Deletions of GABPI from the amino-terminus showed the ability to activate the β4GalNAcTB as long as they displayed at least the last four TMDs. The initial two TMDs were dispensable for the activation. This is remarkable, since they comprise a cysteine rich domain, which is conserved in the human ortholog. By analysis of the generated mutants, we were able to identify the luminal loops between TMD 3 and 4 and between TMD 5 and 6 to be responsible for GABPI's influence on β4GalNAcTB. Alignments of GABPI, the human ortholog zDHHC23 and further insect homologs revealed for these regions a significant insect specific conservation, which clearly differs from the mammalian family members. The following analysis of these conserved motifs by generation of single point mutations revealed glutamic acid 177 and 178 as well as histidine 335 and proline 346 as absolutely essential for the activation of βGalNAcTB, and further LL348/349 to be only inactive as double mutant. In parallel, β4GalNAcTB was analyzed. Hybrids between β4GalNAcTB and the GABPI independent β4GalNAcTA had already shown the importance of β4GalNAcTB's stem region for GABPI interaction. By analysis of further hybrids, the responsible region could be narrowed down to the first nine amino acids close to the TMD.

Again, an insect specific conservation of hydrophobic amino acids was observed in this region. Accordingly, we addressed four amino acids in point mutation experiments. We could show phenylalanine 34 and isoleucine 41 as well as the combination of tyrosine 38 and 40 to be essential for interaction with GABI. Experiments in which GABPI and β4GalNAcTB were fused revealed additional mechanistic insights into the role of GABPI in activating β4GalNAcTB. In summary, were able to show that β4GalNAcTB requires two luminal loops of GABPI and a region in its own stem region as 'add on' domains. Whereas the domains in GABPI were important for interaction with βGalNAcTB, the amino acids in the stem of the transferase are further involved in accomplishing the enzymatic activity.

Chapter 1 - General Introduction

1.1 Glycosylation

The diversity of glycosylated compounds in biological systems is immense. Glycosylated proteins and lipids are involved in various biological processes of signaling, cell-cell adhesion and pathogen-host recognition. To obtain this variety, hundreds of glycosyltransferases are organized in the complex glycosylation machinery, which is localized in the Endoplasmic Reticulum (ER) and the Golgi apparatus (Golgi). Glycosylation is performed while protein and lipid acceptors run through the secretory pathway. The enormous complexity of synthesized glycoconjugates of course requires a demanding synthesizing system. The responsible enzymes to accomplish this are the glycosyltransferases (Coutinho *et al.*, 2003). The comprehension of the organization and interaction of glycosyltransferases and their distinct regulation is of major interest, since the modulations of this system might present powerful tools to influence various cell-cell interaction based processes.

1.2 Glycosyltransferases

Glycosyltransferases catalyze the synthesis of glycans by transferring metabolically activated monosaccharides, mostly in the form of nucleotide sugars, to growing carbohydrate chains on glycoproteins or glycolipids. The majority of glycosyltransferases is localized in the Golgi and exhibits a type II transmembrane protein topology, displaying a short N-terminal tail in the cytosol, a transmembrane domain (TMD), and a luminal stem region followed by the C-terminal catalytic domain (Paulson and Colley, 1989). To ensure the correct biosynthesis of the complex and vast number of glycoconjugates, the glycosylation machinery is not only depending on large numbers of glycosyltransferases, but also on sophisticated mechanisms to ensure the specificity of these enzymes. This is generated by their position in the secretory pathway and by acceptor and donor specificity. Since several glycosyltransferases can differentiate between identical decorated intermediates, there have to be additional features besides the recognition of the terminal monosaccharides. It has been described that generation of substrate specificity may also involve recognition of additional motifs like specific peptide sequences in the elongated glycoprotein (Smith and Baenziger, 1988;Okajima *et al.*, 2005). Although the distinctive domain for the recognition of the substrate and nucleotide sugar binding is located within the catalytic domain of the glycosyltransferase (Kapitonov and Yu, 1999), there is also the possibility of a third protein factor’s influence to alter the specificity of glycosyltransferases. For the human β4galactosyltransferase I, the presence of α-lactalbumine

alters the specificity from N-acetylglucosamine to glucose, resulting in the synthesis of lactose in the mammary gland (Ramakrishnan *et al.*, 2001;Ramakrishnan and Qasba, 2001a;Ramakrishnan and Qasba, 2001b;Ramakrishnan *et al.*, 2002). Such additional motifs, which are required to establish the correct function or specificity of a glycosyltransferase have also been referred to as “add on” domains (Qasba and Ramakrishnan, 2007), and can be found on the glycosyltransferase itself as well as on additional proteins. An additional factor in generation of substrate specificity may also be the local organization. Glycosyltransferases have been shown to be organized in multi enzyme complexes (Giraudo *et al.*, 2001;van Meer, 2001;Giraudo and Maccioni, 2003) which could promote substrate channeling. A peculiar helper factor has been found for the core 1 β3galactosyltransferase. The chaperone Cosmc has been described to be crucial for correct folding of the transferase in the ER, which is required for the transport to the Golgi (Ju and Cummings, 2002).

1.3 Glycosylation of proteins

Proteins can in general be glycosylated in an N-linked or O-linked manner. In the first case, the glycan is coupled to an asparagine residue, which is part of the sequence Asn-X-Ser/Thr. A 14mer oligosaccharide modification is added co-translationally by the oligosaccharyltransferase, directly after the protein leaves the ribosome in the ER (Kelleher and Gilmore, 2006). This initial modification is then followed by processing in the downstream Golgi compartments, leading to characteristic structures of N-linked glycans for each type of cell. In case of O-linked glycosylation the initial attachment is only a monosaccharide which is coupled to the hydroxyl-residue of serine, hydroxyproline, threonine or tyrosine. In some cases, distinct sequence motifs are recognized by the various O-glycan initiating glycosyltransferases, but in other cases recognition is based on non linear structures, not easily predicted from the primary sequence (Jensen *et al.*, 2010). Further saccharides are added in a successive manner, resulting in oligosaccharides of variable length and degree of branching. According to the initiating sugar residue, O-linked glycoproteins are divided into subgroups, which also descent from different cell compartments. Whereas O-mannose, -fucose and -glucose are initiated in the ER, the O-GalNAc is rather occurring in the cis-Golgi (Spiro, 2002).

1.4 Glycosylation of lipids

Glycolipid biosynthesis is initiated by linkage of galactose or glucose to ceramide in the ER. Further elongation is then carried out in the Golgi. The mechanism by which the glycosyltransferases, acting on glycolipids, generate their specificity is rather poorly understood. It is, however, likely that this mechanism is in some way depending on the glycolipid moiety. For the *Drosophila* GlcNAc-transferase brainiac it has been shown that it is able to function as well on vertebrate glycolipids, where it uses Manβ1-4Glcβ1-Cer, as well as on invertebrate glycolipids, where it uses Galβ1,4Glcβ1-Cer. Also knock out flies for egghead, the enzyme transferring the mannose, could be rescued by expression of vertebrate β4galT6, a lactosyl-ceramide synthase, indicating that the downstream enzymes can also act on Galβ1,4Glcβ1-Cer instead of Manβ1,4Glcβ1-Cer (Wandall *et al.*, 2005). A possible mechanism for generating substrate specificity has been proposed earlier and is introducing an accessory factor (Ramakrishnan *et al.*, 2002;Qasba *et al.*, 2008). A model of such membrane associated cofactor is also supported by the analogy to the glycolipid degradation machinery, where the sphingolipid activation protein (SAP or GM2-AP) presents the glycolipid substrate to the glycosidases in the lysosomes. The sphingolipid is in this case bound to a carbohydrate

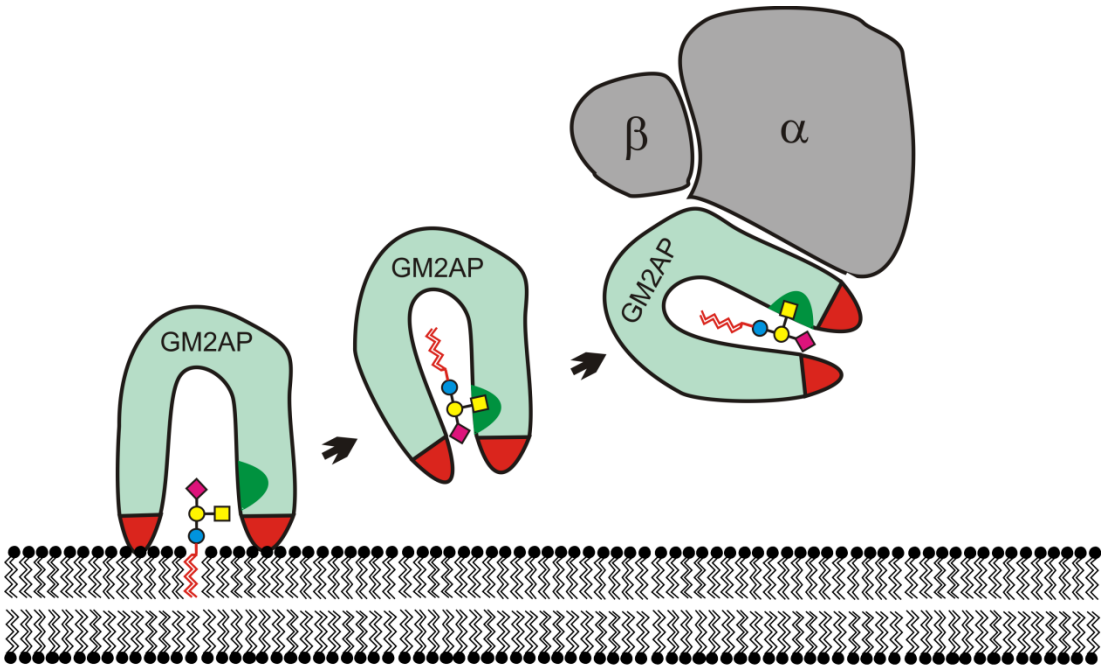

Figure 1: **Model of GM2-activator stimulated hydrolysis of gangliosides by β-hexosaminidase A**. The GM2-activator protein (GM2-AP) can bind to the membrane by hydrophobic loops (red). GM2 is bound to the carbohydrate recognition site (green) and lifted into the hydrophobic cavity of GM2-AP. The GM2 binding induces a conformational change and the more hydrophilic closed conformation of the complex leaves the membrane to present GM2 to the glycosidase (grey). Figure adapted and modified from T. Kolter, K. Sandhoff 2010 (Kolter and Sandhoff, 2010)

recognition site of the GM2-AP and then lifted into a hydrophobic cavity of the protein. In the loaded state, GM2-AP changes its conformation and transports the sphingolipid to the glycosidases (Kolter and Sandhoff, 2005;Kolter and Sandhoff, 2006;Kolter and Sandhoff, 2010). In all described models a prerequisite for enzymes to act on the initial sugars of glycolipids is the capability to dislocate them from the membrane, either by themselves or by help of a cofactor. For the initiating steps of N-glycans and O-glycans, as well as for glycolipid biosynthesis, specialized enzymes are deployed. However, as chains grow, enzymes requiring only a specific sugar structure as acceptor might act on both types of glycoproteins and on glycolipids.

1.5 The β4galactosyltransferase gene family

The metazoan β4GalT family consists of mainly galactosyltransferases (GalTs) and N-acetylgalactosaminyltransferases (GalNAcTs) (Narimatsu, 2006). They transfer their respective monosaccharides from UDP-activated sugars in β,4 linkage to terminal GlcNAc residues, generating Galβ1,4GlcNAc (lacNAc) in case of GalTs or GalNAcβ1,4GlcNAc (lacdiNAc) for GalNAcTs. All six human family members, as well as all other mammalian enzymes, are β4GalTs, whereas all invertebrate members have been shown to be β4GalNAcTs. LacdiNAc is seldom found in vertebrates, where it only appears on specific proteins, like for instance the luteinizing hormone or glycodelin (Roseman and Baenziger, 2000;Seppala *et al.*, 2007). It is found more frequently on glycoproteins and glycolipids in invertebrates. Ramakrishnan and Qasba have shown that only one single amino acid is necessary to distinguish between GalT and GalNAcT activity (Ramakrishnan and Qasba, 2002). The crystal structure for the catalytic domain of bovine β4GalT I in complex with UDP-Gal, in conjugation with α-lactalbumin and as free enzyme has been solved (Gastinel *et al.*, 1999;Ramakrishnan and Qasba, 2001b). The earlier identified conserved sequence motifs FNRA, NVG, DVD and WGW(G/R)EDD(D/E)) (van Die *et al.*, 1997;Amado *et al.*, 1999) were found in the binding pocket, being involved in UDP-Gal and substrate binding. Of six mammalian β4GalTs, β4GalT V and VI have a specific function in glycolipid biosynthesis (Nomura *et al.*, 1998;Sato *et al.*, 2000). Both synthesize the formation of lactosylceramide from glucosylceramide, hence similar to β4GalT I forming free lactose in mammary glands in presence of α-lactalbumin. In addition β4GalT V and VI can also act on terminal GlcNAc-residues, exactly like the four other mammalian β4GalTs (van Die *et al.*, 1999;Sato *et al.*, 2000), indicating that glycolipid specificity might also require a potentially membrane bound cofactor to present the glycolipid to the enzymes.

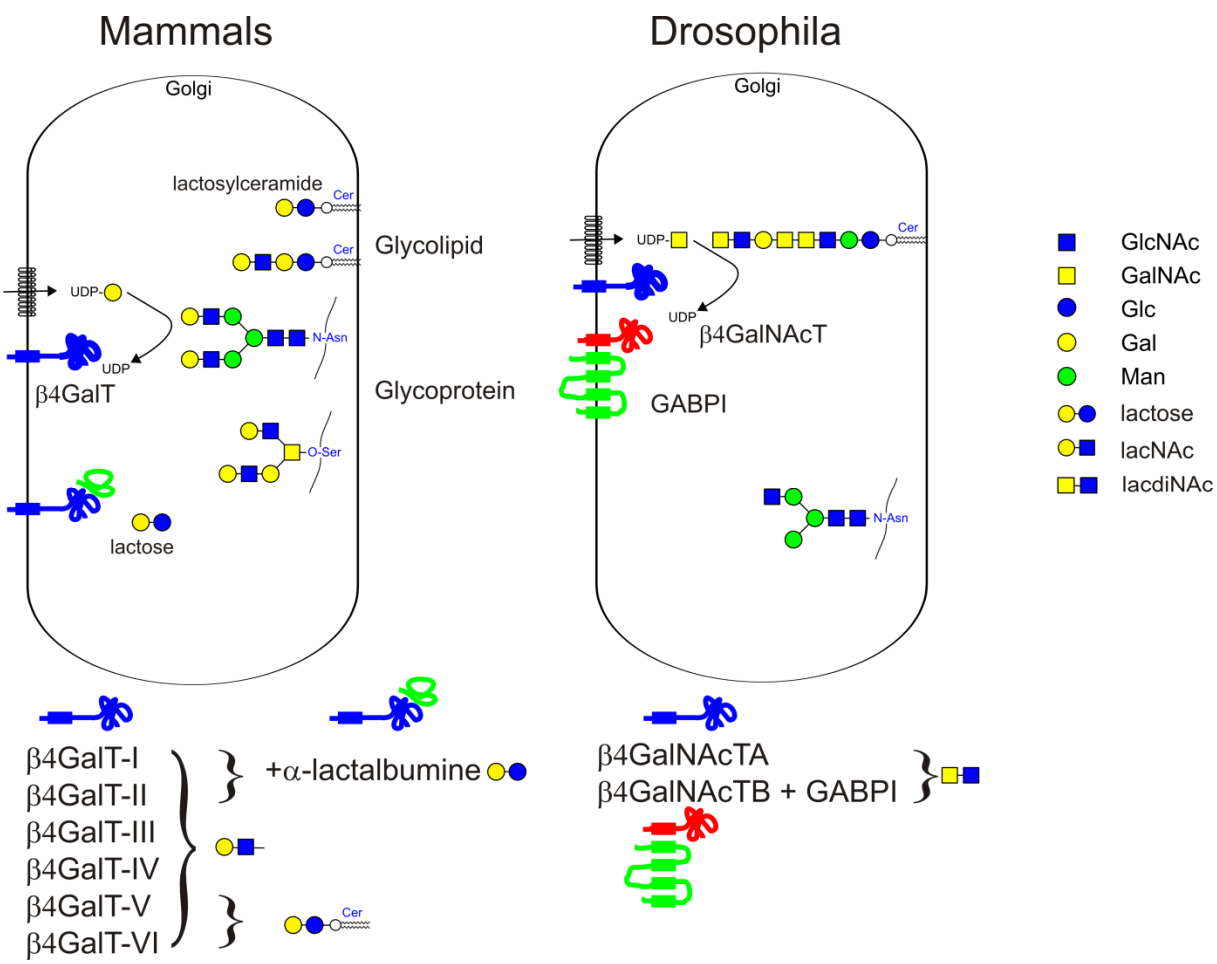

Figure 2: **Members of the β4GalT family**. In mammals, six different galactosyltransferases (β4GalTI-VI) can synthesise the lacNAc epitope. β4GalTI and II in addition are able to function as lactose synthase in conjugation with α-lactalbumine. β4GalTV and VI synthesise lactosylceramide by an unknown mechanism. Two β4GalNAcTs, homologs of the mammalian β4GalTs, generate the lacdiNAc disaccharide in *Drosophila*. The glycolipid specific β4GalNAcTB requires GABPI as a cofactor, whereas β4GalNAcTA is an autonomous enzyme. Figure adapted and modified from Bakker, 2009

1.6 GalNAc-transferases in *Drosophila melanogaster*

There have been three members of the β4GalT family identified in *Drosophila*. One is an ortholog of the mammalian β4GalT VII, transferring galactose to xylosylated proteins to form glycosaminoglycans (Nakamura *et al.*, 2002;Vadaie *et al.*, 2002). The other two enzymes have been described as β4GalNAc-transferases (Haines and Irvine, 2005;Stolz *et al.*, 2007;Johswich *et al.*, 2009), responsible for the formation of lacdiNAc structures. In *Drosophila* the lacdiNAc epitope has so far only been described on glycolipids. *Drosophila* β4GalNAcT was cloned in a heterologous expression cloning system by Dr. Hans Bakker in the group of Prof. Dr. Rita Gerardy-Schahn. A cDNA library was therefore expressed in CHO cells which do not generate the lacdiNAc epitope. Transfected cells were screened for

lacdiNAc expression, using an antibody originally raised against *Schistosoma mansoni* surface glycoconjugates (van Remoortere *et al.*, 2000). In a subsequent sibling selection procedure, Dr. Hans Bakker was able to show that lacdiNAc synthesis depends on the expression of not only one, but of two gene products. One clone encoded the sought after β4GalNAcTB (flybase CG14517), the other clone, however, encoded for a type III membrane protein (flybase CG17257) which was identified as member of the DHHC protein family (Mitchell *et al.*, 2006). The second, homologous, β4GalNAcTA (flybase CG8536) was cloned via polymerase chain reaction (PCR). In contrast to β4GalNAcTB, this enzyme is active independently of any cofactor, also if expressed in a soluble form. For β4GalNAcTB activity was never detected neither for the full length protein, nor for the solubly expressed form (Haines and Irvine, 2005). *Drosophila* mutants, lacking the upstream mannosyltransferase egghead and the GlcNAc-transferase brainiac display a lethal phenotype (Schwientek *et al.*, 2002;Muller *et al.*, 2002;Wandall *et al.*, 2003;Wandall *et al.*, 2005). They show defects in epithelial morphogenesis during oogenesis and embryogenesis (Goode *et al.*, 1992;Goode *et al.*, 1996a;Goode *et al.*, 1996b). Mutants lacking β4GalNAcTA show a presumably neuromuscular phenotype, resulting in abnormal locomotion (Haines and Irvine, 2005;Haines and Stewart, 2007). For the βGalNAcTB mutant, however, only a minor fraction of flies displays abnormal oogenesis. In contrast to the mutants for upstream enzymes, both β4GalNAcT knock outs remain viable and fertile.

