Recognition of a glycosylation substrate by the O-GlcNAc transferase TPR repeats

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O-linked N-acetylglucosamine (O-GlcNAc) is an essential and dynamic post-translational modification found on hundreds of nucleocytoplasmic proteins in metazoa. Although a single enzyme, O-GlcNAc transferase (OGT), generates the entire cytosolic O-GlcNAc proteome, it is not understood how it recognizes its protein substrates, targeting only a fraction of serines/threonines in the metazoan proteome for glycosylation. We describe a trapped complex of human OGT with the C-terminal domain of TAB1, a key innate immunity-signalling O-GlcNAc protein, revealing extensive interactions with the tetratricopeptide repeats of OGT. Confirmed by mutagenesis, this interaction suggests that glycosylation substrate specificity is achieved by recognition of a degenerate sequon in the active site combined with an extended conformation C-terminal of the O-GlcNAc target site.

1. Introduction

The attachment of a single β-N-acetylglucosamine (O-GlcNAc) sugar onto serine and threonine residues of nucleocytoplasmic proteins is a dynamic and abundant post-translational modification found in higher eukaryotes [1–3]. Remarkably, this modification is regulated by only two antagonistic enzymes: the O-GlcNAc transferase (OGT), which transfers the GlcNAc moiety onto acceptor residues from the donor sugar nucleotide UDP-GlcNAc, and the O-GlcNAc hydrolase (OGA), which removes it. To date more than 1000 O-GlcNAc proteins have been identified by mass spectrometry [4–10]. These proteins cover a wide range of cellular processes such as transcription and translation [11–13], trafficking and localization [14,15], as well as cell cycle progression [16–19]. However, it remains unclear how a single OGT enzyme is able to specifically recognize a limited number of serines/threonines on such a large number of substrates.

OGT is a multi-domain protein with a catalytic core at the C-terminus and 13 tetratricopeptide (TPR) repeats at the N-terminus, making up about half of the enzyme. Early experiments suggested that the TPR domain is involved in substrate recognition and/or protein–protein interactions [20–27]. The structure of the isolated OGT TPR domain revealed topological similarity to other helical repeat proteins and led to speculation that this domain might bind substrates in an extended conformation [20]. The first structural insights into the OGT catalytic domain came from an OGT orthologue in the bacterium Xanthomonas campestris [28,29]. This structure revealed that the sugar donor binding site is made up of the two lobes of the glycosyl transferase B (GT-B) fold, tightly fused to the superhelical TPR domain [28]. The subsequent structure of human OGT [30] revealed a very similar fold with the addition of an intervening domain of unknown function between the two catalytic lobes [30]. The structure suggested an ordered bi-bi mechanism of substrate binding, in which UDP-GlcNAc binds before the acceptor substrate [30]. Initial structural studies exploring Michaelis/substrate complexes with short acceptor peptides have
revealed limited substrate interactions with the enzyme [31,32]. More recently, Pathak et al. investigated the common binding modes of acceptor peptides to OGT [33]. Starting from a peptide library, they identified preference for certain acceptor peptide sequences, leading to definition of a degenerate sequon of OGT peptide substrates ([ITS][PT][VT][S/T][RLV][ASY]). Crystal structures of complexes of OGT with some of these peptides revealed that OGT binds all the acceptor peptides studied so far in an extended conformation with similar conformation of the residues in the −3 to +2 position around the acceptor serine/threonine. Although the C-termini of these peptides point towards the TPR domain, these structural data do not explain how OGT recognizes larger protein substrates for glycosylation. The short sequence patterns alone are not sufficient to accurately predict the O-GlcNAc proteome, suggesting other mechanisms contribute to substrate recognition.

A clue to how this might work came from the unusual OGT substrate host cell factor 1 (HCF1). HCF1 is a ubiquitously expressed chromatin-associated protein and a major transcriptional co-regulator involved in numerous cellular processes such as cell cycle progression (reviewed in [34]), which has also been shown to be heavily O-GlcNAcylated [35]. HCF1 is initially expressed as an approximately 210 kDa protein that is activated by limited proteolysis (protease maturation) within the proteolytic processing domain (PPD), consisting of multiple 20-residue repeats [36,37]. Strikingly, in 2011 it was discovered that OGT not only glycosylates HCF1 but is also needed for its proteolytic maturation [13,35]. A depletion of OGT leads to an accumulation of full-length HCF1 protein and the PPD is proteolytically cleaved by OGT via an unusual glycosylated glutamate interactor [36,37].

