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Polypeptide GalNAc-Ts: from redundancy to specificity

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Mucin-type *O*-glycosylation is a post-translational modification (PTM) that is predicted to occur in more than the 80% of the proteins that pass through the Golgi apparatus. This PTM is initiated by a family of polypeptide GalNAc-transferases (GalNAc-Ts) that modify Ser and Thr residues of proteins through the addition of a GalNAc moiety. These enzymes are type II membrane proteins that consist of a Golgi luminal catalytic domain connected by a flexible linker to a ricin type lectin domain. Together, both domains account for the different glycosylation preferences observed among isoenzymes. Although it is well accepted that most of the family members share some degree of redundancy toward their protein and glycoprotein substrates, it has been recently found that several GalNAc-Ts also possess activity toward specific targets. Despite the high similarity between isoenzymes, structural differences have recently been reported that are key to understanding the molecular basis of both their redundancy and specificity. The present review focuses on the molecular aspects of the protein substrate recognition and the different glycosylation preferences of these enzymes, which in turn will serve as a roadmap to the rational design of specific modulators of mucin-type *O*-glycosylation.

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Introduction

Polypeptide GalNAc-transferases (GalNAc-Ts) are a family of Golgi resident enzymes (20 in humans) that transfer a GalNAc moiety from UDP-GalNAc onto Ser or Thr residues of their protein substrates. This process results in the synthesis of the Tn antigen (GalNAcα1-*O*-Ser/Thr), which can be further elongated by the action of subsequent glycosyltransferases (GTs) [1•,2]. Historically, this modification is known as mucin-type *O*-glycosylation (henceforth *O*-glycosylation) as these glycans are abundant (>50% by weight) in mucins. As the GalNAc-Ts initiate and thus define sites of *O*-glycosylation in densely *O*-glycosylated proteins such as mucins, these enzymes must possess a range of properties in order to properly glycosylate their targets [2,3]. It is now known that the GalNAc-T isoenzymes have different glycosylation preferences that allow them to be classified as: a) glycopeptide/peptide-preferring isoforms (e.g. GalNAc-T1 and GalNAc-T2); b) (glyco) peptide-preferring isoenzymes (e.g. GalNAc-T4); and c) strict glycopeptide-preferring isoenzymes (e.g. GalNAc-T7 and GalNAc-T10) [4••]. This distinction is based on their activity against substrates lacking or containing one or more prior GalNAc-*O*-Ser/Thr moieties which allows them to be classified into early, intermediate or late GTs, representing a range in activities against naked peptides/proteins to already highly glycosylated peptides/proteins (e.g. GalNAc-T2, GalNAc-T4 and GalNAc-T10 are early, intermediate and late GTs, respectively) [1•,4••,5] (See Figure 1).

The glycopeptide activities of the GalNAc-Ts have been further classified into two classes, based on their short-range (or neighboring) and long-range (or remote) glycosylating capabilities. The short-range glycosylation preferences account for the glycosylation of glycopeptide substrates where the sugar moiety is bound to the catalytic domain (thus glycosylating 1–3 residues from the sugar), while the long-range glycosylation preferences comprise glycopeptide sugar binding to the lectin domain which subsequently directs distant acceptor sites (6 to ~ 17 residues away) onto the catalytic domain for glycosylation as depicted in Figure 1 [1•,6••]. It has been found that both the long-range and short-range glycopeptide preferences can operate in an N-terminal or C-terminal direction depending on isoenzyme and furthermore some isoenzymes possess both the long-range and short-range glycopeptide activities (Figure 1). Together, these properties explain how a highly coordinated repertoire of GalNAc-Ts are capable of readily generating multiple closely spaced Tn antigens, as occurs in mucins, as well as glycosylating proteins containing only a few acceptor