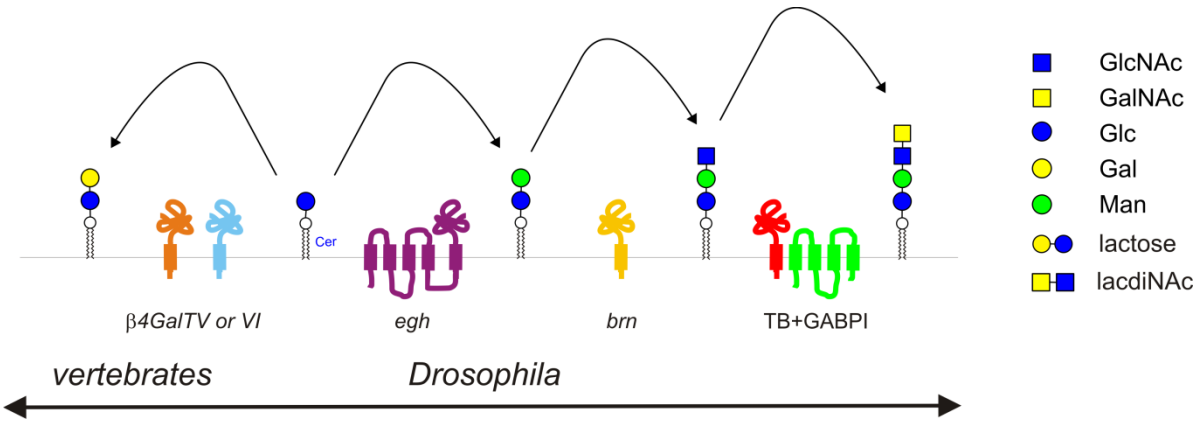

Figure 3: **Initial glycosphingolipid synthesis in *Drosophila melanogaster* and vertebrates.** In *Drosophila*, the initial glucosylceramide is decorated with mannose by the β4mannosyltransferase egghead (*egh*). The β3-N-acetylglucosaminyltransferase brainiac (*brn*) synthesizes the transfer of GalNAc on which the β4-N-acetylgalactosaminyltransferase B (TB) in complex with GABPI adds the GalNAc moiety. In vertebrates, instead of mannose, a galactose is added by the lactosylceramide synthases β4GalTV or VI.

1.7 DHHC protein family

The DHHC protein family in mammalia comprises 23 members. All of them display the eponymous Asp-His-His-Cys peptide motif, which is embedded in a cysteine rich domain (CRD) (Bartels *et al.*, 1999;Lobo *et al.*, 2002;Roth *et al.*, 2002). This motif is a variation of the zinc finger domain.

Figure 4: **Conservation of the DHHC motif amongst GABPI/zDHHC23 orthologs of different species**. Whereas the DHHC motif (red frame) is highly conserved in mammals, there are various alterations of the motif in other species. The homologue in *Schistosoma mansoni* was not found until a BLAST search, focussing on luminal loop regions, was performed. Identical residues: background black, similar residues: grey; threshold: 0,5.

Lobo et al. characterized the *Saccharomyces* ERF2/ERF4 complex to be a palmitoyltransferase (PAT) for the GTPase Ras2. ERF2/ERF4 deficient mutants show a drastic decrease of palmitoylation for Ras2 which, as a result, does not reach the plasma membrane (Jung *et al.*, 1995; Bartels *et al.*, 1999). Since then, an increasing number of DHHC proteins was described to show palmitoyltransferase activity (Linder and Deschenes, 2004; Babu *et al.*, 2004; Mitchell *et al.*, 2006). Palmitoylation may not only increase the membrane affinity of proteins, but also influence the targeting of those. The integrity of the eponymous motif, especially the presence of the cysteine has been shown to be essential for the activity as PAT (Roth *et al.*, 2002;Fukata *et al.*, 2004;Fernandez-Hernando *et al.*, 2006;Mitchell *et al.*, 2006). Despite the essence of this motif for PAT activity, there are also members of the DHHC-family which display alterations of the eponymous motif. *Anopheles gambiae* for instance displays a DHHN and *Apis melliferia* an EYHC. In *Drosophila melanogaster* the recently identified cofactor for β4GalNAcTB, β4GalNAcTB pilot (GABPI) exhibits an alteration to DHHS, which by itself already indicates that a palmitoyltransferase function is rather unlikely. It is in this context noteworthy that the alterations of the DHHC motif are restricted to invertebrates, all vertebrate family members display DHHC.

1.8 Objectives

The mechanism by which glycosyltransferases establish their substrate specificity goes far beyond recognition of the first acceptor saccharides. In several cases there is indication for additional recognition of specific motifs apart from sugars and also for involvement of cofactors. Especially for the glycosyltransferases which synthesize the initial sugars of glycolipids, the recognition is still unsolved. Since the majority of the acceptor molecule, according to its hydrophobic composition, is embedded in the membrane, a possible model for substrate recognition is a hydrophobic patch on the responsible transferase itself, or a hydrophobic cofactor. The glycolipid specific *Drosophila* β4GalNAcTB depends on GABPI for activation. In the first study of this thesis we therefore addressed the effect of GABPI on β4GalNAcTB in *Drosophila* and mammalian cells, applying immunofluorescent staining techniques and MALDI mass spectrometry. The detailed mechanism of the GABPI-β4GalNAcTB interaction remained, however, elusive. Since GABPI and β4GalNAcTB both display areas of conserved hydrophobic amino acids, these motifs and their involvement in the protein-protein interaction were the objective of the second study, being addressed by point mutational analysis.

Chapter 2

Golgi targeting of Drosophila melanogaster β4GalNAcTB requires a DHHC protein family-related protein as a pilot

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Preface – About this manuscript

The *Drosophila melanogaster* β4GalNAcTB is the predominant enzyme for the formation of GalNAcβ1,4GlcNAc residues on Glycoshingolipids. The enzyme itself, however, had not been cloned in an active state. Here we describe the finding of an activating cofactor, which was found during a heterologous expression cloning approach for a functional β4GalNAcTB. We show that the cofactor GABPI is not only essential for activation, but also for correct translocalization of the transferase to the Golgi, which otherwise sticks to the ER. This influence was observed in *Drosophila* S2 cells as well as in a mammalian cell system. We were also able to show that GABPI and β4GalNAcTB form a stable complex by addition of an ER retention signal to GABPI, which leads to retention of active β4GalNAcTB in the ER. My contributions to this study comprise the initial experiments in gene silencing via siRNA in *Drosophila* Schneider cells. These cells were analyzed by surface staining and MALDI MS. I also performed the localization analysis of β4GalNAcTA and β4GalNAcTB in siRNA GABPI downregulated S2 cells. Further I generated the KKTN containing GABPI construct and performed the immunofluorescent analysis.

Golgi targeting of *Drosophila melanogaster* β4GalNAcTB requires a DHHC protein family-related protein as a pilot

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***Drosophila melanogaster* β4GalNAcTB mutant flies revealed that this particular N-acetylgalactosaminyltransferase is predominant in the formation of lacdiNAc (GalNAc β1,4GlcNAc)-modified glycolipids, but enzymatic activity could not be confirmed for the cloned enzyme. Using a heterologous expression cloning approach, we isolated β4GalNAcTB together with β4Gal-NAcTB pilot (GABPI), a multimembrane-spanning protein related to Asp-His-His-Cys (DHHC) proteins but lacking the DHHC consensus sequence. In the absence of GABPI, inactive β4GalNAcTB is trapped in the endoplasmic reticulum (ER). Coexpression of β4GalNAcTB and GABPI generates the active enzyme that is localized together with GABPI in the Golgi. GABPI associates with β4GalNAcTB and, when expressed with an ER retention signal, holds active β4GalNAcTB in the ER. Importantly, treatment of isolated membrane vesicles with Triton X-100 disturbs β4GalNAcTB activity. This phenomenon occurs with multimembrane-spanning glycosyltransferases but is normally not a property of glycosyltransferases with one membrane anchor. In summary, our data provide evidence that GABPI is required for ER export and activity of β4GalNAcTB.**

Introduction

Glycosylation in the secretory pathway is a complex process in which hundreds of glycosyltransferases are involved (Taniguchi et al., 2002). Many glycosyltransferases appear in gene families specified mainly by the nature of the nucleotide sugar donor (Coutinho et al., 2003). Within a given family, individual glycosyltransferases differ regarding the recognized acceptor structures. To understand and modulate cellular glycosylation pathways, it is important to know how this substrate specificity is generated (de Graffenried and Bertozzi, 2004). Important in this respect is the observation that acceptor specificity in many glycosyltransferases is not restricted to recognition of one or a few specifically linked mono-saccharides. Some proteinspecific glycosyl-transferases obtain additional selectivity by recognizing specific peptide motifs in the acceptor. A classic example is the N–acetyl-galactosaminyltransferase (βGalNAcT), which modifies glycoprotein hormones with high selectivity (Smith and Baenziger, 1988). Some glycosyltransferases require other proteins that are not part of the acceptor structure for their specific activity. β1,4-galactosyltransferase (β4GalT) acts on terminally positioned N-acetylglucosamine (GlcNAc) residues conjugated to proteins or lipids. Its specificity changes if it builds a complex with α-lactalbumin. In the complex, free glucose is used as an acceptor, and lactose is formed (Brew et al., 1968). In the case of core 1 β3-galactosyltransferase (C1β3GalT), a molecular chaperone called Cosmc, with specificity for this single client, is required for folding and transportation to the Golgi (Ju and Cummings, 2002, 2005; Ju et al., 2008). Also, for O-mannosylation, two proteins, POMT1 and POMT2, are required (Manya et al., 2004). However, in this case, a two-protein enzymatic complex is proposed. The same is true in heparin sulfate biosynthesis in which two different exostosins are required for efficient biosynthesis (McCormick et al., 2000). For several glycosyltransferases involved in glycolipid biosynthesis, data indicate that factors other than

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the enzyme and the acceptor substrate play a role. This is the case for β4GalT-V and -VI, which are homologues of the β4GalT mentioned in the previous paragraph and of *Drosophila melanogaster* β4GalNAcTB (the subject of this study). Under in vitro conditions, β4GalT-V and -VI transfer galactose (Gal) into β1-4 linkage to terminally expressed GlcNAc residues on glycoproteins (van Die et al.,1999; Guo et al., 2001). However, their involvement in the biosynthesis of lactosyl ceramide (Cer) by Gal transfer onto glucosyl Cer has been demonstrated as well (Nomura et al., 1998 ;Sato et al., 2000; Kolmakova and Chatterjee, 2005). At least in the case of galactosyltransferase V, this latter activity depends on the enzyme's anchorage in the membrane (van Die et al., 1999 ;Sato et al., 2000). Other enzymes involved in glycolipid biosynthesis have been shown to exhibit very low (de Vries et al., 1995 ; Zhu et al., 1998 ; Togayachi et al., 2001) or no (Steffensen et al., 2000; Schwientek et al., 2002) activity if expressed as soluble proteins. In general, very little is known about how lipid acceptors are recognized by glycosyltransferases. However, it has been suggested that a membrane-bound activator protein is required to present glycolipid acceptors to the modifying glycosyltransferases (Ramakrishnan et al., 2002). This hypothesis is substantiated by analogy to the lysosomal sphingolipid degradation machinery in which the sphingolipid activator protein presents the glycolipid substrates to glycosidases (Kolter and Sandhoff, 2005). In this study, we describe a novel mechanism of glycosyltransferase maturation and functionalization for the glycolipidspecific β4GalNAcTB from *Drosophila*. This enzyme, which has been described as an inactive homologue of β4GalNAcTA in a previous study (Haines and Irvine, 2005), is a member of the invertebrate branch of the β4GalT family involved in the biosynthesis of the lacdiNAc (GalNAc β1,4GlcNAc) epitope (Kawar et al., 2002; Vadaie et al., 2002; Haines and Irvine, 2005; Stolz et al., 2008). Because β4GalNAcT had not been cloned when this study was started, we searched for the corresponding activity using expression cloning (Bakker et al., 1997 , 2005; Münster et al., 1998). In a heterologous approach, a cDNA library from *Drosophila* was used for expression in CHO cells, whereas formation of the lacdiNAc epitope was monitored with a specific monoclonal antibody (van Remoortere et al., 2000). As will be demonstrated in this study, the expression of two cDNA clones was required to install the functionally active enzyme.

Results

Expression cloning of a Drosophila β4GalNAcT

Other than in many invertebrates, the lacdiNAc element has been identified on only a few glycoconjugates in mammals (Sato et al., 2003). We ascertained that CHO cells are negative for lacdiNAc. Considering that in mammalian cells terminal GlcNAc residues are recognized by several galactosyltransferases, we additionally hypothesized that signals in the complementation cloning approach could be improved by the use of CHO Lec8 cells. They lack the Golgi UDP-Gal transporter and, consequently, show drastically reduced incorporation of Gal in glycans (Deutscher and Hirschberg, 1986). A cDNA library was constructed from *Drosophila* , subdivided into pools, and, in an established sibling selection procedure (Bakker et al., 1997), used to search for clones that rendered cells positive for lacdiNAc. Cell surface lacdiNAc expression was monitored with antibody 259-2A1, which was originally raised from *Schistosoma mansoni* – infected mice (van Remoortere et al., 2000). In this procedure, it became obvious that two cDNA clones were required for the expression of the lacdiNAc epitope. Although clone one was a member of the β1,4GalT family (van Die et al., 1997), the second clone (flybase CG17257) encoded a type III membrane protein that was related to a gene family referred to as Asp-His-His-Cys (DHHC) proteins (Mitchell et al., 2006). This protein was termed β4GalNAcTB pilot (GABPI) to describe its crucial role in generating a functionally active β4GalNAcTB as shown in the following experiments. The identified β4GalNAcT was identical to the inactive β4GalNAcTB recently cloned by Haines and Irvine (2005) in a homology-based approach. In agreement with this study, we found no in vivo activity for full-length β4GalNAcTB expressed in mammalian cells. It is remarkable that expression cloning identified β4GalNAcTB and not the homologous β4GalNAcTA, which was shown in an earlier study (Haines and Irvine, 2005) to be an active enzyme. To resolve the controversial finding, β4GalNAcTA was cloned by PCR and expressed in comparison with β4GalNAcTB– GABPI in CHO and HEK293 cells. These experiments demonstrated that the cell surface lacdiNAc expression detected with antibody 259-2A1 as a result of β4GalNAcTA was much lower than the lacdiNAc formation after the combined expression of β4GalNAcTB and GABPI. Thus, the data demonstrate the existence of two functionally active β4GalNAcTs (β4GalNAcTA and β4GalNAcTB) in *Drosophila* of which β4GalNAcTB needs the cooperation of GABPI. Because CHO cells demonstrated a low tolerance to expression of the *Drosophila N* -acetylgalactosamine (GalNAc) transferases and HEK293 cells turned out to be a more suitable expression system, subsequent experiments were performed exclusively in HEK293 cells.

β4GalNAcTB specifically modifies glycolipids

Despite elaborated analyses of *Drosophila* glycoproteins (North et al., 2006), the lacdiNAc structure has so far only been found as a modification of glycolipids (Seppo et al., 2000). With both cloned enzymes at hand, we evaluated the question of acceptor specificity. HEK293 cells were

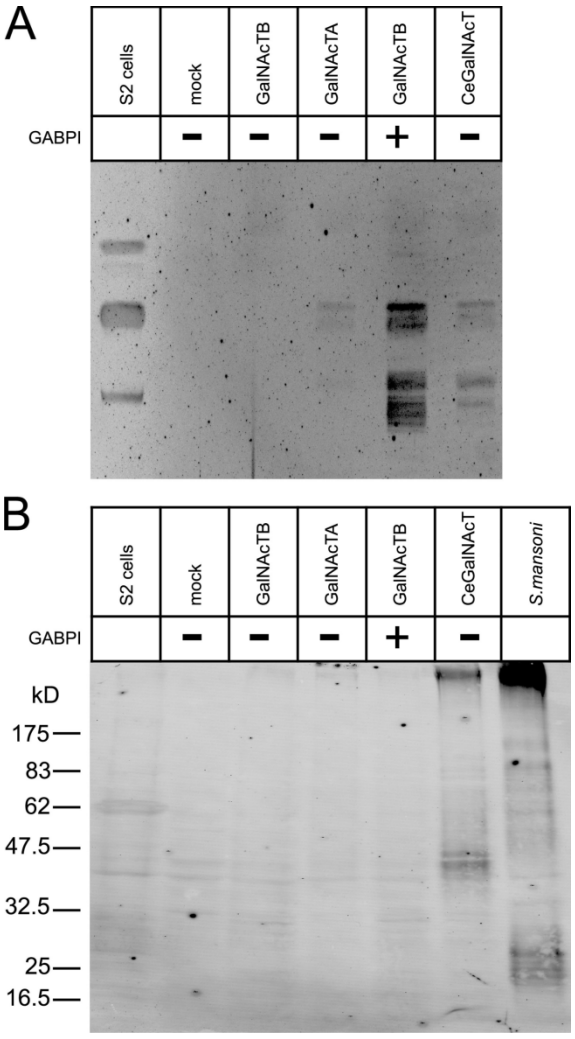

Figure 1. ***Drosophila* -derived β4GalNAcTs are specific for glycolipids.** (A) Glycolipid extracts from S2 cells and HEK293 cells after transfection with empty vector (mock) and GalNAcTs were separated on TLC and immunooverlayed with antibody 259-2A1. (B) Protein extracts from cells as described in A were analyzed by Western blotting with 259-2A1. A protein extract from *S. mansoni* eggs was loaded as a control.

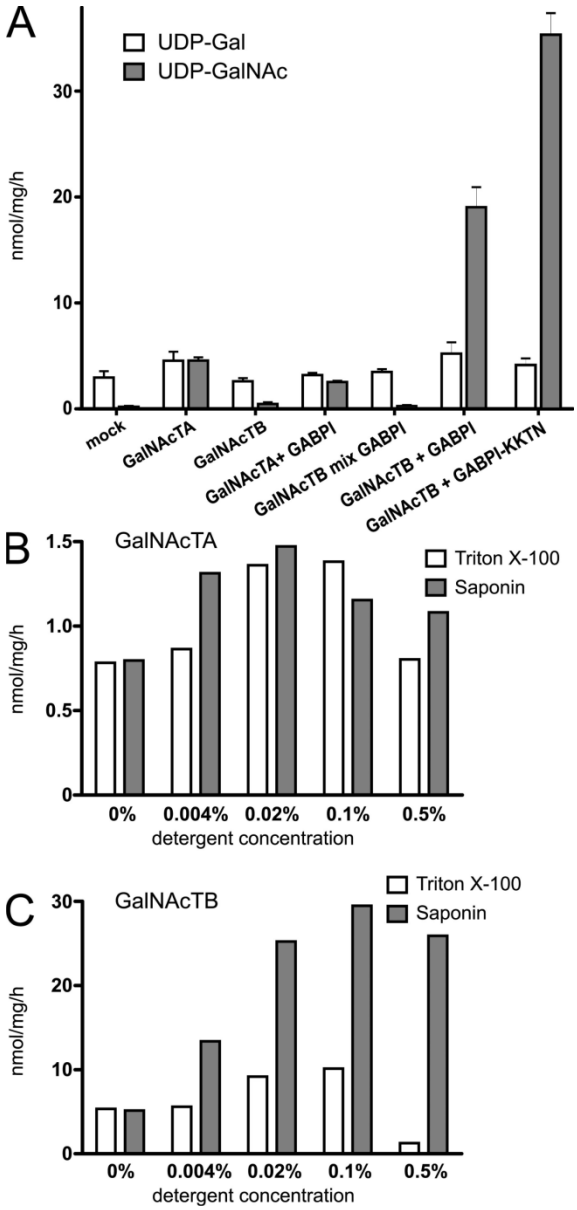

Figure 2. **Activity of β4GalNAcTB is disrupted by Triton X-100.** (A) Microsomal preparations of HEK293 transfected with _ 4GalNAcTs and GABPI as indicated were assayed for activity with GlcNAc-pNP as an acceptor and [3 H]UDP-GalNAc or [3 H]UDP-Gal (endogenous activity as an internal control) as donor substrates. Each value represents the mean of three independent vesicle preparations with standard deviation. GalNAcTB mix GABPI indicates that the proteins were expressed separately but mixed afterward for assays. (B and C) Microsomal fractions of HEK293 cells transfected with β4GalNAcTA (B) or the combination β4GalNAcTB – GABPI (C) were treated with Triton X-100 and saponin in various concentrations and assayed for GalNAcT activity as in A.

transfected with β4GalNAcTA, β4GalNAcTB, or the combination β4GalNAcTB – GABPI and analyzed for the presence of lipid- and protein-bound lacdiNAc using TLC followed by immunooverlay (Fig. 1 A) and Western blotting (Fig. 1 B), respectively. In both systems, *Drosophila* S2 cells, which are naturally positive for the antibody epitope, and HEK293 cells transfected with *Caenorhabditis elegans* β4GalNAcT (Kawar et al., 2002) were used as controls. Although expression of the *C. elegans* enzyme confirmed the availability of β4GalNAcT acceptors on proteins, the absence of specific signals in both HEK293 cells transfected with the β4GalNAcTs and in S2 cells confirmed the earlier observations in flies. In contrast, immunostaining of the lipid extracts resulted in positive signals for S2 cells as well as for HEK293 cells transfected with the β4GalNAcTB–GABPI pair. Expression of β4GalNAcTB alone was not sufficient to produce a signal, whereas faint signals were reliably obtained with β4GalNAcTA. It is important to mention that lipid specificity is preserved, although the glycolipid acceptor structures are different in *Drosophila* and HEK293 cells.