The O-GlcNAcylation sites on the OGT substrates TAB1 [45], colapsin response mediator 2 protein (CRMP2) [9] and casein kinase 2 (CK2) [46] are located in disordered regions close to the C-terminus (figure 1a). Although short peptides derived from these sites can be co-crystallized with OGT [31–33], we have been unsuccessful in using this approach with longer sequences/intact proteins to explore the role of the OGT TPR domain in substrate recognition. Aligning the sequences around the O-GlcNAc sites reveals similarities near the site of modification (figure 1b). Remarkably, this is also similar to the proteolytic cleavage site of a HCF1PRO repeat, with the major difference being a glutamate at the acceptor position (figure 1b).

2. Results and discussion

2.1. The TAB1 O-GlcNAC site resides in a disordered region with similarity to other OGT targets

The O-GlcNACylation sites on the OGT substrates TAB1 [45], colapsin response mediator 2 protein (CRMP2) [9] and casein kinase 2 (CK2) [46] are located in disordered regions close to the C-terminus (figure 1a). Although short peptides derived from these sites can be co-crystallized with OGT [31–33], we have been unsuccessful in using this approach with longer sequences/intact proteins to explore the role of the OGT TPR domain in substrate recognition. Aligning the sequences around the O-GlcNAc sites reveals similarities near the site of modification (figure 1b). Remarkably, this is also similar to the proteolytic cleavage site of a HCF1PRO repeat, with the major difference being a glutamate at the acceptor position (figure 1b).

2.2. A linear fusion of OGT and HCF1PRO reproduces the HCF1PRO binding mode

To explore whether a fusion of the C-terminus of a peptide substrate to the N-terminus of a truncated OGT (312–1031) would generate physiological OGT-substrate complexes, we explored this approach first with HCF1PRO. A construct was designed where an 18-mer HCF1PRO repeat peptide (PPCETHETGTTNTERATAT) was fused to the N-terminal Thr315 of OGT via a three glycine (3xGly) linker (figure 2b). The peptide interacts with the TPRs through some of its side chains, but intriguingly five regularly spaced asparagine side chains in OGT form hydrogen bonds with the HCF1PRO peptide backbone in a sequence-independent manner (figure 2b). We noted the fortuitous proximity of the HCF1PRO C-terminus to the OGT N-terminus (CGQHCF1PRO3xGlyOOGT1322 approx. 12 Å; figure 2b), and wondered whether this would enable the direct tethering of substrates to OGT via a fusion linker to allow us to explore OGT-glycosylation substrate complexes.

2.3. A linear TAB1:OGT fusion suggests that TAB1 makes extensive interactions with the OGT TPRs

We next explored the OGT-substrate fusion approach as a means of trapping complexes of OGT with TAB1. We
generated a TAB1:OGT fusion construct matching the HCF1PRO:OGT fusion, using an 18-mer TAB1 peptide derived from the S395 glycosylation site (VPYSSAQSTSKTSVTLSL; figure 2a). The chimaeric protein was overexpressed as a His6-fusion construct in *E. coli* and purified as described for the HCF1PRO:OGT fusion protein (figure 2a). We were able to generate crystals of the TAB1:OGT fusion protein, solve the structure by molecular replacement and refine the complex against 2.5 Å synchrotron diffraction data to *R*$_{work}$/*R*$_{free}$ = 0.22/0.25 (electronic supplementary material, table S1). The unbiased $|F_o|-|F_c|$ density allowed unambiguous building of the linker and peptide (figure 2b). The first eight amino acids of the TAB1 peptide (VPYSSAQ), covering the glycosylation site, were found in a similar conformation in the active site to the free TAB1 peptide in complex with OGT reported previously [31] (figure 2b, RMSD on C$_{α}$ = 1.4 Å). The electron density revealed Ser395 to be glycosylated as a result of self-glycosylation during expression in *E. coli*, which was confirmed by western blot analysis (figure 3a). The sugar occupies the same position as observed in a complex with a short synthetic TAB1 glycopeptide [31] (maximum atomic shift = 0.1 Å). Intriguingly, there appears to be some extra electron density near Ser396 and Ser399 suggestive of additional glycosylation sites (electronic supplementary material, figure S1) that could be an artefact of the very high (local) concentrations of the fused substrate peptide, or glycosylation occurring in *trans* as a result of the high protein concentrations (approx. 10 mg ml$^{-1}$) used in the crystallization experiments. In the TAB1:OGT fusion structure, the TAB1 peptide forms two side-chain-mediated interactions (Ser404/Thr406) with the TPR domain of OGT (Asp386/Asp420) (figure 2b). These are remarkably similar to the interactions between the same OGT residues and Thr1090 and Thr1092 of the HCF1PRO repeat (figure 2b). Similarly, the interactions between the TAB1/HCF1 peptide backbones and the five regularly spaced OGT TPR asparagines are conserved (figure 2b). Furthermore, the overall conformations of the TAB1 and HCF1 peptides in the respective fusion constructs is similar (RMSD on C$_{α}$ = 1.3 Å). Thus, a linear TAB1:OGT fusion suggests that the TAB1 OGT substrate makes extensive interactions with the OGT TPRs.