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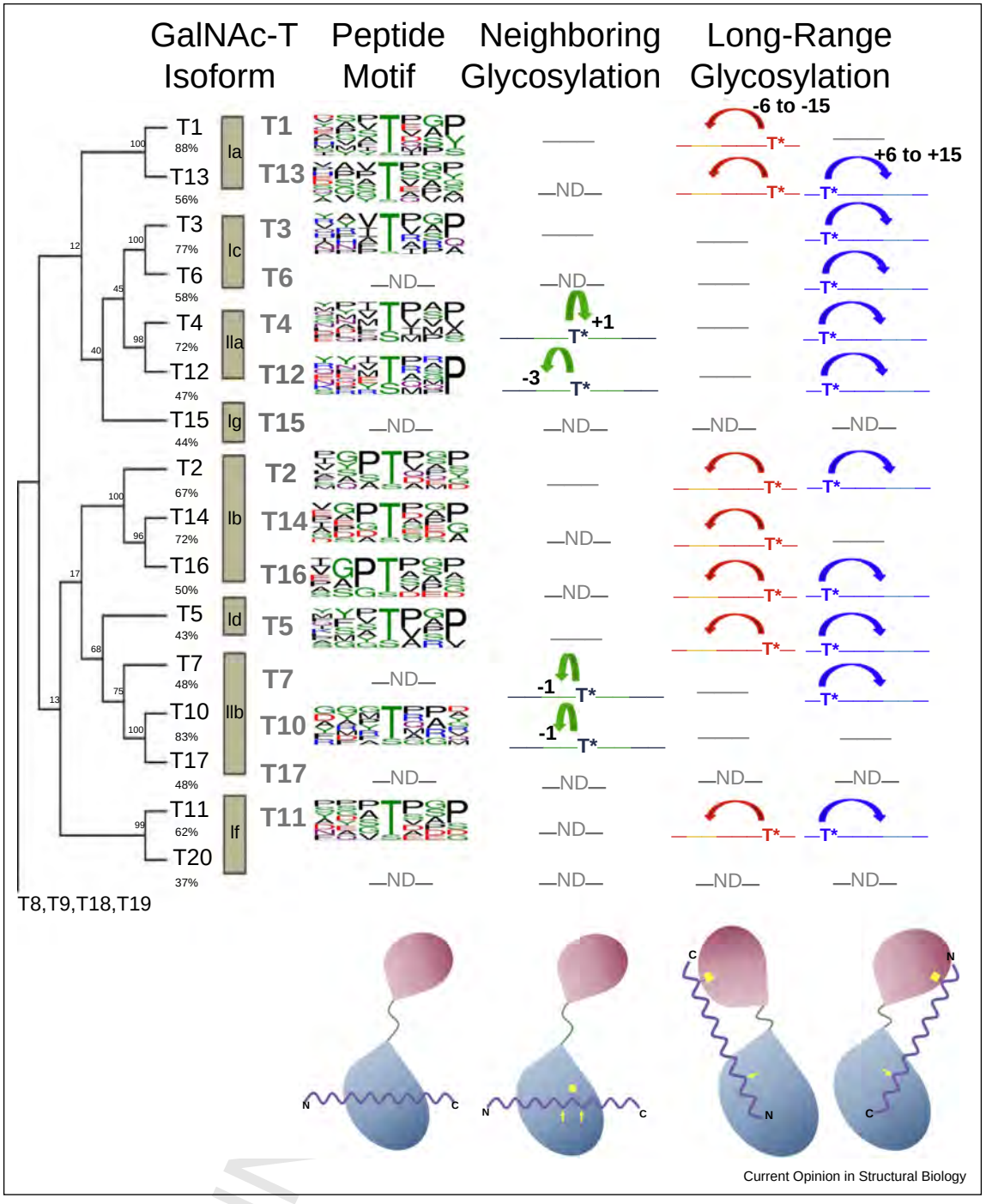
Figure 1



Summary of the known peptide and glycopeptide specificities of the GalNAc-T family.
Phylogenetic tree of the GalNAc-Ts showing their 1) random peptide derived peptide substrate motifs as Sequence Logos [7••,23••,32••,46] 2) neighboring prior glycosylation preferences (1–3 residues) due to catalytic domain interactions [4••], and 3) their long-range prior glycosylation preferences (∼6 to ∼17 residues) due to lectin domain binding [23••,40••]. Note that '-ND-' stands for not determined while '---' indicates no or weak activity. Also note that GalNAc-T8, GalNAc-T9, GalNAc-T18 and GalNAc-T19 exhibit nearly undetectable activities against most substrates [47] and have not been well characterized. The models at the bottom show the different substrate binding modes that lead to the indicated specificities. The catalytic and lectin domains are shown as oval-shaped figures in blue and red, respectively. (Glyco)peptides are indicated in purple while the yellow squares denote the position of prior GalNAc moieties in the glycopeptides. Arrows indicate the position of GalNAc transfer to the acceptor site.

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sites. These combined properties must also be involved in the targeting of specific substrates.

The fact that most isoenzymes of this family are capable of glycosylating common acceptor substrates, particularly those containing the (Thr/Ser)ProXPro motif (where 'X' usually stands for a small hydrophobic residue; see Figure 1), suggests that they may also serve redundant functions [4••,7••]. Paradoxically, in recent years an incremental number of reports have demonstrated that several GalNAc-T isoenzymes are highly specific for certain protein substrates, as identified by the Simple Cell (SC) approach developed by the Clausen group [8••,9]. Using this strategy, ApoC-III was identified as a specific substrate of GalNAc-T2 [9] and GalNAc-T11 was reported to be specifically involved in glycosylation of the peptide linkers between class A repeats of the LDL receptor family [10,11]. One common theme found for isoenzyme-specific glycosylation is that such a glycosylation can interfere with the proprotein processing of neighboring sites thus controlling such a processing [12]. Two of the most studied examples are GalNAc-T3 and its substrate, the fibroblast growth factor 23 (FGF23) [13], as well as GalNAc-T2 and angiopoietin-like Protein 3 (ANGPTL3) [14], where miss-regulation of *O*-glycosylation can lead to familial tumoral calcinosis and dyslipidemia, respectively [13,14]. In addition, the aberrant expression or mutation of several GalNAc-T isoenzymes and the overexpression of the Tn-antigen is directly associated with many cancers [15,16•,17], the mechanisms of which are still not understood, although Tn–Tn self-association may play a role [16•]. Finally, the GalNAc-Ts are involved or implicated in many other biological functions including development, receptor trafficking and modulation and protein secretion, which are beyond the scope of this review [18].