In vitro activity of β4GalNAcTB – GABPI is detergent sensitive

The observations that β4GalNAcTB is inactive if it is separately expressed in a heterologous cell system or if it is tested as a recombinant soluble protein

(Haines and Irvine, 2005) encouraged further analyses to examine at which step β4GalNAcTB and GABPI interact with each other in the biosynthesis. In the first experiment, it was established that a soluble secreted construct of β4GalNAcTB was still inactive when coexpressed with GABPI. Subsequently, we wondered whether the two proteins expressed in separate cells have the capacity to form an active enzyme. Microsomal fractions of HEK293 cells transfected with either β4GalNAcTB or GABPI were isolated, mildly treated with detergent (saponin 0.01%), and functionally tested in mixtures. The assay system used to follow β4GalNAcT activity was adapted from an established assay (Palcic et al., 1988). In this assay system, [^{3}H]UDP-GalNAc is the donor, and GlcNAc – *p*-nitrophenyl (pNP) is the acceptor substrate. No GalNAc transfer was measured in mixed vesicles (Fig. 2 A), whereas controls with microsomes of β4GalNAcTA – or β4GalNAcTB – GABPI-transfected HEK293 cells were active after identical treatment with detergent. In accordance with lacdiNAc formation in intact cells, β4GalNAcTB was not active when expressed alone, but it showed higher activity than β4GalNAcTA when expressed in combination with GABPI. In contrast to β4GalNAcTA, the β4GalNAcTB activity strongly depended on the detergent used. Only background levels were measured if membranes were treated with Triton X-100 (Fig. 2 B) or NP-40 (not depicted) at 0.5%, which is routinely used in glycosyltransferase assays. The milder detergent saponin increased activity over a wide concentration range (Fig. 2 B). The rather low activity measured in the absence of detergent was probably a result of limited transport of the substrates over the vesicle membranes. As saponin is known to perforate and not disrupt membranes (Schulz, 1990), these data suggest that the maintenance of protein complexes in intact membrane patches is required for 4GalNAcTB activity.

ER export of β4GalNAcTB requires GABPI

The data presented so far for the interaction between β4GalNAcTB and GABPI are highly reminiscent of the interactions between the human C1β3GalT generating the T antigen (core 1 O -glycan Gal 1-3GalNAc α1-Ser/Thr) and its client-specific molecular chaperone, Cosmc (Ju and Cummings, 2002). Cosmc supports functional folding of C1β3GalT in the ER but then dissociates and releases C1β3GalT (Ju et al., 2002b; Ju et al., 2008). Therefore, the following experiments addressed the subcellular localization of GABPI and β4GalNAcTs. Flag-β4GalNAcTs and Myc-GABPI were separately expressed in HEK293 cells and, after selection of stable clones, were detected by indirect immunofluorescence. Flag-β4GalNAcTA and Myc-GABPI colocalized with the Golgi marker α-mannosidase II (Fig. 3, A and C).Only the signal generated by Flag-β4GalNAcTB overlapped with the ER marker calnexin (Fig. 3 B). However, when GABPI was cotransfected (Fig. 3, D – F), the immunofluorescence images showed a clear shift of β4GalNAcTB to the Golgi. Moreover, as shown in Fig. 3 F, GABPI and β4GalNAcTB colocalized in this compartment. This experiment demonstrated that ER export of β4GalNAcTB needs piloting by GABPI, which by itself is an autonomous protein fully equipped with the information required for folding and transport to the Golgi. As GABPI moves with β4GalNAcTB, the question was raised whether both proteins remain associated in the Golgi. To answer this question, GABPI was tagged with a C-terminal KKTN dilysine signal (Zerangue et al., 2001), which retains proteins in the ER. GABPI was indeed successfully localized in the ER using this approach (Fig. 3 G). More importantly, β4GalNAcTB was also retained in

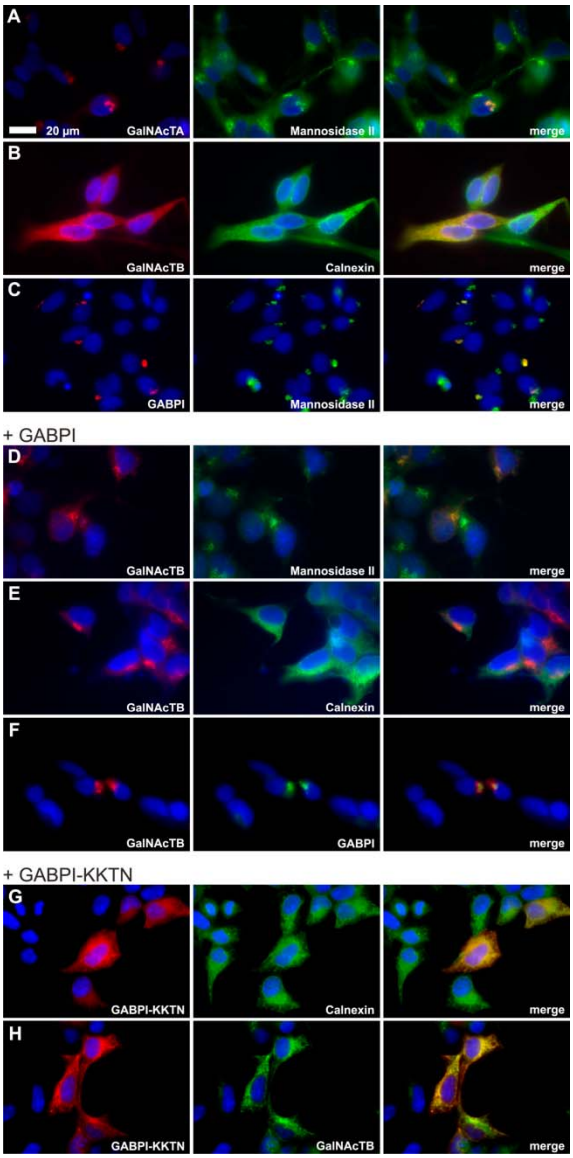

Figure 3. **The Golgi resident protein GABPI pilots β4GalNAcTB to the Golgi apparatus.** The subcellular localization of β4GalNAcTs and GABPI was analyzed with N-terminally tagged proteins expressed in HEK293 cells. (A – C) Flag- β4GalNAcTA, -B, and Myc-GABPI were visualized by indirect immunofluorescence (red). Subcellular compartments were labeled with anti –α-mannosidase II (Golgi) or calnexin (ER; green). (D – F) Flag-β4GalNAcTB (red) coexpressed with Myc-GABPI (F, green). (G) Subcellular localization of Myc-GABPI containing an N-terminal KKTN sequence. (H) Flag-β4GalNAcTB (green) coexpressed with Myc-GABPI – KKTN (red). Nuclei were stained with Hoechst 33258 (blue).

the ER in cells expressing KKTN-tagged GABPI (Fig. 3 H). In vitro enzymatic activity of β4GalNAcTB was about two times as high as the non retained construct (Fig. 2 A, right bars), and cell surface lacdiNAc was also detectable in these cells. Although the latter probably required cycling to the Golgi of at least part of the enzyme, ER retention might have allowed a higher protein expression level that was enzymatically active in vitro. Together, these data demonstrated that the DHHC family – related protein has, in contrast to Cosmc, functions that go beyond those of a client specific chaperone.

Depletion of GABPI in Drosophila S2 cells delocalizes GalNAcTB and reduces lacdiNAc-containing glycolipid formation

To additionally evaluate the influence of GABPI on β4GalNAcTB localization in the natural environment, RNAi experiments were performed. S2 cells were transiently transfected with N-terminally tagged β4GalNAcTs, and localization of the enzymes was monitored. As shown in Fig. 4 A, both β4GalNAcTs were colocalized in vesicular structures presumed to be the Golgi. β4GalNAcTB did not show any overlap with the ER-specific antibody anti-HDEL (Fig. 4 B). Incubation of cells with doublestranded RNA (dsRNA; Clemens et al., 2000) corresponding to a central coding region of GABPI dissected the HA-β4GalNAcTB signal from Flag-β4GalNAcTA (Fig. 4 C) and shifted the signal to structures that are part of the ER (Fig. 4 D). To answer whether the RNAi-induced redistribution of the enzyme is also followed by a change in activity, a second knockdown experiment was performed in which dsRNAs were designed to down-regulate β4GalNAcTA, β4GalNAcTB, or GABPI. S2 cells were cultured for 3 d in the absence or presence of dsRNA, after which the expression of lacdiNAc was displayed by immunocytochemistry and matrix-assisted laser desorption/ionization (MALDI) time of flight (TOF) mass spectrometry (MS), as illustrated in Fig. 5 . The intense staining of control cells with antibody 259-2A1 was in accordance with the detection of lacdiNAc-containing glycolipid structures by negative-ion mode MALDI-TOF-MS. β4GalNAcTA knockdown did not change the signal pattern in comparison with control cells, whereas depletion of either 4GalNAcTB or GABPI had comparably strong effects on lacdiNAc expression. In the negative-ion mode, reduction of glycolipids carrying lacdiNAc repeats was accompanied by an enrichment of GlcNAcβ,3Galβ,3GalNAcα, 4GalNAcβ,4(PE-6) GlcNAcβ,3Manβ,4GlcβCer species with a molecular mass of 1,958.4 D, an acceptor structure for β4GalNAcT. The positive-ion mode analyses clearly demonstrated the accumulation of a second β4GalNAcT acceptor structure, GlcNAcβ,3Manβ,4GlcβCer, having a molecular mass of 1,087.6 D. Changes in the glycolipid structures are very similar to changes observed in β4GalNAcTB knockout flies (Stolz et al., 2008) and thus are not further addressed in this paper. In addition, a new glycolipid species carrying

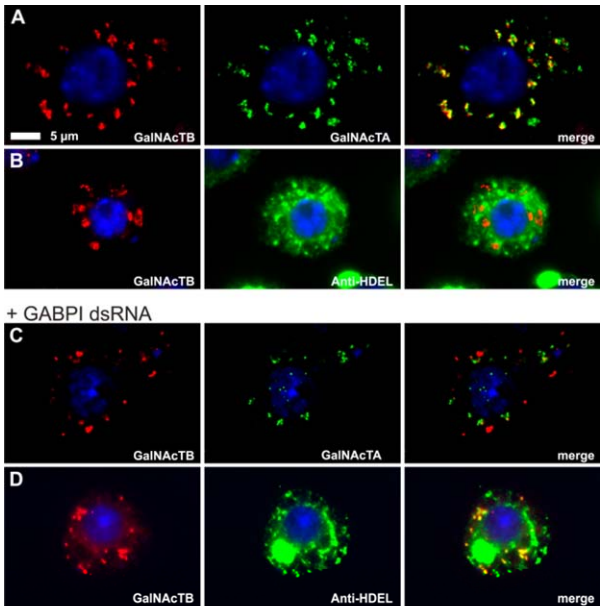

Figure 4. **Knockdown of GABPI in S2 cells interferes with Golgi localization of β4GalNAcTB.** (A – D) S2 cells after 3 d of culture in the absence (A and B) or presence (C and D) of dsRNA directed against GABPI were transiently transfected with Flag-β4GalNAcTA and HA-β4GalNAcTB. The Flag and HA epitopes were detected 2 d after transfection using respective antibodies, and the ER was marked with anti-HDEL. Nuclei were stained with Hoechst 33258.

lacdiNAc repeats with a molecular mass of 1,796.3 D has been identified and characterized by MALDI-TOF/TOF-MS (Table S1 and Figs. S1 and S2, available at http://www.jcb.org/cgi/content/full/jcb.200801071/DC1) as well as two extended species (Table S1 and Fig. S3). In summary, the results presented in Figs. 4 and 5 allow the conclusions that (a) β4GalNAcTB is the major lacdiNAc-synthesizing enzyme in S2 cells as it is in the fly, (b) GABPI enables Golgi targeting of β4GalNAcTB, and (c) β4GalNAcTB is essentially required to convey functionality.

Pull down of the complex formed between β4GalNAcTB and GABPI

Because the data shown so far consistently argue for the existence of β4GalNAcTB and GABPI as a complex in the Golgi, we examined this contact in pull-down experiments. HEK293 cells transiently transfected with cDNA constructs encoding Myc-GABPI – HA and Flag-β4GalNAcTA and -B were lysed with buffer containing 1% NP-40. The anti-HA antibody 12CA5 coupled to Sepharose beads was used to precipitate Myc-GABPI – HA. To control the expression of recombinant proteins, total cell lysates were analyzed by Western blotting in parallel to precipitated proteins (Fig. 6). Both proteins were well expressed, as shown in the total cell lysates, whereas only Flag-β4GalNAcTB and not Flag-β4GalNAcTA was precipitated via Myc-GABPI – HA. This provided additional evidence for a tight interaction between β4GalNAcTB and GABPI.

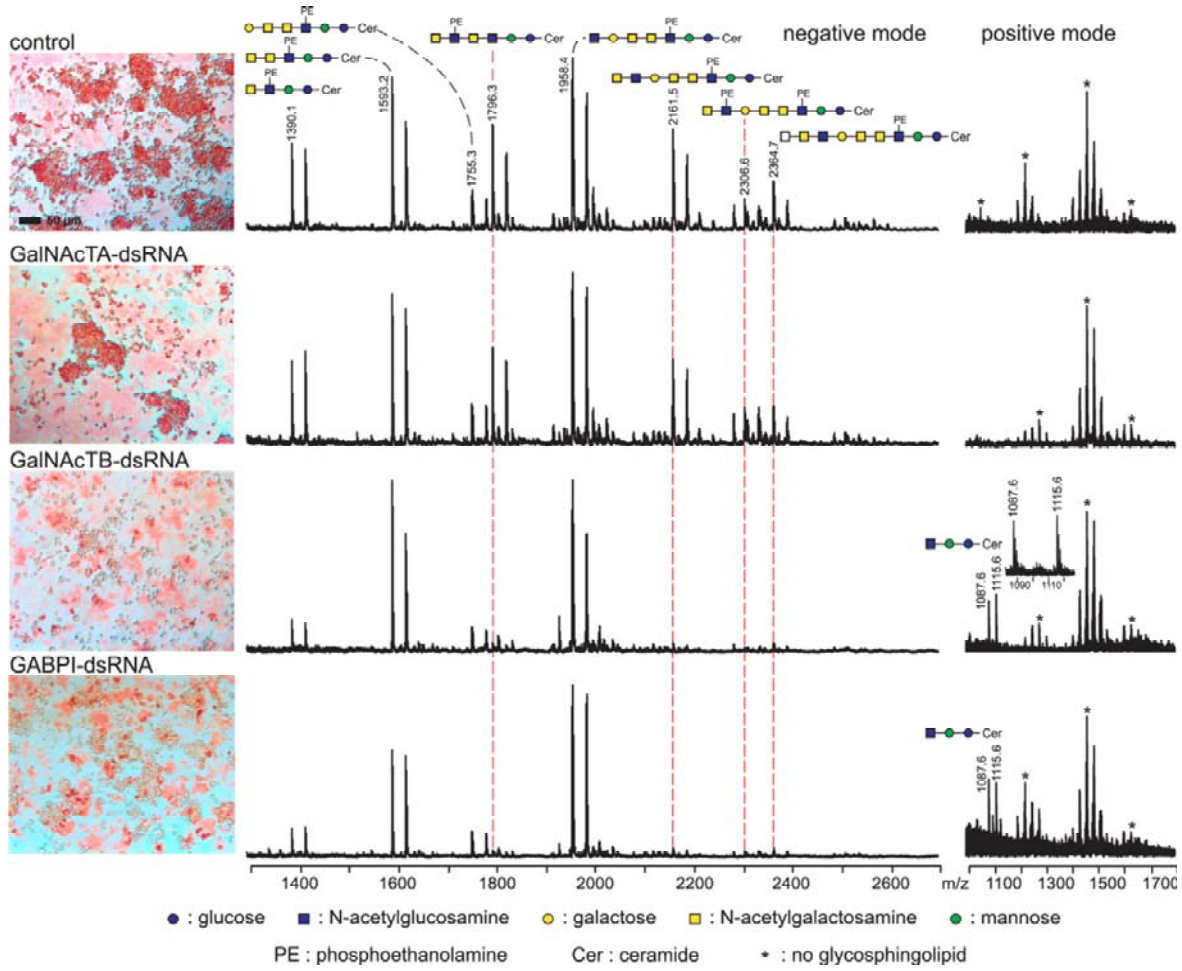

Figure 5. **Knockdown of GABPI in S2 cells abrogates β4GalNAcTB activity.** S2 cells before and after dsRNA down-regulation of β4GalNAcTs and GABPI as indicated were stained with antibody 259-2A1 to display lacdiNAc structures on the surface. Extracted glycolipid fractions were analyzed by MALDITOF-MS in negative- and positive-ion mode. Glycolipid species carrying multiple lacdiNAc structures that show differential expression are connected by dashed red lines. The positive-mode spectra show accumulation of the β4GalNAcT trisaccharide precursor.

The DHHC family – related protein GABPI is not an acyltransferase

Characterized DHHC protein family members are palmitoyltransferases in which the cysteine residue in the conserved DHHC motif is essential for activity (Lobo et al., 2002; Roth et al., 2002; Valdez-Taubas and Pelham, 2005). GABPI, in contrast to all mammalian and the other *Drosophila* members in the family, has exchanged this motif from DHHC to DHHS. This was already an argument against its function as acyltransferase. To validate this assumption, a series of mutants was constructed with which a potential involvement of the DHHS motif in GABPI functions could be tested. The ability of GABPI to install a functional β4GalNAcTB was not abolished by reconstruction of the DHHC motif, by replacement of the serine by alanine, or by successive replacement to AAAA (unpublished data). In addition, the critical cysteine residue (C29) that may serve as acyl residue acceptor in β4GalNAcTB was mutated. Again, no effect on GABPI – β4GalNAcTB Golgi localization and activity was found (unpublished data).

The stem region of β4GalNAcTB is needed for activation by GABPI

Because the β4GalNAcTs isolated from *Drosophila* are highly homologous proteins, it was of relevance to identify primary sequence elements responsible for the strict GABPI dependency of β4GalNAcTB. The aligned primary sequences indicated the stem region to be the domain of highest variability. Consequently, hybrids were made by domain swapping, as shown in Fig. 7 . The chimera in which cytoplasmic and transmembrane domains of β4GalNAcTA were added to stem and catalytic regions of β4GalNAcTB (hybrid A-B-B) remained GABPI dependent for Golgi localization (Fig. 7, A and B) and activity (not depicted). However, additional replacement of the stem region destroyed activation by GABPI. The resulting protein was inactive and retained in the ER (Fig. 7, C and D). Because the stem region in β4GalNAcTA is considerably longer than in β4GalNAcTB, additional constructs were prepared in which the size was trimmed from the N and C termini to the exact length of the β4GalNAcTB stem region. All constructs remained inactive (unpublished data), allowing the conclusion that information contained in the stem region of β4GalNAcTB is essential for

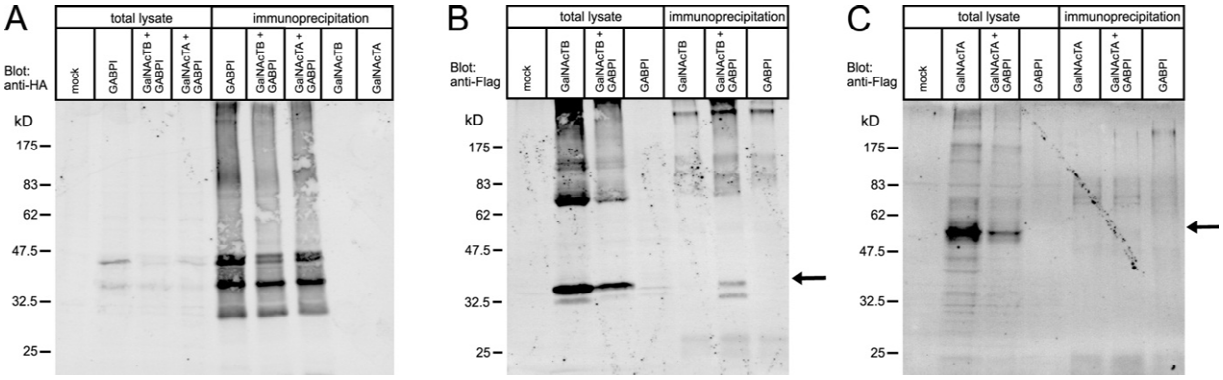

Figure 6. **Myc-GABPI – HA coprecipitates Flag-β4GalNAcTB.** HEK293 cells transiently transfected with Myc-GABPI – HA and Flag-β4GalNAcTA or Flag-β4GalNAcTB as indicated were lysed and precipitated with mouse monoclonal anti-HA (12CA5) coupled to Sepharose A beads. (A – C) Precipitated proteins as well as total cell lysates were displayed on Western blots using rabbit anti-HA (A) or mouse anti-Flag antibody (B and C). In spite of similar expression levels seen in total cell lysates for the Flag-tagged β4GalNAcTs, immunoprecipitation of Myc-GABPI – HA pulled down only Flag- β4GalNAcTB. Arrows indicate the running position of Flag- β4GalNAcTB and Flag- β4GalNAcTA in B and C, respectively.

its function. In contrast, β4GalNAcTA remained Golgi localized and active independently of GABPI when fused to the cytoplasmic and stem region of β4GalNAcTB (construct B-B-A; Fig. 7 E). This is in agreement with the fact that the catalytic domain of β4GalNAcTA can be produced as soluble enzyme and, therefore, is an independent active entity.

Discussion

Using a heterologous expression cloning approach, we isolated β4GalNAcTB as the major enzyme responsible for the biosynthesis of lacdiNAc structures in *Drosophila* . In this study, this enzyme was demonstrated to depend on the cooperation of a multimembrane-spanning protein related to the DHHC protein family. To point out the complexity of its involvement in forming a functionally active β4GalNAcTB, it was called GABPI. GABPI was cloned simultaneously to β4GalNAcTB in a classical expression cloning approach. This demonstrates the power of this technique, which exclusively screens for activity. The fact that the second β4GalNAc transferase in *Drosophila*, β4GalNAcTA (Haines and Irvine, 2005), was not detected in the expression cloning approach is a result of the much lower activity of this enzyme, which we confirmed in in vitro and in cellular test systems as well as on the systemic level (Stolz et al., 2008). Although β4GalNAcTA has been shown to act on protein acceptors in vitro (Sasaki et al., 2007), the low activity of β4GalNAcTA in HEK293 cells observed in this study does not allow a conclusion on the nature of the acceptor. Obviously, β4GalNAcTB is strictly lipid specific. This specificity is remarkable because glycan structures added to lipid anchors are different between mammals and flies. This suggests that selectivity is at least partly established through the lipid anchors. GABPI might be involved in the lipid specifi city, but β4GalNAcTB also requires GABPI for in vitro activity with the synthetic acceptor substrate GlcNAc-pNP. In particular, these types of small hydrophobic aglycon-linked monosaccharide acceptors usually overcome the restricted specificity of glycosyltransferases; even the glycoprotein hormone-specific β4GalNAcT is reactive with such acceptors (Smith and Baenziger, 1988). Trials to assemble an active enzyme by combining vesicle preparations containing β4GalNAcTB and GABPI separately failed. This was also the case for the O-mannosyltransferases (Manya et al., 2004). This shows that GABPI and β4GalNAcTB do not act in a sequential reaction mechanism. Combined with the experiments in which it was shown that β4GalNAcTB remains in the ER in an inactive state if expressed alone and can only reach the Golgi in the presence of GABPI, it can be concluded that interaction between β4GalNAcTB and GABPI most likely starts in the ER and requires coexpression of the two proteins. Most importantly, GABPI is an autonomous protein equipped with all of the information needed for Golgi destination. In this respect, GABPI seems to be different from Cosmc, the client-specific molecular chaperone required to activate C1 β3GalT. A soluble, active form of recombinant C1β3GalT can be produced (Ju et al., 2002a), although Cosmc is not associated with this enzyme (Ju and Cummings, 2002). Purified rat liver C1β3GalT was also devoid of Cosmc (Ju et al., 2002b). According to the classical definition of a chaperone, Cosmc releases an active C1β3GalT (Ju et al., 2002b , 2008). In contrast, GABPI moves with β4GalNAcTB to the Golgi and retains β4GalNAcTB in the ER if it is retained itself. This and the fact that the proteins can be coimmunoprecipitated argue for a stable complex of both. An insertion of the complex in an intact membrane patch is indispensable for functionality. Proof of this is provided by the fact that β4GalNAcTB activity tested with GlcNAc-pNP was almost completely abolished after addition of Triton X-100 or NP-40. Although these are rather mild detergents that normally do not dissociate protein

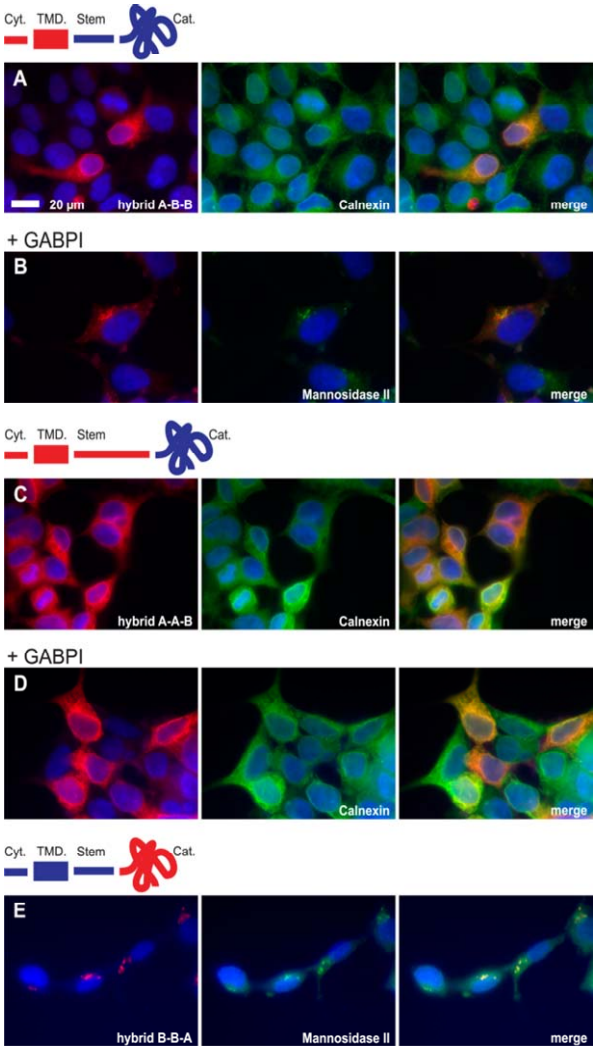

Figure 7. **Protein domains of β4GalNAcTB involved in the interaction with GABPI.** Hybrids of Flag- β4GalNAcTA (red) and -B (blue) were cloned by domain swapping of the cytoplasmic (Cyt) and transmembrane domain (TMD), stem region, and catalytic domain (Cat) as illustrated and expressed in HEK293 with and without GABPI. The intracellular localization was analyzed by indirect immunofl uorescence using α-mannosidase II and calnexin as markers for Golgi and ER, respectively.

complexes, their presence interferes with membrane integrity. This in turn may cause de formation of associated complexes. In contrast, saponin, which only perforates membranes, most likely increased activity by allowing the substrates to enter the vesicles without disturbing the proper embedding of the enzyme in the membrane. Detergent sensitivity is a property of many mannosyltransferases in the ER (Schutzbach, 1997), including the protein O mannosyltransferase complex (POMT1 and POMT2), which is inactivated by Triton X-100 (Manya et al., 2004), and egghead, the mannosyltransferase acting two steps upstream of β4GalNAcTB in *Drosophila* glycolipid biosynthesis (Wandall et al., 2003). These enzymes are multitransmembrane-spanning proteins. Glycosyltransferases of the Golgi containing one transmembrane domain are usually not sensitive to detergents. As β4GalNAcTB is a typical member of the Golgi type II transmembrane glycosyltransferases, the observed detergent sensitivity is expected to be conveyed by disturbance of GABPI or the GABPI – β4GalNAcTB complex. In line with the experiments in HEK293 cells, dsRNA induced knockdown of GABPI in *Drosophila* S2 cells separated β4GalNAcTB from β4GalNAcTA, depleted cell surface expression of the lacdiNAc epitope, and provoked an accumulation of the β4GalNAcT glycolipid acceptor structures. These effects observed at the cellular level were exactly phenocopied in a *Drosophila* mutant with an inactivated β4GalNAcTB gene (Stolz et al., 2008). The knowledge that all functionally characterized DHHC family proteins are palmitoyltransferases prompted experiments designed to determine whether GABPI could function as an acyltransferase. All residues critical for a potential acyltransferase activity in GABPI (Mitchell et al., 2006) as well as the only cysteine residue that may serve as acyl acceptors in β4GalNAcTB were point mutated. None influenced the functionality of GABPI or activity of β4GalNAcTB. The functionally crucial cysteine in the name-giving DHHC motif is exchanged by serine in GABPI, which argues against its function as a palmitoyltransferase. In experiments aimed at understanding how β4GalNAcTB and GABPI interact, we demonstrated that the selectivity with which GABPI activates β4GalNAcTB and not the highly homologous β4GalNAcTA is attributed to a structural element in the stem region. However, this area cannot be the solely responsible element. Additional sequences in the catalytic domain must be involved in determining GABPI dependency. The exact function of GABPI in priming activity of β4GalNAcTB in the Golgi is difficult to address. However, several glycosyltransferases acting exclusively in the glycolipid biosynthetic pathways need membrane anchorage and cannot be expressed as soluble recombinant proteins (Amado et al., 1998 ; Steffensen et al., 2000; Schwientek et al., 2002). One of these enzymes is brainiac (Schwientek et al., 2002), a β3GlcNAc transferase acting right upstream of β4GalNAcTB in glycolipid biosynthesis of *Drosophila*. The factors determining membrane dependency of brainiac are not yet identified. Because we found the product of brainiac accumulated in S2 cells treated with RNAi against GABPI (Fig. 5), an involvement of GABPI for brainiac function in vivo is not likely. However, because mammalian lipid-modifying enzymes have been suggested to form multienzyme complexes (Giraudo and Maccioni, 2003), GABPI, being an essential part of β4GalNAcTB, might be an anchor position in the pathway without being essential for the activity of all enzymes. A striking parallel exists between β4GalNAcTB and β4GalT-V and -VI described in the Introduction. These mammalian galactosyltransferases are members of the same gene family and are essentially dependent on membrane contact for transfer of Gal onto glucosyl Cer (van Die et al., 1999; Sato et al., 2000). As soluble enzymes, β4GalT-V and -VI recognize terminal GlcNAc residues instead of glucose.

Therefore, it can be speculated that these enzymes require a cofactor similar to GABPI, which mediates glycolipid acceptor recognition. Orthologues of GABPI are found in arthropod and vertebrate species but not in nematodes, indicating that GABPI homologues might play a role in higher eukaryotes as well. In summary, it can be concluded that the identification of GABPI reveals a novel mechanism to generate specificity in the complex glycosylation pathway.

Materials and methods

Expression cloning
A cDNA library from *Drosophila* larval poly(A) + RNA (Clontech Laboratories, Inc.) was constructed in pCMV-Script using the pCMV-ScriptXR cDNA library construction kit (Agilent Technologies). The library was divided into pools of 10,000 independent clones and used for expression cloning after the sibling selection strategy described previously (Bakker et al., 1997 , 2005). The CHO cell line Lec8 (Deutscher and Hirschberg, 1986) grown in α-MEM supplemented with 10% FCS (both obtained from Biochrom AG) was used as the host. Pools or clones of the cDNA library were transfected into Lec8 cells using Metafectane (Biontex). After 2 d, cells grown in 6-well plates were fixed with 1.5% glutaraldehyde, incubated with the anti lacdiNAc monoclonal antibody 259-2A1 (van Remoortere et al., 2000) followed by HRP-conjugated goat anti – mouse antibody (Jackson Immuno-Research Laboratories), and detected by tyramide signal amplification using biotin-tyramide (Speel et al., 2006), streptavidin-AP (Invitrogen), and Fast-Red (Sigma-Aldrich) as chromogenic substrate.

Plasmid constructs
All tagged mammalian expression constructs were made in pcDNA3 (Invitrogen). Myc-GABPI (flybase gene number CG17257) contains an N-terminal Myc tag (MAQKLISEEDLNLRPLE [antibody-bound sequence underlined]) and Myc-GABPI – HA, an additional C-terminal HA tag (SR YPYDVPDYASL). Flag- β4GalNAcTB (CG14517), Flag- β4GalNAcTA (CG8536), and *C. elegans* GalNAcT (Kawar et al., 2002) contain N-terminal Flag tags (MDYKDDDDKGS). The Myc-GABPI – KKTN construct was cloned by PCR using Myc-GABPI as a template. For expression in *Drosophila* S2 cells, Flag- β4GalNAcTA and HA-β4GalNAcTB (N-terminal HA tag; MYPYDVPDYAGS) were cloned in pIB/V5-His (Invitrogen). Hybrids of β4GalNAcTA and β4GalNAcTB are identified by a three-letter code, whereby the first letter indicates the cytoplasmic plus transmembrane region, the second letter indicates the stem region, and the third letter indicates the catalytic domain (e.g., A-B-B). Borders between the three regions are after amino acids 29 and 135 in β4GalNAcTA and after 33 and 65 in β4GalNAcTB. Flag- or Myc-tagged constructs were used for all experiments unless indicated.

Preparation of ER and Golgi fractions from transfected HEK293 cells
HEK293 cells were grown in DME/HAM' s F-12 supplemented with 10% FCS (both obtained from Biochrom AG). Cells transiently transfected as described in the Expression cloning section for CHO cells were washed with PBS and collected by centrifugation (5 min at 1,500 *g*). The cell pellets from three 175-cm 2 plates (9 × 10 7 cells) were resuspended in 7 ml of lysis buffer (10 mM Hepes-Tris, pH 7.4, 0.8 M sorbitol, and 1 mM EDTA) containing an EDTA-free protease inhibitor mixture (Roche). After 10 strokes in a Dounce homogenizer, the lysate was centrifuged (10 min at 1,500 *g*). The supernatant was collected, and the pellet was subjected to a second homogenization/centrifugation round. The ER/Golgi-rich fraction was obtained by centrifugation of the combined supernatants at 100,000 *g* for 1 h. Pelleted vesicles were resuspended in 500 µ l of assay buffer (0.1 M MOPS, pH 7.5) and 20- µ l aliquots kept at _ 80 ° C. Protein concentrations were determined using a bicinchoninic acid kit (Thermo Fisher Scientific).

In vitro _ 4GalNAcT assays
Standard enzyme assays were performed with 20 µl of the ER/Golgi preparations in 50 µ l of assay buffer (0.1 MOPS, pH 7.5, 20 mM $MnCl_2$, 10 mM ATP, 100 mM GalNAc, 0.1% BSA, and 0.01% saponin). Therefore, 20- µl aliquots of the ER/Golgi vesicle preparation were supplemented to obtain the appropriate buffer composition and 0.5 mM of the radio-labeled nucleotide sugars UDP-6[^{3}H]Gal (specific activity of 32 Bq/nmol; GE Healthcare) or UDP-1[^{3}H]GalNAc (specific activity of 36 Bq/nmol [PerkinElmer]; diluted with cold nucleotide sugars [Sigma-Aldrich]). Reactions were started by adding the acceptor substrate GlcNAc-pNP (Sigma-Aldrich) at 1 mM and were incubated for 2 h at 28 ° C. Control samples were incubated in the absence of GlcNAc- *O* -pNP and subtracted from measured values. Reactions were stopped by addition of 1 ml of ice-cold water, and products were isolated on columns (Sep Pak Plus C 18; Waters Corporation) as described previously (Palcic et al., 1988). The elutes were dried and counted in 2 ml of scintillation cocktail (Luma Safe Plus; Lumac LSC). Incorporated radioactivity was measured in a counter
(LS 6500; Beckman Coulter).

Analyses of glycosphingolipids and proteins from transfected HEK293 cells
Transiently transfected HEK293 cells were washed with PBS, scraped off the plates, and collected by centrifugation (10 min at 1,500 *g*). *Drosophila* S2 cells were harvested by centrifugation and extracted in the same way. The cell pellets (10^7 cells) were resuspended in 300 µl of water and sonicated for 5 min in a bath sonicator. 2-propanol and hexane were added to obtain a solvent ratio of 55:25:20 (2-propanol/hexane/water), and the mixtures were sonicated again for 5 min. Samples were centrifuged for 10 min at 1,500 *g* , and supernatants were dried under nitrogen. The extracts were resuspended in chloroform/methanol/water (3:47:48) and desalted by reverse-phase chromatography (Sep Pak Plus C 18 columns; Williams and McCluer, 1980). The eluted glycosphingolipids were dried under nitrogen, and one fourth of each sample was spotted onto a TLC plate (Nano-Durasil-20; Macherey-Nagel) and developed in running solvent composed of chloroform/methanol/0.25% aqueous KCl (5:4:1). For immunostaining, the silica plate was fi xed in 0.1% polyisobutylmethylacrylate (Sigma-Aldrich) in aceton. The plate was blocked overnight with 1% BSA in TBS at 4 ° C followed by incubation with primary antibody (mouse antilacdiNAc 259-2A1) for 2 h at room temperature and with secondary antibody goat anti – mouse IRDye 800 (LI-COR Biosciences) for 30 min. After washing, the plate was analyzed on an infrared imaging system (Odyssey; LI-COR Biosciences). Protein samples for Western blotting were isolated from the same cells by dissolving 10 7 cells in 750 µ l of lysis buffer (2 mM EDTA, 50 mM Tris-HCl, pH 8.0, 1 mM MgCl 2 , and 1% NP-40 supplemented with a protease inhibitor mixture [Roche]) and analyzing 20 µ l of these samples by standard Western blotting techniques. The blot was incubated with the same antibodies as the TLC plate and analyzed in the same way.

Subcellular localization studies by immunofluorescence
Subcellular localizations of recombinant Flag- β4GalNAcTA, Flag- β4GalNAcTB, and Myc-GABPI were performed with stably transfected HEK293 cells (without selecting clones). Therefore, transfected cells were cultured for 3 wk in the presence of G-418 (EMD). For staining, cells were seeded onto glass coverslips, fi xed in 4% PFA, and permeabilized for 30 min with 0.1% saponin in PBS containing 0.1% BSA. Samples were incubated with the respective primary antibodies (anti-Flag tag M5, anti-Flag tag F7425, anti-HA tag 12CA5 or anti-Myc tag 9E10, and rabbit anti – α-mannosidase II or -calnexin as a Golgi or ER marker) for 1.5 h at room temperature. After three washings (in PBS, 0.1% BSA, and 0.1% Tween 20), cells were incubated with anti – mouse IgCy3 and anti – rabbit IgG Alexa Fluor 488 for 1 h at room temperature. After staining with the nuclear dye (Hoechst 33258; Hoechst Pharmaceuticals), the slides were washed with water, mounted (Dako), and analyzed under a microscope (Axiovert 200M; Carl Zeiss, Inc.) using a Plan Apochromat 63 × /1.40 oil differential interference contrast objective (M27; Carl Zeiss, Inc.) at room temperature. Images (1,388 × 1,040 pixels) were taken using a camera (AxioCam MRm; Carl Zeiss, Inc.) and Axiovision 4.4 software (Carl Zeiss, Inc.). Images taken in an automatic exposure setting with filter sets for Hoechst 33258, Alexa Fluor 488, and Cy3 were converted in blue, green, and red, respectively; intensities were adapted to be equal for the three colors, and images were reduced to 600 dots per inch for display in Figs. 3 and 7 .