How this family of GTs can glycosylate multiple common sites in some proteins and at the same time be highly isoenzyme-specific for sites in other proteins remains unanswered. Herein, we review the recent advances that have begun to unravel the substrate-recognition mechanism of several of the most representative isoenzymes, as well as presenting the structural and kinetic basis for both their overlapping and selective activities.

Structural similarities between GalNAc-Ts isoenzymes

To obtain a complete understanding of the mechanism underlying the substrate specificity of this family of enzymes, crystal structures of GalNAc-T isoenzymes have been solved, either in the apo form or in complex with (glyco)peptide substrates and products. To date, the following structures have been reported (see Figure 2a and b): a) *Mus musculus* GalNAc-T1 with Mn^{2+} (*Mm*GalNAc-T1) (PDB entry 1XHB) [19•]; b) human GalNAc-T2 (*Hs*GalNAc-T2) complexed with UDP-Mn^{2+} (PDB entry 2FFV), a 'naked' peptide (PDB entry 2FFU) [20•] and three glycopeptides (PDB entries 5AJP, 5AJO and 5AJN) [21••]; c) *Hs*GalNAc-T10 complexed with UDP-Mn^{2+} and Ser-GalNAc (PDB entries 2D7I and 2D7R) [22•]; d) *Hs*GalNAc-T4 complexed with UDP, Mn^{2+} and a glycopeptide (PDB entry 5NQA) [6••]; and e) the recently reported structures of *Hs*GalNAc-T4 complexed with UDP, Mn^{2+} and a diglycopeptide (PDB entry 6H0B) [23••] and two splice variants of the fly PGANT9A/B with UDP-Mn^{2+} and a peptide substrate (PDB entries 6E4Q and 6E4R; Figure 2b) [24••].

These structures all show an N-terminal catalytic domain adopting the typical GT-A fold, characterized by two abutting Rossmann-like folds which is linked by a short flexible linker to a C-terminal ricin-like lectin domain [6••,19•,20•,21••,22•] (Figure 2a). The lectin domain, a unique structural feature only present in this family of eukaryotic GTs, has a β-trefoil fold built from three repeat units (α, β and γ) that are potentially capable to bind a GalNAc moiety [25,26•]. It should be noted that these repeats are not necessarily all active binders based on their sequence motif [4••] and by experiment [7••,27].

GalNAc-T catalytic domain: UDP-GalNAc binding site and flexible loop

The first crystal structure of a GalNAc-T was for *Mm*GalNAc-T1 [19•]. It provided the initial picture of the active site for which subsequent GalNAc-T structures highly resemble, particularly in the architecture of the critical and conserved Mn^{+2} binding site (formed by Asp209, His211 (the DXH motif) and His344). This structure also showed that the catalytic and lectin domains were closely associated [19•] (Figure 2b). Subsequent crystal structures of GalNAc-T2 complexed to both UDP-Mn^{2+} and to UDP-Mn^{2+} and the EA2 peptide (AspSerThrThrProAlaProThrThrLys) [20•], together with the GalNAc-T10 crystal structure complexed with hydrolyzed UDP-GalNAc and Mn^{2+} [22•], further defined the GT-A fold active site residues that tethered the uridine diphosphate of the UDP-GalNAc donor substrate [20•,22•]. In addition, the structure of GalNAc-T10 catalytic domain revealed the GalNAc moiety bound in the UDP-GalNAc-binding pocket [22•,28]. These structures, as well as the crystal structures of the first pre-Michaelis and Michaelis complexes of *Hs*GalNAc-T2 [29••], were of fundamental importance for defining models of the dynamics of GalNAc-T2 during its catalytic cycle, which consists of an ordered bi–bi kinetic mechanism [29••,30,31]. In addition, the Michaelis complex revealed that these GTs follow a front-face S_Ni-type reaction mechanism [29••].

Structures of GalNAc-Ts, both with and without bound peptide substrate, have revealed a dynamic flexible loop at the surface of the catalytic domain substrate binding site as an important structural feature of the GalNAc-Ts [20•,21••,29••]. This flexible loop, formed by residues