Knockdown experiments in *Drosophila* S2 cells
dsRNA was made using the MEGAscript T7 transcription kit (Applied Biosystems). Each primer used in the PCR contained a 5 _ T7 RNA polymerase – binding site followed by sequences specifi c for the target genes: GABPI (5 _ -CCGGCACCTCCAATTTTCTTTC-3´ and 5´-GTCCATATCCCCCACCTCGTCA-3´), β4GalNAcTA (5´-ATGTACCTCTTCACCAAGGCGA-3´ and 5´ ATAACCAATGTTCATCATGGCA- 3´), and β4GalNAcTB (5´-TCAACTTTTCCTGCCAACAATG-3´ and 5´ ACCACGCCGCCGAAAAGACC-3´). *Drosophila* Schneider (S2) cells were grown in Schneider ' s *Drosophila* medium (Invitrogen) supplemented with 10% FCS and 4 mM L- glutamine (Biochrom AG). For RNAi knockdown experiments (Clemens et al., 2000), 10^6 cells were plated per 6 wells in serum-free medium, and dsRNA of β4GalNAcTA, _ GalNAcTB, and/or GABPI was added directly to the media in a final concentration of 37 nM (15 µ g). After 30 min at room temperature, 2 ml of Schneider ' s medium containing FCS was added, and incubation was continued for 3 d at 27 ° C. For the immunocytochemical analysis of surface-expressed lacdiNAc structures, the protocol described in the Expression cloning section for CHO cells was used. Light microscopic images of Fig. 5 were taken using the aforementioned microscope and software using a camera (AxioCam MRc; Carl Zeiss, Inc.) and a Plan Apochromat 10 × /0.45 objective (Ph1M27; Carl Zeiss, Inc.). To determine the subcellular localization of β4GalNAcTA and _ 4GalNAcTB in GABPI dsRNA – treated cells, the N-terminally Flag- and HA-tagged enzymes were transiently transfected with Fugene (Roche) into cultures that had been treated for 3 d with dsRNA. The day after transfection, cells were washed with serum-free medium, and RNAi treatment was repeated with a concentration of 18.5 nM dsRNA. 2 d after transfection, cells were transferred to concanavalin A – coated coverslips for 1 h, fixed in 4% PFA, and further processed as described in the previous section for HEK293 cells except that 0.1% saponin was kept in all incubation and washing solutions. Mouse anti-Flag M5 (Sigma-Aldrich) in combination with rabbit anti-HA (Sigma-Aldrich) was used to visualize the tagged β4GalNAc transferases, whereas the ER was stained with mouse anti-HDEL (Santa Cruz Biotechnology, Inc.). Secondary antibodies used were goat anti – mouse, Alexa Fluor 488, and goat anti – rabbit Cy3. Fluorescent images were made using a microscope (Axiovert 200M) as for the aforementioned HEK293 cells except that the ApoTome mode was used and fi ve images were averaged. Fig. 4 shows 330 × 236-pixel sections of the original images. In addition, glycosphingolipid extracts (prepared as described in Analyses of glycosphingolipids and proteins) from S2 cells before and after dsRNA treatment were analyzed by MALDI-TOF-MS in a TOF/TOF mass spectrometer (Ultraflex II; Bruker Daltonics) as described previously (Wuhrer and Deelder, 2005 ; Stolz et al., 2008).

Immunoprecipitation
Transiently transfected HEK293 cells were lysed for 30 min at 4 ° C using 750 µ l of lysis buffer (2 mM EDTA, 50 mM Tris-HCl, pH 8.0, 1 mM $MgCl_2$, and 1% NP-40 supplemented with protease inhibitor mixture). After centrifugation for 30 min at 12,000 *g* , anti-HA antibody 12CA5 coupled to Sepharose A beads was added to supernatants and incubated for 3 h at 4 ° C on a rotating wheel. Immunocomplexes were pelleted by centrifugation (300 *g* for 5 min) and washed twice with 50 mM Tris-HCl, pH 8.0, and 1% NP-40, twice with 50 mM Tris-HCl, pH 8.0, 500 mM NaCl, and 1% NP-40, and once with the fi rst washing buffer. Immunoprecipitated proteins were separated in SDS-PAGE, blotted onto polyvinylidenedifluoride membranes (Waters Corporation), and stained with mouse anti-Flag M5 or rat anti-HA antibody.

Online supplemental material
Table S1 shows newly registered zwitterionic glycosphingolipid species. Fig. S1 shows negative-mode MALDI-TOF-MS of S2 cell glycosphingolipids.
Fig. S2 shows MALDI-TOF/TOF-MS fragmentation analysis of two zwitterionic glycolipid species containing lacdiNAc tandem repeats.
Fig. S3 shows MALDI-TOF/TOF-MS analysis of two zwitterionic glycolipid species.

Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.200801071/DC1.

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Supplemental material

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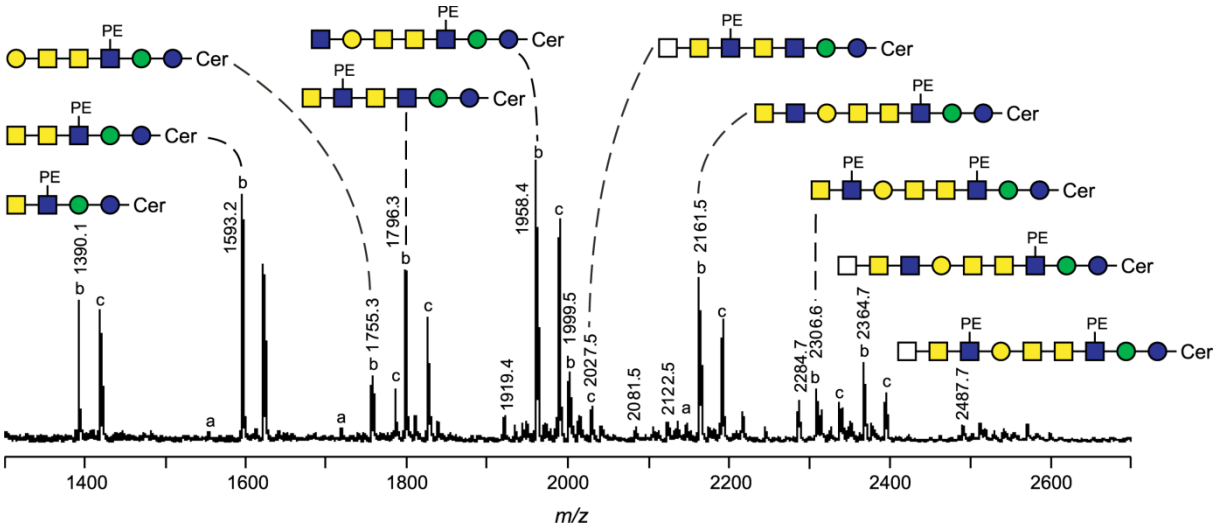

Figure S1. **Negative-mode MALDI-TOF-MS of S2 cell glycosphingolipids.** Glycosphingolipid extracts of nondepleted S2 cells were analyzed by negative-ion mode MALDI-TOF-MS in the reflectron mode using 2,5-dihydroxybenzoic acid as matrix for sample preparation. A list of newly registered masses and assigned glycosphingolipid structures compared with known GSL structures in *Drosophila* is given in Table S1. b, glycosphingolipid with a Cer composition of C14:1 tetradecasphingenine and C20:0 arachidic acid; a and c, glycosphingolipid with Cer masses that are 28 D (two methylene groups, C2H4) lower (a) or higher (c) than for b. Blue circles, glucose; green circles, mannose; yellow circles, galactose; yellow squares, GalNAc; blue squares, GlcNAc; white square, *N*-acetylhexosamine; PE, phosphoethanolamine; m/z, mass/charge.

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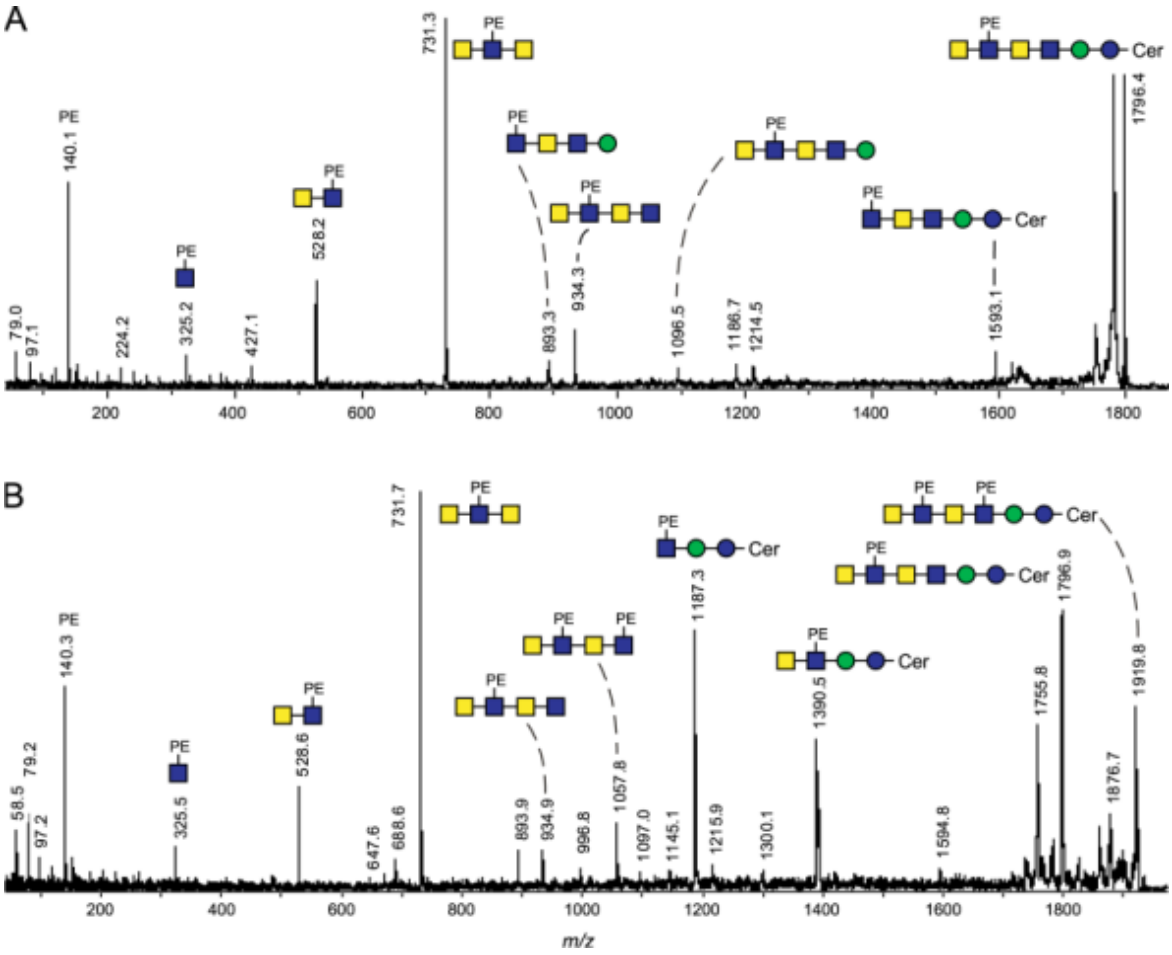

Figure S2. **MALDI-TOF/TOF-MS fragmentation analysis of two zwitterionic glycolipid species containing lacdiNAc tandem repeats.** (A and B) Zwitterionic glycosphingolipid species with a hexasaccharide glycan moiety (mass/charge = 1,796) with one phosphoethanolamine (PE) modification (A) and the hexasaccharide glycan moiety (mass/charge [m/z] = 1,919) containing two phosphoethanolamine residues (B) were predicted to contain two hexose and four *N*-acetylhexosamine residues. Fragmentation patterns by MALDI-TOF/TOF-MS (in deprotonated form using 2,5-dihydroxybenzoic acid as matrix) confirmed the presence of a tetra *N*-acetylhexosamine sequence with one or two phosphoethanolamine modifications, which can only be interpreted as a repeat of two lacdiNAc units. Blue circles, glucose; green circles, mannose; blue squares, GlcNAc.

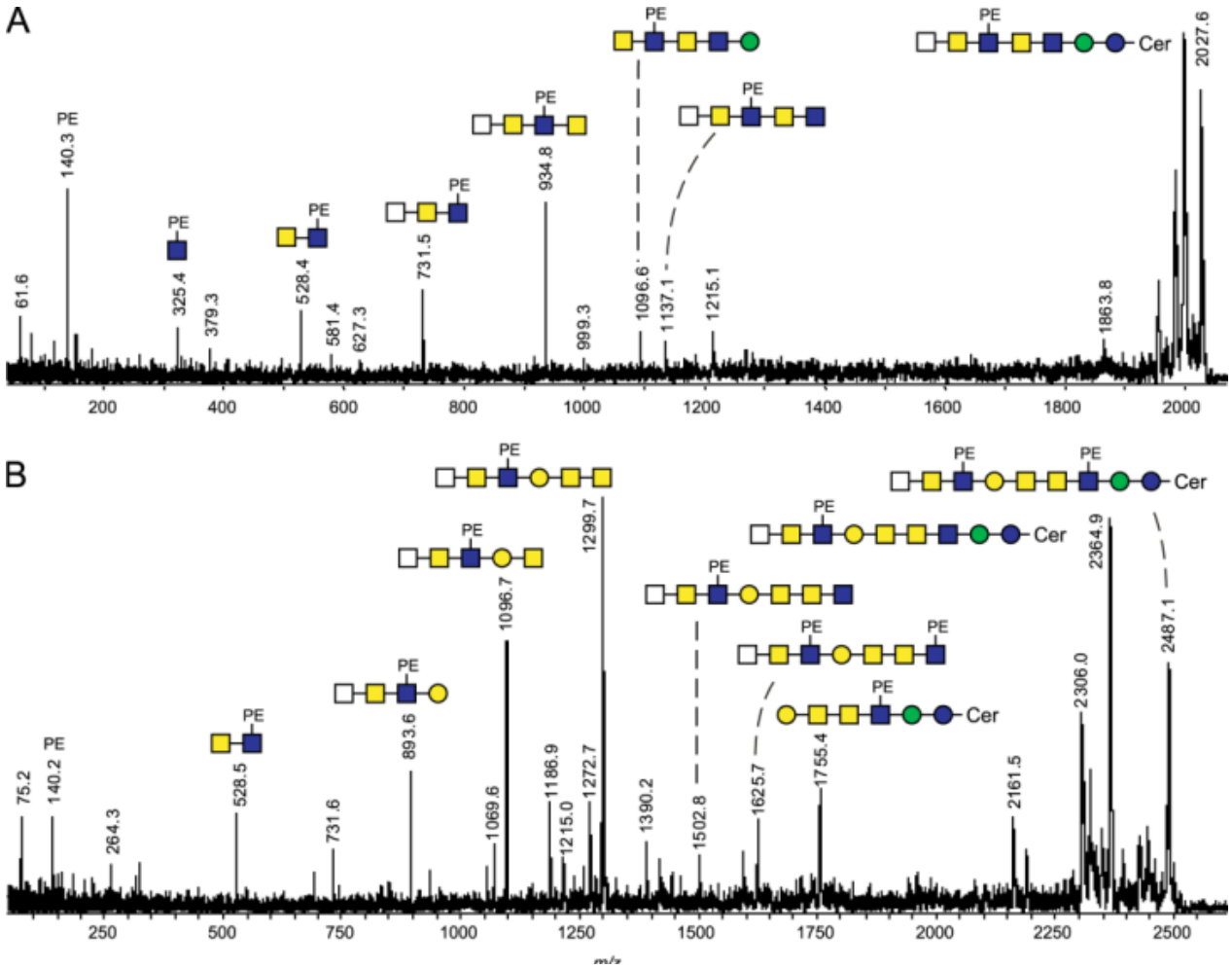

Figure S3. **MALDI-TOF/TOF-MS analysis of two zwitterionic glycolipid species.** (A and B) Zwitterionic glycosphingolipid species with five *N*-acetylhexosamine residues (mass/charge [m/z] = 2,027; A) and six *N*-acetylhexosamine sugars (mass/charge = 2,487; B) were analyzed by MALDITOF/ TOF-MS in a deprotonated form using 2,5-dihydroxybenzoic acid as a matrix. Fragmentation patterns confirmed the presence of an additional terminal *N*-acetylhexosamine residue compared with known structures present in *Drosophila*, probably a GalNAc residue in a-1,4 linkage in analogy to other *Drosophila* zwitterionic glycosphingolipid structures. Blue circles, glucose; green circles, mannose; yellow circles, galactose; yellow squares, GalNAc; blue squares, GlcNAc; white square, *N*-acetylhexosamine; PE, phosphoethanolamine.

Table S1. **Newly registered zwitterionic glycosphingolipid species**

Zwitterionic GSL	Proposed structure	Registered mass
		D
Nz6*	GalNAcβ,4(PE-6)GlcNAcβ,3GalNAcβ,4GlcNAcβ,3Manβ,4GlcβCer, GalNAcβ,4GlcNAcβ,3GalNAcβ,4(PE-6)GlcNAcβ,3Manβ,4GlcβCer	1,796.3/1,824.4[a]
$Nz_2$6*	GalNAcβ,4(PE-6)GlcNAcβ,3GalNAcβ,4(PE-6)GlcNAcβ,3Manβ,4GlcβCer	1,919.4
Nz7*	(HexNAc1-)GalNAcβ,4GlcNAcβ,3GalNAcβ,4(PE-6)GlcNAcβ,3Manβ,4GlcβCer	1,999.5/2,027.5[a]
$Nz_2$7*	(HexNAc1-)GalNAcβ,4(PE-6)GlcNAcβ,3GalNAcβ,4(PE-6)GlcNAcβ,3Manβ,4GlcβCer	2,122.5
Nz9*	(HexNAc1-)GalNAcβ,4(PE-6)GlcNAcβ,3Galβ,3GalNAcα,4GalNAcβ,4(PE-6)GlcNAcβ,3Manβ,4GlcβCer	2,364.7
$Nz_2$9*	(HexNAc1-)GalNAcβ,4(PE-6)GlcNAcβ,3Galβ,3GalNAcα,4GalNAcβ,4(PE-6)GlcNAcβ,3Manβ,4GlcβCer	2,487.7

GSL, glycosphingolipid. Zwitterionic glycosphingolipids were registered as $[M-H]^-$ species and are alternative structures to those described by Seppo et al. (2000).

[a]Glycosphingolipid with a Cer species with a 28-D higher mass, which reflects two additional methylene groups in the Cer moiety.

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Preface – About this manuscript

The glycolipid specific *Drosophila melanogaster* β4GalNAcTB, responsible for the formation of GalNAcβ1,4GlcNAc on glycosphingolipids is not an autonomous enzyme. It depends on the previously described cofactor GABPI for translocation as well as for activity. GABPI's function is assumed to go beyond the classical chaperone function, since it was shown to interact with β4GalNAcTB not only in the ER, but also to pilot the transferase to the Golgi apparatus. Here we further investigate the character of the GABPI β4GalNAcTB interaction. We analyze GABPI's membrane topology and describe three insect conserved, remarkably hydrophobic domains. Two of them are found on GABPI, positioned in the luminal loop regions between transmembrane domains 3 and 4 and also between transmembrane domains 5 and 6. The third motif is part of β4GalNAcTB's stem, near the transmembrane domain. According to a generated functional fusion protein of both enzymes, these domains might be found in close proximity in the active complex of both enzymes. In all three motifs, we were able to find specific amino acids, essential for activity by single point mutation. We therefore propose these regions to be "add on" domains – motifs, essential for the function of the enzyme complex, but afar the catalytic domain. My contribution to this study comprises the experiments to solve GABPI's membrane topology and orientation. Further, I performed the analysis of truncation constructs to find GABPI's minimal functional entity, and also the experiments addressing the conserved GABPI loop domains and their involvement in β4GalNAcTB activation. Finally Dr. Hans Bakker and I wrote the manuscript.

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Chapter 3

'Add-on' Domains of Drosophila β1,4-N-Acetylgalactosaminyltransferase B in the Stem Region and its Pilot Protein

Condensed title: 'Add-on' Domains of β4GalNAcTB

Keywords: DHHC /GABPI/glycolipid/glycosyltransferase

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Summary

The glycolipid specific *Drosophila melanogaster* β1,4-N-Acetylgalactosaminyltransferase B (β4GalNAcTB) depends on a zinc finger DHHC Protein family member named GalNAcTB pilot (GABPI) for activity and translocation to the Golgi. The 6 membrane spanning protein actually lacks the cysteine in the cytoplasmic DHHC motif, displaying DHHS instead. We show here that the whole conserved region around the DHHS sequence, which is essential for palmitoylation in DHHC proteins, is not required for GABPI to interact with β4GalNAcTB. In contrast, the two luminal loops between transmembrane domain 3-4 and 5-6 contain conserved amino acids which are crucial for activity. Besides the dependence on GABPI, β4GalNAcTB requires its exceptional short stem region for activity. A few hydrophobic amino acids positioned close to the transmembrane domain are essential for the interaction with GABPI. Along with its catalytic domain, β4GalNAcTB, thus, requires an area in its own stem region and two small luminal loops of GABPI as "add-on" domains. Moreover, some inactive GABPI mutants could be rescued by fusion with β4GalNAcTB, indicating their importance in direct GABPI-β4GalNAcTB interaction.

Introduction

Little is known on what motivates Golgi localized glycosyltransferases to act on glycolipid acceptors. It has been observed that several glycosyltransferases require membrane anchoring to act on glycolipids (Amado *et al.*, 1998;Steffensen *et al.*, 2000;Schwientek *et al.*, 2002). We have recently reported that this is also the case for *Drosophila melanogaster* β1,4-N-aceteylgalactosaminyltransferase B (β4GalNAcTB), which in addition requires a second protein named GalNAcTB pilot (GABPI) for activity. Without GABPI, β4GalNAcTB remains in the endoplasmic reticulum (ER) in an inactive state and upon co-expression resides as a complex with GABPI in the Golgi (Johswich *et al.*, 2009). β4GalNAcTB acts exclusively on glycolipids, whereas a second, homologous *Drosophila* enzyme, β4GalNAcTA, also shows activity on glycoprotein acceptors and works independently from GABPI or its transmembrane domain (Haines and Irvine, 2005;Sasaki *et al.*, 2007;Johswich *et al.*, 2009). Although both enzymes are able to generate the lacdiNAc (GalNAcβ1,4GlcNAc) glycotope on glycolipids, β4GalNAcTB does this, in the presence of GABPI, more efficiently than β4GalNAcTA. Also mutant flies have revealed that β4GalNAcTB is the predominant enzyme for glycolipid biosynthesis (Stolz *et al.*, 2007). Both enzymes show the typical type II membrane topology for Golgi glycosyltransferases, with a short N-terminal cytoplasmic domain and a stem region between transmembrane and C-terminal catalytic domain. The most obvious difference between the two GalNAc-transferases is the length of the stem region. β4GalNAcTB has an extraordinary short stem region and is inactive when it is exchanged for the stem of β4GalNAcTA.

GABPI is a member of the DHHC protein family. This family consists of multi-transmembrane proteins which present a variant of the zinc finger domain (Putilina *et al.*, 1999) in a cytoplasmic loop. It is defined by the eponymous Asp-His-His-Cys (DHHC) motif, embedded in a cysteine-rich domain. Characterized DHHC protein family members have been shown to be palmitoyltransferases, acting on cysteine residues in the cytoplasmic part of proteins (S-palmitoylation) (Linder and Deschenes, 2007). The DHHC motif is required for *in vitro* and *in vivo* activity. Mutations in this amino acid sequence, especially replacement of the cysteine residue, abolish protein acyltransferase activity (Lobo *et al.*, 2002;Roth *et al.*, 2002;Linder and Deschenes, 2004;Fernandez-Hernando *et al.*, 2006;Nadolski and Linder, 2007). The DHHC sequence and the cysteine-rich domain are conserved throughout eukaryots and multiple members of the DHHC protein family exist in each species (Dietrich and Ungermann, 2004;Mitchell *et al.*, 2006). Although clearly a member of the DHHC protein family, GABPI shows a unique alteration of the eponymous DHHC motif to DHHS. It is striking that the DHHC motif on the one hand is highly conserved (all 23 human and all other 16 *Drosophila* family members show the DHHC sequence), but in most of GABPI's insect orthologs mutated in various ways. Since the motif itself has been shown to be crucial for palmitoyltransferase function, such activity was rather unlikely for GABPI. Experiments in which the complete DHHS sequence was changed to AAAA without loss of function and the lack of cysteines in the cytoplasmic domain of β4GalNAcTB strengthened the view that GABPI is not acting as a palmitoyltransferase for β4GalNAcTB (Johswich *et al.*, 2009). GABPI can be considered an 'add-on' protein for a glycosyltransferase. An 'add-on' domain has been

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defined as a protein or part of a protein outside of the catalytic domain of a glycosyltransferase that is required for activity (Qasba and Ramakrishnan, 2007). This can be on a separate protein, like for α-lactalbumine, which modifies the acceptor specificity of β4galactosyltransferase (Ramakrishnan *et al.*, 2001), or on the same protein, for example a lectin binding domain in α-GalNAc transferases (Fritz *et al.*, 2006;Kubota *et al.*, 2006).
To narrow down the regions of GABPI which are directly involved in interaction with β4GalNAcTB, we present the generation of several truncations and point mutations. Analysis of those indicated that two of the six transmembrane domains could be omitted and that two small luminal loops of GABPI were essential for activity, whereas the whole cysteine rich region in the cytoplasmic loop was expendable. Similarly, a small region close to the transmembrane domain in the stem region of β4GalNAcTB was shown to be required for activity. This indicated that β4GalNAcTB requires an "add on" domain as well on GABPI as in its own stem region. In addition, we obtained indications that certain amino acids are directly involved in the interaction between GABPI and β4GalNAcTB as some defective mutants could be rescued by fusion of the two proteins.

Results

Topology of GABPI in the Golgi membrane
Topology analysis of GABPI using TopPred II (Claros and von Heijne, 1994) proposed a topology model of six transmembrane domains (TMDs), predicting the DHHS motif on the cytoplasmic face of the membrane. To investigate and confirm the membrane topology of GABPI, an epitope-insertion approach was used. Therefore, the full length GABPI protein with an amino-terminal Myc and carboxy-terminal HA tag was generated (Myc-GABPI-HA) and expressed in HEK293 cells. Additionally, based on the topology prediction, truncation constructs, in which GABPI was shortened from the N-terminal site between the TMDs, were produced (Figure 1, 1-4).
Immunofluorescence was used to map the orientation of introduced tags. Transfected cells were treated either with the detergent saponin (Figure 1, 1-4 A and 1-4 C) to permeabilize all cellular membranes (Seeman, 1967) or digitonin (Figure 1, 1-4 B and 1-4 D), which is only able to perforate the plasma membrane (Katz and Wals, 1985) and therefore only allows the detection of cytoplasmic tags. These experiments revealed that as well the C-terminus as the N-terminus were cytoplasmic (Figure 4, 1 A-D). A truncation construct lacking the first 30 AA in front of the first predicted TMD (Myc-NΔ90GABPI-HA) remained detectable with saponin and digitonin (Figure 1, 2 A-D), whereas the truncation of the first N-terminal TMD (Myc-NΔ117GABPI-HA) was exclusively visible in saponin treated cells (Figure 1, 3 A-D), indicating a lumenal orientation of the N-terminus in the Golgi-apparatus. By further deletion of the second TMD, the Myc-tag of the construct (Myc-NΔ141GABPI-HA) proved to be cytosoplasmically oriented (Figure 1, 4 A and 4 B). To investigate the orientation of predicted loop regions, GABPI constructs containing internal HA-tags and N-terminal Myc-tags were generated (Figure 1, 5-8). As described above, the tags were detected immunologically in transfected HEK293 cells. Internal HA-tags in the predicted loops 3-4 and 5-6 were shown to be lumenally oriented (Figure 1, 6 A-D and 8 A-D). In contrast, the HA-tags in predicted loop regions 2-3 and 4-5 close to the DHHS motif demonstrated cytosolic orientation of these parts (Figure 1, 5 A-D and 7 A-D).
Taken together, all immunofluorescence experiments confirmed the predicted transmembrane topology model of GABPI. The protein consists of six transmembrane regions with three lumenal and two cytoplasmic loops. The amino- and carboxyterminal end, as well as the DHHS motif is localised in the cytoplasm (see also Figure 2).

GABPI requires four TMDs as minimal functional entity
To determine the minimal functional entity of GABPI, the generated deletion constructs and additional C-terminal deletion constructs (Figure 1, 1-4) were tested for their ability to activate β4GalNAcTB by immunocytochemical staining against the product of β4GalNAcTB, the GalNAcβ1,4-GlcNAc or lacdiNAc glycotope by mAb 259-2A1 (van Remoortere *et al.*, 2000) on the cell surface of transfected HEK293 cells. All deletion constructs containing not less than the last 4 TMDs remained fully functional, whereas further deletions from either side destroyed the ability to activate β4GalNAcTB. The four TMDs also need to be continuous. Expression of TMDs 3+4 and TMDs 5+6 separated in different proteins and co-expressed in HEK293 cells did not restore activity.

Lumenal loop regions are insect conserved and essential for GABPI-β4GalNAcTB interaction
Having determined the membrane orientation and minimal active domain of GABPI, we continued to mutate specific domains. Functional tests of the internal HA-tagged constructs demonstrated that insertions of HA-tags between TMDs 3 and 4, as well as between TMDs 5 and 6 resulted in proteins which were not able to activate β4GalNAcTB any

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more. In contrast, the insertion of an HA-tag in the DHHS loop region did not affect functionality. A sequence alignment of these luminal loop regions of GABPI and related insect DHHC proteins showed high similarity. In contrast, the human ortholog zDHHC23 is significantly different, especially in loop 5-6, which is shorter than GABPI's. For detailed functional analyses addressing the loop regions of GABPI, insect-conserved amino acids, which differ from the human ortholog (Figure 2), were selected for site-directed mutagenesis and constructs were tested for functionality by cell surface staining, using the anti lacdiNAc antibody, and for their ability to pilot β4GalNAcTB to the Golgi in immunofluorescence (Table 1). Additionally, localization studies of GABPI itself were performed to distinguish properly Golgi localized from misfolded ER localized proteins and therefore malfunctioning proteins. All point-mutated constructs, with the exception of the P193A mutation, were still able to reach the Golgi (Table 1). Several of the mutated amino acids in the luminal loops 3-4 and 5-6 of GABPI comprise essential elements for β4GalNAcTB activation. In contrast, mutations of any amino acid of the DHHS motif, as well as depletion of the whole cysteine rich domain did not affect functionality. This confirmed that this sequence, unlike for other DHHC family members, has no relevance for this specific GABPI function (Johswich *et al.*, 2009). Moreover, all inactive mutants, as revealed by cell surface staining for the produced lacdiNAc epitope and by in vitro enzyme assays (according to Johswich *et al.*, 2009; data not shown), where not able to pilot β4GalNAcTB to the Golgi (Table 1).

Some defective GABPI-mutants can be rescued by direct fusion to β4GalNAcTB

When GABPI is directly C-terminally fused to β4GalNAcTB it forms an active complex, which remains, like the separately expressed proteins, sensitive to high detergent concentrations in *in vitro* assays (data not shown). The fusion protein reaches the Golgi apparatus (Figure 3 A) and is able to carry out LacdiNAc synthesis. However, when this protein was co-expressed with a separate β4GalNAcTB construct, it was not able to pilot it to the Golgi any more (Figure 3 B). Thus, GABPI can interact with the fused β4GalNAcTB, but is then occupied and cannot bind a second transferase.

Defective GABPI constructs were fused to β4GalNAcTB as well to estimate if the mutant's defect could be overcome by forcing the two proteins together. The functionality of the H335A and P346A mutants could be rescued by fusion to GalNAcTB, whereas other defective mutants remained inactive after fusion (Figure 4). This indicates that histidine 335 and proline 346 are essential for GABPI-β4GalNAcTB binding but not required for activity if the two proteins are forced together. The other GABPI mutants, which remained inactive as fusion protein, were still able to reach the Golgi, indicating that delivery of β4GalNAcTB to the Golgi by itself does not lead to an active enzyme but that GABPI is in addition required for activity.

A hydrophobic area in the stem of β4GalNAcTB is crucial for GABPI interaction

Previous experiments on β4GalNAcTB had revealed the importance of the luminal stem region for GABPI interaction. Hybrid proteins of β4GalNAcTA, the GABPI independent enzyme, and GalNAcTB were autonomously active as long as they carried the catalytic domain of β4GalNAcTA. As soon as they possessed the catalytic domain of β4GalNAcTB, however, they only showed activity if co-expressed with GABPI. In addition, β4GalNAcTB required its own stem region, but not its own cytoplasmic and transmembrane domain to be active (Johswich *et al.*, 2009). A sequence alignment of the stem region with related insect β4GalNAcTs showed high similarity in the area of the stem region close to the transmembrane domain (Figure 2). Initial indication that this area is important was obtained by generating an additional hybrid of N-terminal β4GalNAcTA and C-terminal β4GalNAcTB. Whereas a hybrid at the border between transmembrane and stem domain showed to be active, the activity was lost when the switch was made 10 amino acids further in the stem region. Interestingly, the conserved amino acids in the β4GalNAcTBs are predominantly of hydrophobic character. Mutation of four amino acids at once (F34, Y38, Y40 and I41) to alanine as well as to serine destroyed the activity of the enzyme. All these amino acids were also addressed in single point mutations to Serine. Here the single mutations F34S and I41S destroyed the activity (Figure 5 C1 and D1), whereas the single mutations Y38S and Y40S did not. In a double mutation of both tyrosines, the activity again vanished. Single point mutations F38A and I41A, however, left the enzyme intact (Figure 5 C3 and D3). Mutation of both tyrosines to the structural more similar phenylalanine did also not harm the activity. After all, the inactive β4GalNAcTB mutants were consequently fused to GABPI. But, whereas activity of some of the GABPI mutants could be restored by generation of a fusion, none of the β4GalNAcTB mutants could be rescued this way (Figure 5 C2 and D2). However, when the sub-cellular localisation was determined, a striking difference was observed. All inactive β4GalNAcTB

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mutants were retained in the ER when co-expressed with GABPI, but in a hybrid the F34/Y38/Y40/I41S mutant was localized in the ER, whereas the quadruple mutant to alanine was reaching the Golgi (Figure 5A and B). Once more, like for the hybrids of inactive GABPIs, a mutant which had an intact catalytic domain was localised to the Golgi, but showed no activity.

Materials and Methods

Generation of N- and C-terminally truncated proteins

N-terminal deletion constructs were amplified from Myc-GABPI (Johswich *et al.*, 2009). Sense and antisense primers were flanked by XhoI and XbaI sites, respectively. PCR products were ligated into XhoI/XbaI sites of the expression vector pcDNA3 containing an N-terminal Myc-tag (MAQKLISEEDLNLRPLE) and a C-terminal HA-tag (SRYPYDVPDYASL).

Generation of proteins containing internal HA-tags and point mutations

Constructs encoding GABPI with internal HA-tags were generated by PCR. The construct Myc-GABPI-HA was used as template for the amplification. PCR products were ligated with each other via the AatII cleavage site within the HA tag and cloned into the XhoI/XbaI sites of the expression vector pcDNA3 containing an N-terminal Myc tag. Single point mutations were generated by overlap extension PCR (Ho *et al.*, 1989) on the original full length constructs of *Drosophila* Myc-GABPI-HA and Flag-β4GalNAcTB.

Generation of GABPI- β4GalNAcTB fusion proteins

Fusion proteins of GABPI and β4GalNAcTB, and mutants thereof, were generated by XhoI/BamHI-restriction of GABPI and BamHI/XbaI-restriction of β4GalNAcTB. Products were ligated into XhoI/XbaI sites of the expression vector pcDNA3 containing an N-terminal Myc-tag.

Subcellular localization and topology studies by immunofluorescence

Subcellular localizations of recombinant Myc-GABPI-HA, truncated constructs and Myc-GABPI containing internal HA tags were carried out in HEK293 cells (Eckhardt *et al.*, 1999). Therefore, cells were transfected in 6 well plates using Metafectane (Biontex, München, Germany). After two days, cells were resuspended and transferred in a 75 cm^2 flask. The cells were cultured for three weeks in the presence of G-418 (Calbiochem). For staining, cells were seeded onto glass coverslips, fixed in 4% PFA and permeabilized for 30 min with 0.1% saponin in PBS containing 0.1% BSA or for 15 min at RT with digitonin solution (5 µg/ml digitonin, 0.3 M Sucrose, 0,1 M KCl, 2.5 mM $MgCl_2$, 1 mM EDTA, 10 mM HEPES, pH 6.9). After digitonin incubation cells were washed three time with PBS and incubated with blocking solution (0.1% BSA in PBS) for 30 min at RT. Samples were incubated with the respective primary antibodies (anti-HA tag 3F10, or anti-Myc tag 9E10, and rabbit anti-giantin (Covance) or –calnexin (Acris) as Golgi or ER marker) for 1.5 hours at room temperature. After three washing steps (PBS, 0.1% BSA, 0.1% Tween 20), cells were incubated with anti-mouse IgCy3 and anti-rabbit Ig Alexa 488 for one hour at room temperature. After staining with the nuclear dye Hoechst 33258 (Hoechst Pharmaceuticals), the slides were washed with water, mounted (DakoCytomation) and analyzed under a Zeiss Axiovert 200m.

Immuncytochemistry staining

In vivo activity tests were carried out with transiently transfected HEK293 cells. Therefore, cells were transfected using Metafectane (Biontex, München, Germany). After two days, cells grown in 6 well plates were fixed with 1.5% glutaraldehyde, incubated with the anti lacdiNAc monoclonal antibody 259-2A1 (van Remoortere *et al.*, 2000), followed by HRP-conjugated goat-anti-mouse (Jackson ImmunoResearch) and detected by tyramide signal amplification using biotin-tyramide (Speel *et al.*, 2006), streptavidine-AP (Caltag) and Fast-Red (Sigma) as chromogenic substrate.

Discussion

Since it was clear that GABPI's influence on β4GalNAcTB could not be explained by a palmitoylation and functional domains of GABPI should rather be positioned on the luminal side of the Golgi membrane, we started resolving the topology of GABPI. The only described topology of a DHHC protein (Akr-1) shows a 6 transmembrane domain protein which presents its eponymous motif to the cytosol (Politis *et al.*, 2005). Although an involvement of GABPI's DHHS-motif in the activation was unlikely, since introduced alterations did not interfere with β4GalNAcTB interaction, a change of orientation could not be excluded without further analysis. However, the orientation of GABPI could clearly be shown to be analogous to Akr-1 and therefore shows no difference to other DHHC proteins. After showing that only transmembrane domains 3 till 6

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were required for interaction with β4GalNAcTB, there remained only two small loops of 12 and 35 amino acids that were exposed to the lumen. We now have shown that these two luminal loops indeed play an essential role in the interaction between β4GalNAcTB and GABPI. Insertion of HA tags in these regions abolished activity and several point mutations in the loops resulted in an inactive enzyme as well.