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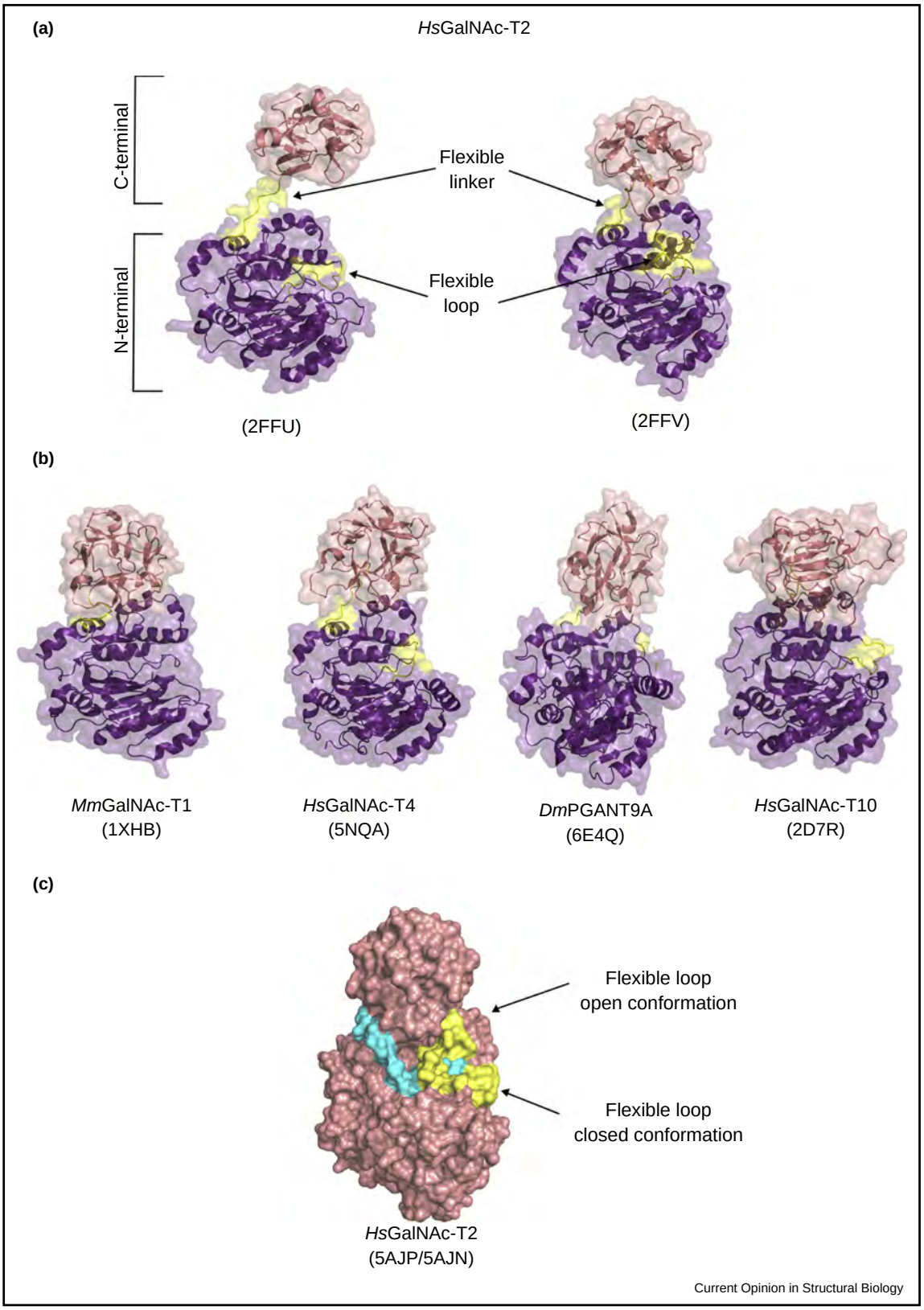
Figure 2



Cartoon and surface representation of GalNAc-Ts structures.
Catalytic and lectin domains are shown in purple and salmon respectively, while flexible loop and linker are displayed in yellow. **(a)** Extended (left

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Arg362 to Ser373 in *Hs*GalNAc-T2, is able to adopt either a closed conformation to form a lid over UPD, rendering the enzyme in an active form, or an open conformation in which the loop folds back to expose UDP to the bulk solvent (inactive form; Figure 2c) [20•,29••]. This interconversion has recently been shown to be dependent of the presence of UDP-GalNAc in *Hs*GalNAc-T2, where this sugar nucleotide stabilizes the closed conformation and consequently allows the binding of the substrate peptide [32••]. The importance of this flexible loop in catalysis is exemplified by the molecular basis of the inactivation of the *Hs*GalNAc-T2-Phe104Ser mutant, which is linked to low levels of high density lipoprotein cholesterol [32••,33]. It was found that Phe104 controls the inactive-to-active transition of the flexible loop due to its hydrophobic interaction with Ala151/Ile256/Val360, as well as a CH-π interaction with the side-chain of Arg362 located in the flexible loop [32••]. The hydrophilic Phe104Ser mutation fails to lock the flexible loop in its active form, thus impeding peptide substrate binding and the failure to glycosylate its targeted peptide substrates (i.e. ApoC-III and ANGPTL3). This results in low levels of HDL [32••,33,34].