Although all eukaryotic species contain numerous DHHC proteins, clear orthologs of GABPI can be identified in most other metazoans. This is zDHHC23 in mammals, for which palmitoylation activity has not yet been established, but which displays an unaltered DHHC motif. This is different in orthologs from insects. Most of them, like *Drosophila* GABPI, have lost the DHHC sequence, but in different ways. Unlike in vertebrates, the two luminal loops, however, are highly conserved (Figure 2). As all these insects also exhibit an ortholog of β4GalNAcTB, it is most likely that the same function is provided by insect orthologs of GABPI, however not by orthologs from mammals, which lack β4GalNAcTB. The longer loop between TMD 5 and 6, which shows more conservation than loop 3-4, displays a preserved structure with two cysteines, several prolines and two stretches of hydrophobic amino acids. Whereas the cysteines are also conserved in mammals, the region in between is remarkably different from insects. Exchange of individual amino acids revealed the loop's necessity, but also some redundancy. Only one of the cysteines was essential and, although one proline mutation was inactive, the most conserved proline could be exchanged by alanine without change. Activity of L348 and L349 mutants was only lost in a double mutant, but a double mutant in the less conserved hydrophobic patch (VV339/340SS) was still active.

Beside the dependency on GABPI, we had already observed that β4GalNAcTB required its own stem region to be active (Johswich *et al.*, 2009). We now have shown that an, once again insect conserved area close to the transmembrane domain contains several essential amino acids. We have mutated three conserved aromatic amino acids and an isoleucine, and could show that they were all relevant for the function of β4GalNAcTB. We could furthermore illustrate that the hydrophobic nature of the amino acids was fundamental. The enzyme was still active when F34 and I41 were changed to alanine, but not when replaced by the more polar serine. Neither was the activity lost when the two tyrosines where replaced by phenylalanines, indicating the significance of the two tyrosines' aromatic rings and not their hydroxyl groups. When all four conserved amino acids were replaced by alanine or serine, the enzyme was in both cases inactive. An interesting observation was, however, made when the inactive β4GalNAcTB was fused to GABPI. The quadruple alanine mutant allowed the enzyme to reach the Golgi, whereas the serine mutant remained in the ER. All mutants of GABPI, on the other hand, targeted β4GalNAcTB to the Golgi in the fusion constructs, independently of the generation of an active complex. We thus have obtained three types of mutants. GABPI mutants (H335A and P346A) that could be fully rescued by generation of a GABPI-β4GalNAcTB fusion, GABPI and β4GalNAcTB mutants that remained inactive as fusion constructs, but reached the Golgi, and the inactive β4GalNAcTB mutant that remained in the ER as fusion protein. The rescue of mutants by fusion indicates a direct involved in GABPI-β4GalNAcTB interaction, but not in enzymatic activity. Amino acids addressed in not rescued mutants, might have a role in catalysis or glycolipid recognition. The hydrophobic stem region of β4GalNAcTB must have a function in both.

We have narrowed down domains on GABPI and in the stem of β4GalNAcTB that are responsible for the joined activity of the two proteins. We have identified them as 'add-on' domains, a term coined by Qasba and Ramakrishnan (Qasba and Ramakrishnan, 2007) to describe important domains for glycosyltransferase activity outside of the catalytic domain. It was also predicted that something likewise was required for glycolipid specific glycosyltransferases (Ramakrishnan *et al.*, 2002;Qasba *et al.*, 2008) Still, the exact way GABPI is involved in activating β4GalNAcTB remains elusive.

Can the situation in *Drosophila* be transferred to mammals? The observation in glycolipid-specific glycosyltransferases never went further than that at least for some enzymes membrane anchorage was required for activity (Amado *et al.*, 1998;Steffensen *et al.*, 2000) and that one enzyme (β3GalT4) shows a very hydrophobic, putative stem region that differs significantly from homologous enzymes not acting on glycolipids (Amado *et al.*, 1998). If a factor like GABPI is required for membrane linked activity is not known. There are, however, two mammalian homologs of β4GalNAcTB which can transfer galactose to glucosylceramide. These enzymes are β4GalT5 and -6, and at least β4GalT5 can do this only when membrane bound (Sato *et al.*, 2000). Interestingly, β4GalT5 and -6 show a conserved YLF sequence in the stem at the same distance to the membrane as the YDYI sequence in *Drosophila* β4GalNAcTB. Such concentration of large hydrophobic amino acids is not found in β4GalT1 till 4. Similar mechanisms could thus play a role in glycolipid biosynthesis in other species than insects. The ortholog of GABPI in mammals,

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zDHHC23, is not a likely candidate to play a role in glycolipid biosynthesis. GABPI's ortholog in *Schistosoma mansoni* however lacks almost the complete DHHC-motif and the surrounding cysteine rich domain (Fig. 2). It nevertheless shows the characteristic conserved luminal loops in a predicted four TMD topology and was not found until we searched for homologous proteins, specifically considering loop 5-6. Complex glycolipids are made by *Schistosoma* in which potential homologs of β4GalNAcTB are involved and thus a similar mechanism as in *Drosophila* could generate specificity for glycolipids in Trematodes.

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Construct	Activity	Subcellular Localization	β4GalNAc-TB-Piloting
GABPI loop 3-4 mutations			
P170A	+	Golgi	+
LL171/172SS	+	Golgi	+
LL171/172AA	+	Golgi	+
E173A	+	Golgi	+
L174S	+	Golgi	+
P176A	-	ER	-
E177A	+	Golgi	+
E178A	+	Golgi	+
EE177/178AA	-	Golgi	-
EE177/178 Fusion	-	Golgi	+
N179A	+	Golgi	+
GABPI loop 5-6 mutations			
C334A	+	Golgi	+
H335A	-	Golgi	-
H335A Fusion	+	Golgi	+
P336A	+	Golgi	+
V339S	+	Golgi	+
V340S	+	Golgi	+
VV339/340SS	+	Golgi	+
P342A	+	Golgi	+
P346A	-	Golgi	-
P346A Fusion	+	Golgi	+
V347A	+	Golgi	+
L348S	+	Golgi	+
L349S	+	Golgi	+
LL348/349SS	-	Golgi	-
LL348/349AA	-	Golgi	-
LL348/349AA Fusion	-	Golgi	+
P350A	+	Golgi	+
DD351/352AA	+	Golgi	+
C353A	-	Golgi	-
C353A Fusion	-	Golgi	+
S354A	+	Golgi	+

Table 1: Impact of point mutations in conserved GABPI loop regions 3-4 and 5-6. Activity was tested by cell surface staining with the mAb 259-2A1. ER or Golgi localization of GABPI and piloting of βGalNAcTB was scored using antibodies against respective Myc and Flag tags. Highlighted by a grey background are the fusion constructs of GABPI and βGalNAcTB.

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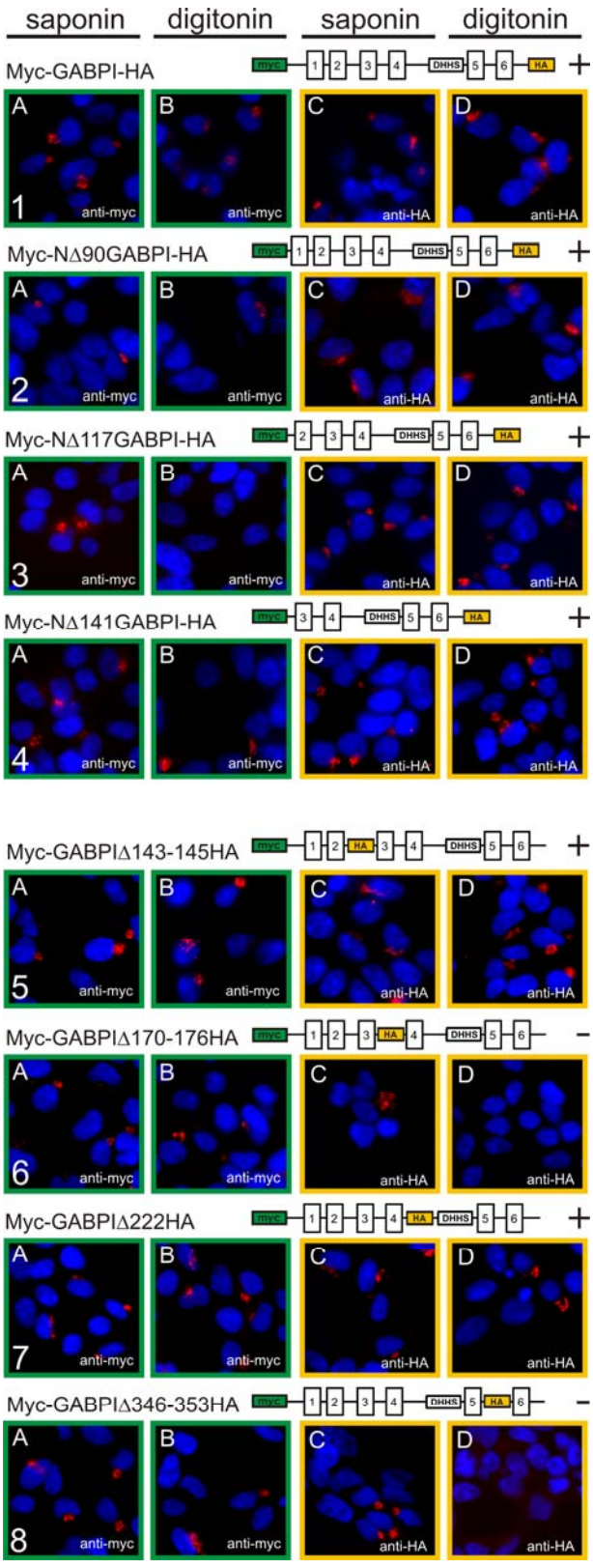

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Figure 2: The Myc-GABPI-HA truncation (1-4) and internal HA (5-8) constructs were expressed in Hek293 cells and stained with anti-Myc (A and B) or anti-HA antibodies (C and D) after either Saponin (A and C) or Digitonin treatment (B and D; tag is only visible, if positioned in the cytosol). The nuclei were stained by Hoechst 33258 (blue). Functionality of constructs, as revealed by cell surface staining of the product after co-expression with βGalNAcTB is indicated by + and - .

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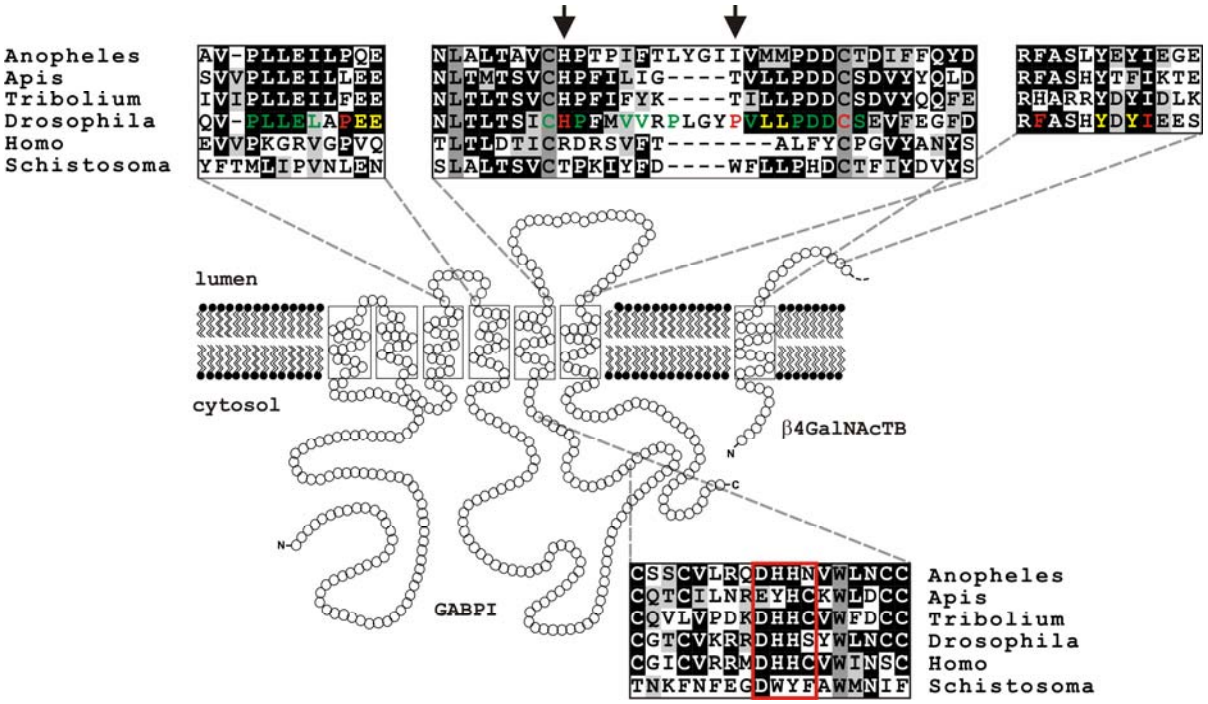

Figure 2: Alignment of insect GABPI orthologs and human zDHHC23 (loop regions 3/4 and 5/6 as well as the DHHC motif) and homologs of β4GalNAcTB (stem region near the TMD). For the *Drosophila* enzymes, effect of mutation is indicated by colour: green = no effect, red = abolished activity, yellow = abolished activity only, if neighbouring amino acid is also mutated. The arrows mark the amino acids, whose effect on activity can be rescued by fusion of GABPI and β4GalNAcTB.

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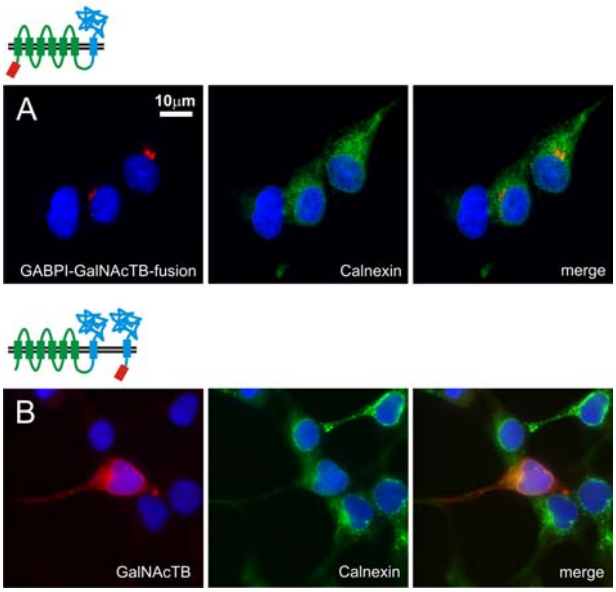

Figure 3: The fusionprotein GABPI-β4GalNAcTB reaches the Golgi (A), whereas β4GalNAcTB co-expressed with GABPI-β4GalNAcTB is still retained in the ER (B). The proteins were expressed in HEK293 cells, and visualised by indirect immunostaining (red) of Myc (A) or Flag (B) tags. The ER was stained with anti-Calnexin (green). The nuclei were stained by Hoechst 33258 (blue).

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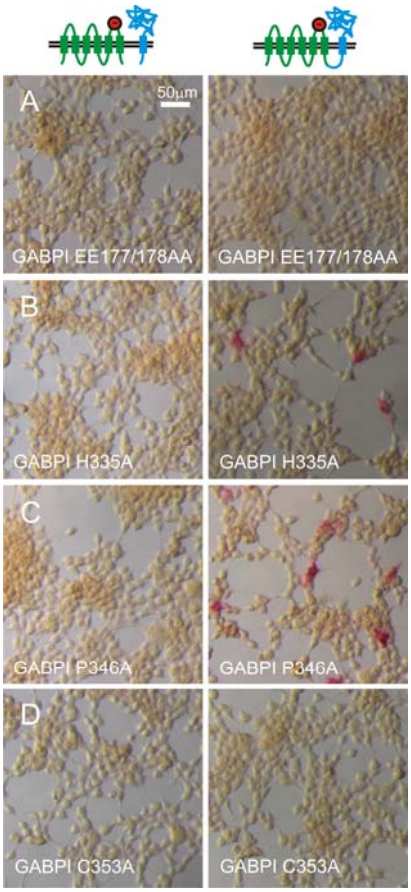

Figure 4: Rescue of inactive GABPI mutants by generation of hybrid GABPI-β4GalNAcTB. In vivo activity was determined by cell surface staining of the lacdiNAc epitope after transient expression in HEK293 cells.

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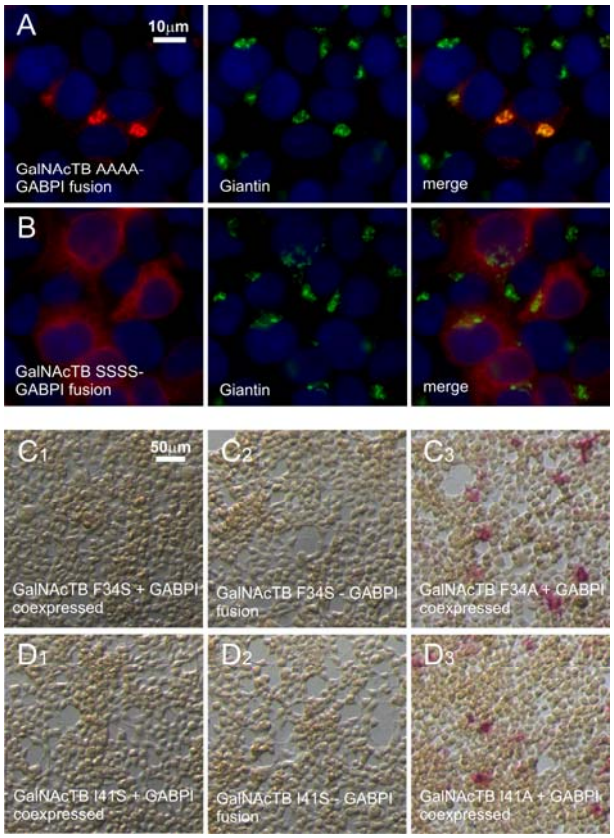

Figure 5: **A-B:** The GABPI-β4GalNAcTB fusion protein displaying quadruple alanine mutations of β4GalNAcTB F34, Y38, Y40 and I41 reaches the Golgi (A), whereas the likewise serine mutant is kept in the ER (B). The subcellular localisation was analysed in HEK293 cells via the N-terminal Myc-tag (red). The Golgi apparatus was stained with anti-Giantin (green). The nuclei were stained by Hoechst 33258 (blue). **C-D:** ***In vivo*** activity of point mutations of β4GalNAcTB F34 and I41 mutants. F34S and I41S mutants show no activity (C1 and D1), activity can not be restored by fusion to GABPI (C2 and D2), and F34A and I41A remain active (C3 and D3).

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Chapter 4 - General Discussion

4.1 GABPI – A DHHC protein that displays common topology and orientation, but clearly has a different function

With one exception (Mizumaru *et al.*, 2009) all characterised members of the DHHC protein family in literature were categorized as palmitoyltransferases. They display the eponymous DHHC motif embedded in a cysteine rich domain. This motif's cysteine is involved in the addition of a palmitate, which is transferred from palmitoyl-CoA to an acceptor cysteine, usually found on the cytosolic side, close to the transmembrane domain of the palmitoylated protein. Such modification can alter the membrane affinity and thereby the location in the cell (Dunphy and Linder, 1998;Linder and Deschenes, 2007;Tsutsumi *et al.*, 2008;Baekkeskov and Kanaani, 2009;Fukata and Fukata, 2010). Alterations of the motif, especially if affecting the cysteine in the DHHC motif diminish the activity (Lobo *et al.*, 2002;Roth *et al.*, 2002;Valdez-Taubas and Pelham, 2005). In this work we describe a *Drosophila* DHHC family member which clearly shows functionality independent from the family's eponymous motif. We state that its influence on the *Drosophila* β4GalNAcTB can not be due to a palmitoyltransferase activity. Not only is the natural appearance of the motif altered to DHHS itself, we also mutated the complete motif to AAAA without influence on GABPI's activity. To exclude an involvement of other neighbouring cysteines, we deleted the whole embedding cysteine rich domain and still found the protein active. Also the mutation of the only possible cytoplasmic cysteine in β4GalNAcTB to alanine did not influence its activation by GABPI. The only described membrane topology for a DHHC protein is that of yeast Akr1 (Politis *et al.*, 2005). In this analysis, 46 and 51 amino acid long invertase segments which carry three NX(S/T) glycosylation sides were inserted in the predicted loops. The glycosylation could then be detected by Western blot analysis. Such attempt always bears the high risk of changing the proteins physical behaviour. Since we also assumed the predicted cytosolic DHHS-motif not to be responsible for the β4GalNAcTB interaction, we followed a different methodology to solve GABPI's membrane topology, using relatively small tags which could then be visualised after immunofluorescent staining of differently detergent treated cells. These experiments clearly revealed GABPI to be a six transmembrane domain protein, displaying a cytosolic DHHS motif and cytosolic N- and C-termini as well. Despite its

functional dissimilarities, the orientation and exterior of GABPI is rather typical for a DHHC protein.

4.2 Comparison of β4GalNAcTA and β4GalNAcTB

β4GalNAcTB was found during an expression cloning attempt. This procedure, selecting for the product of a protein's enzymatic activity - in this case the expression of the lacdiNAc epitope on the cell surface - happened to be the ideal tool for the identification of β4GalNAcTB. Several identified and cloned putative glycosyltransferases show rather low or even no activity. Also β4GalNAcTB had been described as inactive paralog of β4GalNAcTA (Haines and Irvine, 2005). Whereas β4GalNAcTB could be shown to be strictly glycolipid specific, β4GalNAcTA is able to perform lacdiNAc-synthesis on lipids as well as on proteins. However for glycolipids, β4GalNAcTA's affinity is rather low, which explains the fact, that it was not identified during expression cloning. The blueprint of both enzymes indicates the separation for lipid and protein specificity. Both enzymes display a rather conserved catalytic domain. In contrast to that, their stem region is quite different. β4GalNAcTA's stem consists of 106 amino acids, which is a common length for glycosyltransferases. β4GalNAcTB, though, displays only 34 amino acids, which is particularly short. Glycosyltransferases involved in the glycolipid biosynthesis have been described to generally display shorter stem regions than those involved in O- and N-linked glycosylation of proteins (Patel and Balaji, 2007). It has also been shown for glycosyltransferases involved in glycolipid biosynthesis that they need membrane anchorage to accomplish their function. If expressed in truncated, soluble form lacking their transmembrane domain, they show either diminished or even no activity (de Vries *et al.*, 1995;Amado *et al.*, 1998;Steffensen *et al.*, 2000;Togayachi *et al.*, 2001;Schwientek *et al.*, 2002).

4.3 Character of interaction between GABPI and β4GalNAcTB

GABPI was found due to its influence on β4GalNAcTB's activity. It nevertheless also shows a crucial influence on the transferase's translocation. In absence of GABPI, β4GalNAcTB is in contrast to β4GalNAcTA found in the ER. It only reaches the Golgi apparatus in co-expression with GABPI. GABPI itself, however, is fully equipped with all the information required for Golgi translocation. It could also be shown that GABPI and β4GalNAcTB form a

stable complex in the ER by co-expression of an ER retained GABPI, which manages to hold the active β4GalNAcTB in the ER. It can be assumed that the formation of the functional GABPI-β4GalNAcTB complex starts in the ER. Its character is likely to be different from a common chaperon interaction, which would enable the influenced protein by assisting its folding to leave the ER, but would remain in the ER itself. This is the case for Cosmc, which releases the activated C1β3GalT (Ju and Cummings, 2002;Ju *et al.*, 2008). GABPI could be shown to remain associated to β4GalNAcTB after leaving the ER by co-immunoprecipitation of both proteins. The fact that an artificially ER retained GABPI also kept β4GalNAcTB in the ER supported a continuous interaction model. This conclusion was also confirmed by an immunofluorescent interaction assay which is based on binding of two primary antibodies to both proteins, and estimates their proximity (DuoLink©, see additional Figure 1; (Nilsson *et al.*, 1994;Baner *et al.*, 1998;Nilsson *et al.*, 2002)). Here GABPI clearly was found to be in close proximity to β4GalNAcTB. The character of the GABPI-β4GalNAcTB interaction, however, is not easily addressed. During our experiments on GABPI's membrane organisation, we found that insertions of HA tags do not affect activity, if applied to the cytosolic loops, which is in contrast to Akr 1, where insertion of invertase segments in the cytosolic, DHHC containing cytoplasmatic loop, destroyed the activity (Politis *et al.*, 2005). In our case, however, as soon as we addressed the luminal loops between transmembrane domains 3 and 4 or 5 and 6 by an insertion, we found GABPI inactive. We further investigated these luminal loops and found that they were highly conserved in insect orthologs of GABPI, but not in mammalian orthologs, as for instance human zDHHC23. They also displayed remarkable stretches of conserved hydrophobic amino acids. When we checked β4GalNAcTB for regions likely to be involved in the GABPI interaction, we found its stem region close to the transmembrane domain to display a rather parallel motif. It is conserved amongst β4GalNAcTB's insect orthologues and consists of several hydrophobic amino acids. Such concentration of hydrophobicity is rather uncommon for the stem region of glycosyltransferases, but has been described for the glycolipid specific β3Gal-T4 as a peculiar difference to its glycoprotein specific paralogs (Amado *et al.*, 1998). Since in direct fusion to GABPI β4GalNAcTB is active, we can assume that the luminal loop between transmembrane domains 5 and 6 is likely to be positioned in close proximity to β4GalNAcTB's stem region. If we now consider both enzymes conservation in these two domains and their predominant hydrophobic character, we can propose this split "add on" domain to be involved in glycolipid recognition. A domain for this function should display features, which enable it to bind to the lipid anchor, and present it to the catalytic domain of the transferase. By the two interacting

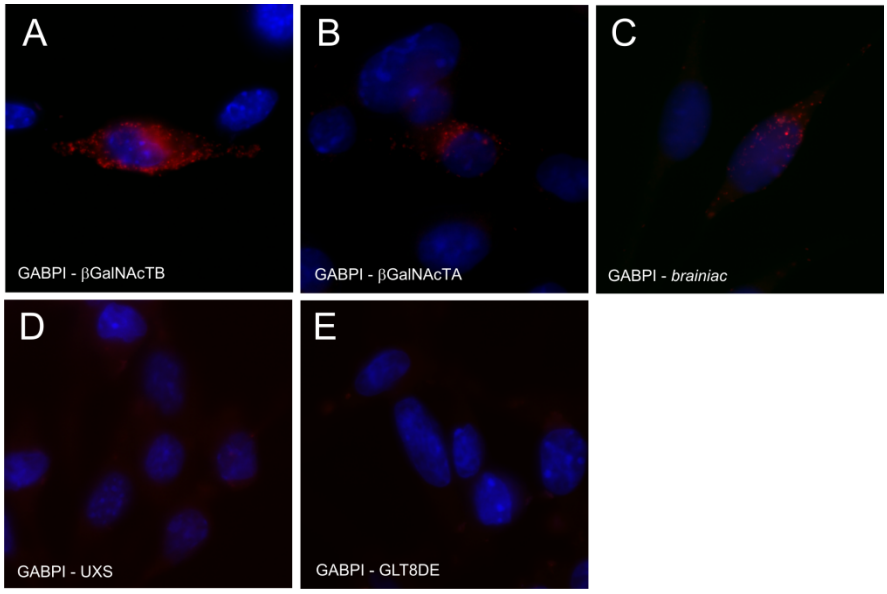
hydrophobic motifs, GABPI and β4GalNAcTB might in combination present exactly this hydrophobic patch which is required to bind to the lipid anchor, and possibly lift it for presentation to the catalytic domain. Such hydrophobic property for glycolipid dislocation has already been described for the GM2-activator protein (GM2-AP). The water-soluble β-Hexosaminidase A, responsible for ganglioside degradation is not able to access gangliosides by itself, unless they extend far enough into the aqueous phase (Conzelmann and Sandhoff, 1979). Cristal structure of the non-glycosylated GM2-AP revealed a hydrophobic cavity suitable for the ceramide moiety (Wright *et al.*, 2000;Wright *et al.*, 2003). GM2-AP also displays hydrophobic loops which enable it to penetrate into the hydrophobic region of the membrane's lipid bilayer. After binding of the ganglioside to a carbohydrate recognition site and lifting it into the cavity, the protein converts to a more water soluble, closed conformation and presents the ganglioside to the β-Hexosaminidase (Kolter and Sandhoff, 2005;Kolter and Sandhoff, 2010) (see also figure 1). Beside the amino acids of GABPI's and β4GalNAcTB's described luminal domains, the embedding of the two proteins into the membrane has been shown to be a prerequisite for functionality. *In vitro* activity assays with Golgi vesicles of double transfected HEK293 cells showed a drastic effect of detergent treatment. The enzyme complex is not able to function in vesicles treated under commonly applied detergent concentrations of NP-40 and Triton-X. Since both have hardly the potential to dissociate protein complexes, but will interfere with membrane organisation, this indicates the necessity of membrane integrity for the functionality of GABPI and β4GalNAcTB. This detergent sensitivity was also found for the GABPI-β4GalNAcTB fusion protein, whose activity is thus clearly dependent on the membrane integrity as well. Since dissociation of both proteins under these conditions is even more unlikely, these findings support the hypothesis of intact membranes to be required for this mechanism. Detergent sensitivity has already been observed for several multi transmembrane domain displaying glycosyltransferases like the mannosyltransferase egghead, acting upstream of β4GalNAcTB on Drosophila glicosphingolipids (Wandall *et al.*, 2003). For a Type II protein glycosyltransferase such effect has not been described yet, and it is likely to be determined by the interference with GABPI and its organisation in the membrane.

4.4 GABPI – A universal blueprint of activating factors for glycolipid specific Glycosyltransferases?

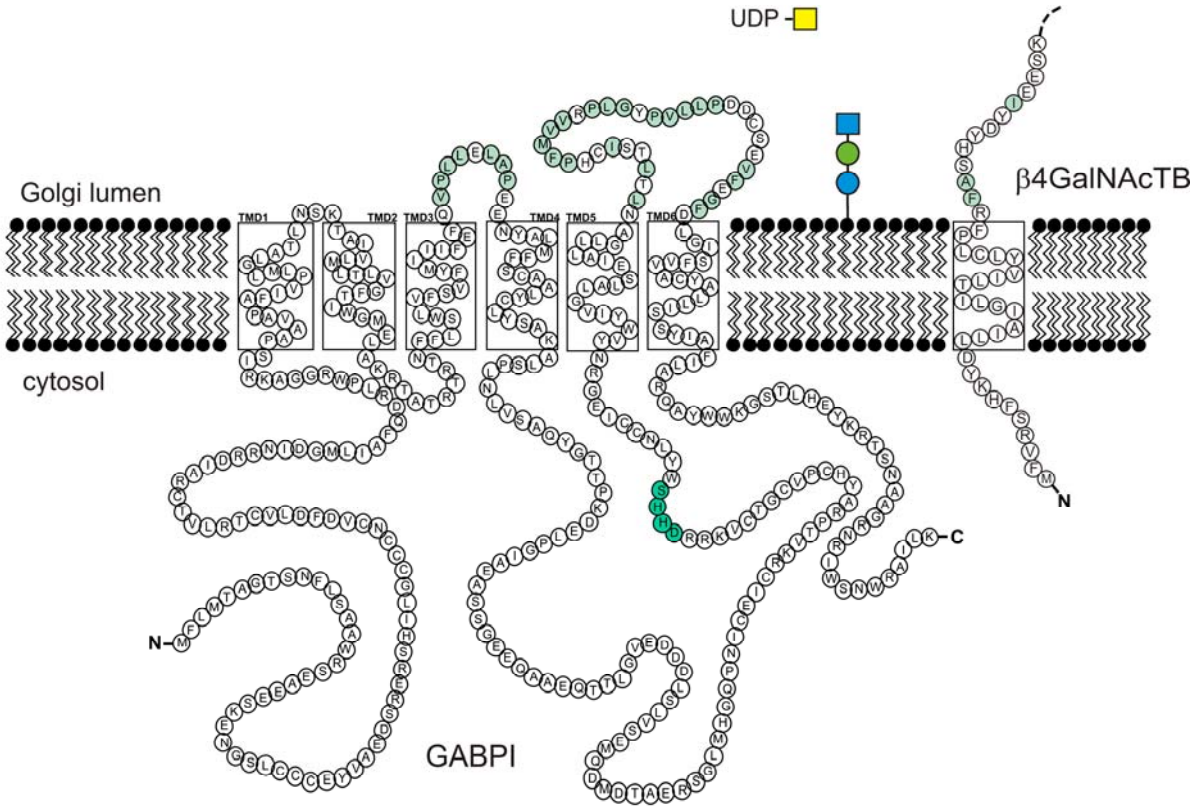
The mechanisms by which glycosyltransferases involved in the glycolipid biosynthesis establish their specificity is not yet understood. In case of β4GalNAcTB it is obvious that this is not alone due to the sugar moiety, since the enzyme can synthesise the lacdiNAc structure on *Drosophila* glycolipids, but also on vertebrate glycolipids, as shown in HEK293 and CHO cell lines, but not on terminal GlcNAc residues on glycoproteins. Vertebrate glycolipids display GlcNAc-Gal-Glc-Cer as acceptor structure for the β4GalNAc-transferase, whereas *Drosophila* glycolipids present a mannose instead of galactose (Seppo and Tiemeyer, 2000) (see also figure 3). Selectivity therefore has to be secured, at least partly, by the glucosylceramide moiety. The biosynthesis of glycolipids is performed basically by two groups of glycosyltransferases - those which act on more membrane distant moieties and can be expressed in soluble form and those which are in close proximity to the membrane. In analogy to the mechanism of glycolipid degradation it was proposed that a membrane bound cofactor might be involved in presentation of glycolipid residues to the transferases (Ramakrishnan *et al.*, 2002;Qasba *et al.*, 2008). This hypothesis perfectly fits to what we have observed for β4GalNAcTB's dependency on its cofactor. GABPI as a multitransmembrane protein co-localises with co-expressed β4GalNAcTB. Both proteins display conserved hydrophobic stretches in their membrane near luminal domains, of which we were able to spot several as indispensable for correct function. The membrane association of β4GalNAcTB is also relevant in co-expression with GABPI. A soluble form of the transferase, consisting of catalytic domain and stem, but lacking the transmembrane domain, could not be activated by GABPI.

4.5 Outlook: GABPI as a platform for GSL synthesis

As the organisation of glycolipid biosynthesis in general is rather poorly understood, also the organisation of the involved enzymes is unclear. Since all synthesis takes place close to the membrane, a platform like organisation of proteins, as it has been described for heparan synthesis is most likely (McCormick *et al.*, 2000;Pinhal *et al.*, 2001). First indication for such form of organisation can be the imperative of GABPI which is indicated by knock out mutant's phenotypes for the described enzymes. *Drosophila* mutants lacking β4GalNAcTB or β4GalNAcTA show defects in oogenesis and also behavioural dysfunctions, but still remain viable (Haines and Irvine, 2005). First analysis of a *Drosophila* knock down of GABPI however shows a temperature dependant lethal phenotype (data not shown). In all performed experiments so far we only addressed β4GalNAcTB and β4GalNAcTA as potential interacting candidates. In co-immunoprecipitation the β4GalNAcTA was not captured by GABPI under conditions of β4GalNAcTB binding. In recently performed assays, we also found β4GalNAcTA to weakly interact with GABPI (supplementary figure 1B). Also for the upstream enzyme brainiac, we found indication for close proximity to GABPI (supplementary figure 1C). Two unrelated enzymes, udp-xylose synthase and a xylosyltransferase (supplementary figure 1D and E) served as negative control. Although brainiac can clearly function in GABPI's absence, which could be shown in GABPI downregulated *Drosophila* Scheider S2 cells, the first enzymes for *Drosophila* glycolipid synthesis are likely candidates to be organised in a platform like complex in the Golgi membrane.



Supplementary Figure 1: **GABPI as a platform for glycosphingolipid biosynthesis**. In a protein-protein interaction assay, GABPI was shown to clearly interact with β4GalNAcTB (A), but also for β4GalNAcTA (B) and the upstream β3GlcNAcTransferase brainiac (C) a weak interaction could be detected. UXS (D) and GLT8D3 (E) are unrelated cytoplasmic and Golgi enzymes and were used as negative control. Proteins were first incubated with antibodies against tags, which where then recognised by oligo linked secondary antibodys. A ligase and polymerase treatment, followed by incubation with dye coupled probes. Dot like signals are evidence for protein proximity of about 40 nm.



Supplementary Figure 2: **GABPI and β4GalNAcTB in glycosphingolipid biosynthesis.** The luminal loop 5-6 and the β4GalNAcTB stem are likely to be positioned in close proximity. Both regions contain conserved patches of hydrophobicity (light blue) and in cooperation might supply a hydrophobic pocket, which allows the dislocation of the glycospingolipid towards the catalytic domain of β4GalNAcTB.

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Abbreviations

AA	amino acid
AP	alkaline phosphatase
Az	acidic glycosphingolipids with one PE residue based on thenomenclatur of Wiegandt
BCA	bichionic acid
BSA	bovine serum albumine
BLAST	basic local alignment search tool
β4GalNAcT	UDP-GalNAc:GlcNAcβ-R β1,4 N-acetyl-galactosaminyltransferase
β4GalT	UDP-GalNAc:GlcNAcβ-R β1,4 galactosyltransferase
cDNA	complementary desoxyribonucleic Acid
C.elegans	*Caenorhabditis elegans*
Cer	ceramide
CHO	chinese hamster ovary
CRD	cysteine-rich domain
DHB	2,5-Dihydroxybenzoic acid
DHHC	Asp-His-His-Cys
DHHS	Asp-His-His-Ser
dsRNA	double-stranded RNA
D.melanogaster	*Drosophila melanogaster*
EDTA	ethylenediaminetetraacetic acid
ER	endoplasmatic reticulum
FCS	Fetal Calf Serum
Flag	amino acid sequence: DYKDDDDK
GABPI	β4GalNAcTB pilot protein
Gal	D-galactose
GalNAc	2-Acetamido-2-deoxy-D-galactose (*N*-acetyl-galactosamine)
GalT	galactosyltransferase
GFP	green fluorescence protein
Glc	D-glucose
GlcA	glucuronic acid
GlcNAc	2-acetamido-2-deoxy-D-glucose (*N* acetyl-glucosamine)
GlcNAc-pNP	4-nitrophenyl N-acetyl-α-D-glucosaminide
GRP	glucose-regulated protein
GSL	glycosphingolipid
HA	amino acid sequence: YPYDVPDYA
HEK	human embryonic kidney

HPTLC	high-performance thin-layer chromatography
KDEL	Lys-Asp-Glut-Leu
lacdiNAc	*N,N´*-diacetyllactosediamine (GalNAcβ4,GlcNAc)
lacNAc	*N´*-acetyllactosediamine (Galβ4,GlcNAc)
mAB	monoclonal anctibody
MALDI-TOF	matrix assisted laser desorption ionization-time of flight
Man	D-mannose
MS	mass spectroscopy
Myc	amino acid sequence: EQKLISEEDL
NP-40	nonylphenyl-polyethylene glycol
Nz	zwitterionic glycosphingolipids with one PE residue based on the nomenclatur of Wiegandt
OST	oligosaccharyltransferase
PAT	palmitate acyl transferase
PAGE	polyacrylamide gelelectrophoresis
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PE	phosphoethanolamine
RNAi	RNA interference
RP	reverse-phase
RT	room temperature
SDS	sodium dodecylsulfate
TMD	transmembrane domain
TMHMM	prediction of transmembrane helices based on a hidden markov model
Tris	trishydroxymethylaminomethane
UDP	uridine diphosphate
v/v	volume/volume
w/v	weight/volume

Curriculum vitae

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Publications

Johswich, A., Kraft, B., Wuhrer, M., Berger, M., Deelder, A.M., Hokke, C.H., Gerardy-Schahn, R., Bakker, H. (2009). Golgi targeting of *Drosophila melanogaster* β4GalNAcTB requires a DHHC protein family - related protein as a pilot. J.Cell Biol. 184, 173-183.

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Oral Presentations

45. Herbsttreffen auf Schloss Hochhausen

Hochhausen, Germany, October 15th – 17th, 2009

Title: "Hilfsfaktoren für glykolipidspezifische Glykosyltransferasen" –

"Helping factors for glycolipid specific glycosyltransferases"

Poster Presentations

19. Joint Meeting

Wageningen, Netherlands, November 30th – December 2nd, 2008

Title: „Mechanism of Interaction between *Drosophila* β4GalNAcTB and the β4GalNAcTB Pilot Protein (GABPI)"

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