GalNAc-T catalytic domain: peptide-binding site

It is noteworthy that several GalNAc-T crystals soaked or cocrystallized with (glyco)peptides show indeterminate/disordered structures for the substrate bound to the catalytic domain [6••,24••]. Nevertheless, structural information of the GalNAc-T-peptide acceptor recognition could be inferred from the series of structures of (glyco)peptides bound to the *Hs*GalNAc-T2 isoenzyme [21••] and very recently the diglycopeptide bound to *Hs*GalNAc-T4 [23••]. In these latter structures the interactions between the transferase and its acceptor substrates are dissimilar, suggesting differences between isoenzymes at the peptide binding groove level. However, this could also be due to the different (glyco)peptides used for both isoenzymes. The *Hs*GalNAc-T2 structures revealed that the EA2 peptide bound in a shallow cleft on the surface of *Hs*GalNAc-T2, being recognized by hydrophobic interactions and to a lesser extent hydrogen bond interactions [20•] (see Figure 3a). It was also observed that the methyl group of the acceptor Thr residue was embedded within a hydrophobic pocket, providing a plausible explanation of why most GalNAc-T isoenzymes prefer to glycosylate Thr over Ser acceptor residues [20•,21••,35] (Figure 3a). Several other crystal structures of *Hs*GalNAc-T2 in complex with UDP-Mn^{2+} and glycopeptides also showed that the glycopeptides acted as bridges between the catalytic and lectin domains, where the latter bound the glycopeptide GalNAc [21••]. In these structures, UDP and the glycopeptides were bound to an adaptable sugar-nucleotide binding site, with the flexible loop adopting either open or closed conformations (Figure 2c). Interestingly, the binding of a mono-glycopeptide to GalNAc-T4 revealed peptide GalNAc binding at the lectin domain but no observable peptide electron density in its catalytic domain [6••] while recently a homologous diglycopeptide showed a well-resolved peptide bound to the catalytic domain in a closed conformation due to GalNAc-T4's neighboring glycopeptide binding activity (discussed below) [23••] (Figure 3b). Interestingly, in the GalNAc-T4 structure, the portion of the peptide spanning the catalytic and lectin domains was found disordered [21••,23••].

At the level of the peptide-binding groove, it was further observed that three highly conserved aromatic residues (namely Phe361, Phe280 and Trp282 in GalNAc-T2), interact with the (Thr/Ser)-Pro-X-Pro substrate sequence [20•]. Thus far the (Thr/Ser)ProXPro sequence is the only substrate consensus motif remotely conserved among most GalNAc-Ts (Figures 1, 3a and b) [1•,21••]. Indeed, all isoenzymes that experimentally display this (Thr/Ser) ProXPro preference possess the homologus Phe and Trp residues [4••,7••,23••] including GalNAc-T4 and GalNAc-T12. GalNAc-T7 and GalNAc-T10, which lack these conserved residues and do not exhibit the (Thr/Ser)ProXPro preference, instead display strong neighbouring glycosylation preferences at the +1 position relative to the acceptor (i.e. (Thr/Ser)(Thr*/Ser*), where * = -*O*-GalNAc) [4••,28]. These latter two isoenzymes are, therefore, expected to contain a GalNAc binding site in place of the ProXPro binding site found in the other isoenzymes. Presently, the structural and molecular bases for the neighboring glycosylation preferences of GalNAc-T7 and GalNAc-T10 remain to be determined. Thus, the near lack of a conserved substrate consensus motif together with their active site flexibility points to the versatility of these enzymes, allowing them to sculpt their binding sites to accommodate a wide range of acceptor substrates.

GalNAc-T catalytic domain: glycopeptide-binding site

Until very recently there were no structures describing how the so-called neighbouring glycosylation activity of the GalNAc-Ts could be accommodated. The recent report of a diglycopeptide (GlyAlaThr*3GlyAlaGlyAlaGlyAlaGlyThr*11Thr12ProGlyProGly, where Thr* = Thr-*O*-

(Figure 2 Legend Continued) panel) and compact (right panel) forms of monomeric *Hs*GalNAc-T2. **(b)** Cartoon and surface representation of *Mm*GalNAc-T1, *Hs*GalNAc-T4, *Dm*PGANT9A and *Hs*GalNAc-T10. **(c)** Surface representation of the *Hs*GalNAc-T2-UDP-MUC5AC-13 complex. The overall structure is shown in salmon, monoglycopeptide MUC5AC-13 and the flexible loop are depicted in cyan and yellow, respectively. The flexible loop of the enzyme is shown in its closed and open conformations.

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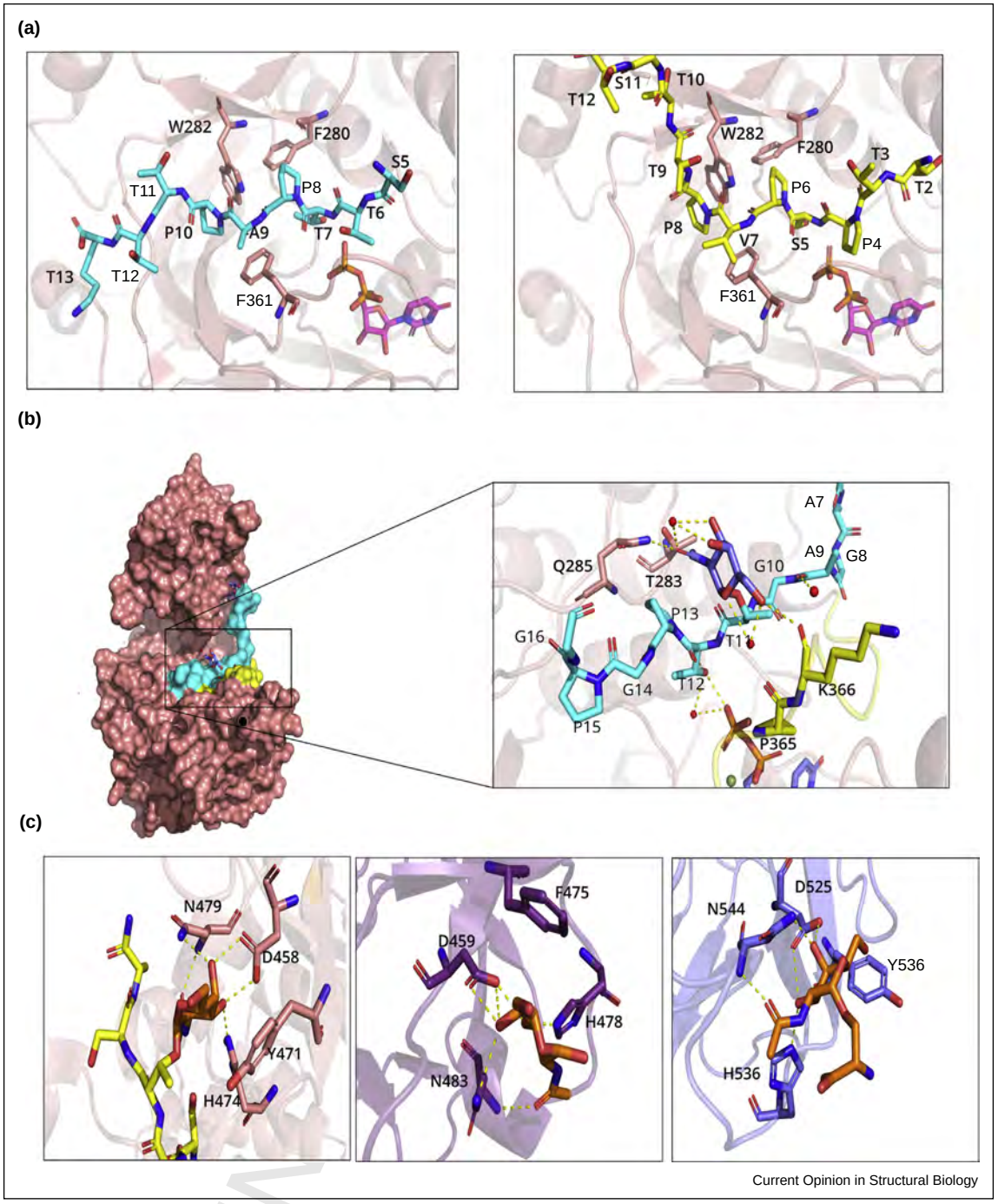
Figure 3



Interactions between GalNAc-Ts and their substrates.
(a) Close-up view of the catalytic domain of *Hs*GalNAc-T2 with two different peptides, EA2 (cyan sticks; left panel) and glycopeptide MUC5AC-13 (GlyThrThrProSerProValProThrThrSerThrThr*SerAlaPro) (yellow sticks; right panel), which are similarly recognized by *Hs*GalNAc-T2 through a hydrophobic patch. UDP is depicted as sticks with magenta carbon atoms and Mn^{2+} is shown as a purple sphere. (**b**) On the left panel, surface representation of the *Hs*GalNAc-T4-UDP-Diglycopeptide 6 (GlyAlaThr*3GlyAlaGlyAlaGlyAlaGlyThr*11Thr12ProGlyProGly) complex. Peptide backbone is depicted in cyan with the two GalNAc groups as blue and red sticks; the enzyme flexible loop is shown in its closed conformation in yellow. On the right panel, close-up view of the main interactions between *Hs*GalNAc-T4 catalytic domain glycopeptide binding-site and the GalNAc group on T^*_{11} of diglycopeptide 6. The GalNAc-T4 residues forming the peptide-binding site are depicted in salmon and yellow and the glycopeptide is depicted in cyan, with the GalNAc groups shown as blue and red sticks. Mn^{2+} and water molecules are depicted as green and red spheres, respectively, and hydrogen bonds appears as dotted yellow lines. Please note that we only show water-mediated interactions in which

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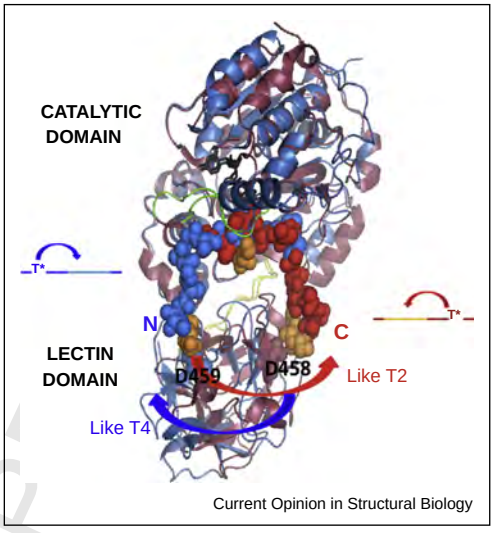
GalNAc) bound to both the lectin and catalytic domains of *Hs*GalNAc-T4 now reveals how this occurs at least in one isoenzyme [23••] (Figure 3b). In this structure the GalNAc of Thr*$_{11}$ is shown tethered by hydrogen bond and hydrophobic interactions to the side chains of Thr283 and Gln285 and the back bone of Lys366 at the surface of the catalytic domain. Importantly, these residues are not conserved among other GalNAc-T isoenzymes and there is no discernible cleft or pocket for the binding of the GalNAc (Figure 3b). Such GalNAc binding presents the adjacent Thr$_{12}$ into the correct orientation to accept GalNAc from the UDP-GalNAc donor. Kinetic studies on a series of glycopeptide substrates further confirmed that the neighbouring GalNAc binding at the catalytic domain was weaker than the remote GalNAc binding of Thr*$_3$ to the lectin domain and further revealed substrate inhibition kinetics on the diglycopeptide, presumably due to competitive binding of the two Thr*'s of the substrate at the lectin domain [23••]. This work is of additional significance as the individual GalNAc-T4 remote and neighboring glycopeptide activities, and both together, could be eliminated or greatly reduced by selective mutagenesis.

GalNAc-Ts lectin domain

The GalNAc-T-glycopeptide recognition at the lectin domain is more easily compared among isoenzymes, as there are crystal structures of *Hs*GalNAc-T2, *Hs*GalNAc-T4 and *Hs*GalNAc-T10 complexed with Ser-*O*-GalNAc as well as longer Thr-*O*-GalNAc containing glycopeptides [6••,21••,22•,23••] (Figure 3c). The GalNAc-T1 lectin domain contains two known functional GalNAc-binding sites out of the possible three (i.e. the α and β subdomains) [36], whereas the GalNAc-T2, GalNAc-T4, and GalNAc-T10 lectin domains contain only one known active site (i.e. α-, α- and β-respectively) [4••]. The first structure of a glycopeptide bound to the lectin domain, that is, Ser-*O*-GalNAc bound to *Hs*GalNAc-T10, revealed the sugar moiety bound to the β-site interacting through several hydrogen bonds (including residues Asp525, Asn544, Tyr536) and one CH-π interaction (His539) (Figure 3c). Subsequent structures of GalNAc-T2 complexed with longer glycopeptides showed no discernible interactions with the peptide backbone of the lectin domain level, while the GalNAc moiety interacted exclusively with residues in the α-subdomain binding site by similar interactions as described for GalNAc-T10 (i.e. via residues Asp458/Asn479/Tyr471 and His474). These residues are conserved in nearly all isoenzymes (Figure 3c) [21••,22•]. Similarly, binding interactions of the peptide GalNAc residue to the lectin α-domain of GalNAc-T4 were recently reported [6••,23••] (Figure 3c); however, a large difference in the orientation of the lectin domains of GalNAc-T4 and GalNAc-T2 relative their catalytic domains was observed. As discussed in the sections below, these differences readily explain the origins of their different long range N-prior or C-prior glycosylation preferences (see Figures 1 and 4).

Figure 4



Superposition of *Hs*GalNAc-T2 and *Hs*GalNAc-T4. Superimposed cartoon representations of *Hs*GalNAc-T2-UDP-MUC5AC-13 glycopeptide (GlyThrThrProSerProValProThrThrSerThrThr*SerAlaPro) complex depicted in red and *Hs*GalNAc-T4-UDP-diglycopeptide 6 (GlyAlaThr*3GlyAlaGlyAlaGlyAlaGlyThr*11Thr12ProGlyProGly) complex depicted in blue. The MUC5AC-13, diglycopeptide 6 and GalNAc moieties are shown in red, blue, and orange atoms, respectively. The arrows indicate the direction of the long-range glycosylation preference of each enzyme, based on the orientation of their respective lectin domains with respect their catalytic domains. Note that the critical Asp residues of the lectin domain GalNAc-binding sites are indicated for clarification purposes.

Earlier work had suggested that the lectin domain of GalNAc-Ts could likely influence substrate specificity by steric hindrance that would depend on the size of the amino acid side chains of the glycopeptide substrate [37], while it has also been suggested that the lectin domains of some GalNAc-Ts could form hetero-dimers and/or homo-dimers that could also alter their specificity [38]. The recent crystal structures of the fly PGANT9-A and PGANT9-B lectin domain splice variants now offers intriguing evidence for something like the former [24••]. In this case, a loop on the lectin domain that protrudes toward the catalytic domain peptide binding site differs in charge between the splice variants. These charge

(Figure 3 Legend Continued) only the water molecule act as a bridge between the residues. **(c)** Main interactions between GalNAc-T2, GalNAc-T4 and GalNAc-T10 isoenzymes lectin domain (shown as salmon, purple and slate, respectively) and the GalNAc moiety (shown as sticks with orange carbon atoms).

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differences correlate with their activities toward highly charged substrates, thus suggesting that at least electrostatic interactions, if not direct peptide substrate binding, of the lectin domain can significantly influence transferase activity [24••]. Biologically, these splice variances are used to properly glycosylate different secretory mucins, whose incomplete glycosylation is shown to alter secretory granule morphology [24••]. Concurrently, structural and molecular dynamics studies on GalNAc-T4 bound to a diglycopeptide have revealed a flexible loop on its lectin domain that can approach the GalNAc residue of catalytic domain bound glycopeptide [23••]. Mutagenesis of this loop was shown to alter the kinetic properties of GalNAc-T4 against both peptide and glycopeptide substrates thus again confirming that additional features of the lectin domain beyond glycan binding will likely play roles in substrate selection of these transferases.

The flexible linker and its role in the remote glycosylation preferences of the GalNAc-Ts

The catalytic and lectin domains of all of the GalNAc-Ts (except for T20 that lacks the lectin domain) are connected by a linker sequence whose length and sequence varies among isoenzymes [6••,19•,20•,21••,22•]. Comparing linkers, the N-terminal regions are more conserved while the C-terminal regions are less conserved [6••]. Previous studies have attributed the relative positioning of the catalytic and lectin domains to the nature of the linker sequence [21••,39], thus the more stretched-out linker of GalNAc-T10 [22•] results in fewer interactions between both domains compared to the more closely spaced domains in GalNAc-T1 [19•] (Figure 2b). This suggested that linker flexibility could function to control the relative orientation of lectin and catalytic domains, therefore, modulating the selection of new GalNAc-modification sites in previously glycosylated substrates [4••,39,40••].

One of the largest questions in the field has been how these enzymes differentially recognize remote prior glycosylation sites in an N-terminal or C-terminal direction. A recent work on *Hs*GalNAc-T2 and *Hs*GalNAc-T4 shows that their flexible linkers display both interdomain rotation and interdomain translational-like motion which could be responsible of their different long range glycopeptide preferences [6••]. The crystal structure of *Hs*GalNAc-T4 with glycopeptide bound to the lectin domain [6••] revealed that its GalNAc-binding site was located on the opposite side of the lectin domain when compared to the homologous site in *Hs*GalNAc-T2 (Figure 4). These different positions of the lectin domain (Figure 4), readily account how GalNAc-T4 promotes the opposite long-range glycosylation preference compared to GalNAc-T2 and other isoenzymes [1•,4••] (see Figure 1). That this rotation is caused by the nature of the flexible linker was supported by molecular dynamics simulations, site-directed mutagenesis, and kinetics experiments [6••]. Indeed, the glycopeptide kinetics of GalNAc-T2 chimeras containing a GalNAc-T3 or GalNAc-T4 flexible linker and a series of flexible linker mutants, demonstrated that its long-range glycosylation preference could be modulated and even reversed simply by modifying its linker [6••]. This suggests that the flexible linker plays a major role in dictating each isoenzyme's long-range glycosylation preference by altering the lectin domain's orientation relative to its catalytic domain [6••]. All together, these findings showed for the first time how a structural feature that is neither in the active site nor in the lectin domain GalNAc-binding site is capable of modifying the activity and the glycosylation preferences of these isoenzymes.

Final remarks

That the GalNAc-Ts are associated with numerous human diseases including cancer [14,15,16•,41] clearly justifies the importance of unravelling the molecular basis that lie beneath their substrate recognition, ranging from redundant overlapping sites [42] to highly specific targets. Here, we have briefly summarized the most important advances at structural level of this family of enzymes that begin to reveal the molecular origins of their unique peptide and glycopeptide specificities. However, additional structures of these isoenzymes in complex with both their redundant and specific (glyco)peptide substrates will be necessary for a thorough mechanistic understanding of their promiscuity, specificity, and distinct glycosylation preferences. In particular, much more needs to be understood regarding their short-range glycosylation preferences as we currently have only one example describing such GalNAc-T-(glyco)peptide recognition. Hence, it is of utmost importance to continue studying this complex family of enzymes to fully understand how they selectively recognize their targets in multiple signaling pathways. Such studies will in turn facilitate the development of GalNAc-T modulators and inhibitors that would certainly be useful for the treatment of many diseases [11,13,16•,43,44]. Finally, one cannot discard the potential for Nature organizing the GalNAc-T's in a cell according to their isoenzyme class (e.g. early, intermediate and late GTs) utilizing their different glycosylation preferences to produce the vast repertoire of glycosylation sites observed *in vitro*. Such organization is clearly present as the retrograde introduction of GalNAc-Ts into the ER (the so called GALA pathway) has been shown to manifestly alter the patterns of *O*-glycosylation and may play a role in cancer [26•,41]. However, this pathway is currently under an intense debate in the Glycobiology community hence its importance has yet to be fully understood [45].

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