2.4. Interactions with the OGT TPRs contribute to TAB1 O-GlcNAcylation

Although the similarity to the HCF1 peptide binding mode and the presence of glycosylation on Ser395 suggests we have trapped a physiologically relevant TAB1:OGT complex, we further tested this model by structure-guided site-directed mutagenesis in the context of truncated OGT (312–1031) and TAB1 (7–409) as separate proteins. Two types of OGT mutants were generated: a single-point mutant in the active site (K842M), known to be essential for catalytic activity [31] and a quintuple mutant where the five key asparagine residues (P4PCETHETG) were changed to alanine. These mutants were expressed as His6-fusion constructs and purified as described for the wild-type OGT. We then tested the ability of the wild-type and mutant OGT constructs to glycosylate the TAB1:OGT fusion protein (figure 2c). The wild-type OGT construct was able to glycosylate the TAB1:OGT fusion protein to a similar extent as the wild-type OGT in the absence of the fusion construct, indicating that the fusion construct did not interfere with the binding and catalytic activity of OGT. In contrast, the mutant OGT constructs were unable to glycosylate the TAB1:OGT fusion protein, indicating that the five asparagine residues are essential for the binding and catalytic activity of OGT. These results suggest that the five asparagine residues are important for the binding and catalytic activity of OGT, and that the TAB1:OGT fusion construct mimics the physiologically relevant binding mode of the HCF1:OGT complex.
residues that form the bulk of interactions in the TPR domain (Asn322, Asn325, Asn356, Asn390 and Asn424, figure 2) were all mutated to alanines (from here on referred to as the 5N5A mutant). Based on the TAB1:OGT fusion protein complex, the 5N5A mutations would be expected to disrupt the binding of the C-terminal region of TAB1 to the TPR domain. Using western blot analysis, we probed OGT activity on TAB1 and blotted for O-GlcNAcylation using an O-GlcNAc Ser395 specific antibody [45]. As demonstrated previously, TAB1 is readily O-GlcNAcylated by WT OGT, whereas no glycosylation is observed with the catalytically inactive K842M mutant [31] (figure 3c). The 5N5A mutant shows significantly reduced activity on a free TAB1 peptide (KKPVSVPYSSAQSTSKTSVTLSL) matching the peptide used in the fusion construct (figure 3b), in agreement with the interactions formed by the key asparagines in the TPR domain of OGT observed in the structure (figure 2b).

![Figure 2](rsob.royalsocietypublishing.org/Content/figure2.png)

**Figure 2.** Design and structures of OGT:substrate fusion proteins. (a) Partial sequence of the fusion proteins showing the His6-tag, the sequence of the substrate peptide, the 3xGly linker and the start of the OGT protein. Construct boundaries are indicated. The reported O-GlcNAc site Ser395 on TAB1 [45] is highlighted with a red arrow. Visible residues in the respective fusion construct structures are highlighted by a green box. (b) Three panels showing the structures of the free HCF1PRO repeat (PDBID 4N39) bound to OGT (i), the fusion protein HCF1PRO:OGT (ii) and the fusion protein TAB1:OGT (iii). OGT is shown in cartoon representation with the TPR and catalytic domains in grey and blue, respectively. The substrate peptides, UDP and the GlcNAc residues are shown as yellow, black and pink sticks, respectively. The 3xGly linker in the fusion constructs is shown as green sticks. OGT residues interacting with the backbone of the substrate peptide are shown as magenta sticks and residues interacting with side chains are shown as orange sticks. The catalytically important K842 residue is shown as orange sticks. The green labels highlight the start and end residues of the substrate part of the fusion constructs. The distance from the N-terminus of OGT and the C-terminus of the free HCF1PRO peptide (top) is shown as a black double-headed arrow. Polar interactions between OGT and the backbone and side chains of substrate peptides are shown as black and blue dashed lines, respectively. The $F_o - F_c$ map for the fusion constructs HCF1PRO:OGT and TAB1:OGT are shown as light-blue mesh contoured to 2.5 $\sigma$. 
suggesting that while interactions of the TAB1 C-terminus with the OGT TPRs are important, further interactions with the globular pseudophosphatase domain of TAB1 may exist. Nevertheless, interactions with the OGT TPRs contribute to TAB1 O-GlcNAcylation.

3. Concluding remarks

The human O-GlcNAc transferase is a multi-domain protein and is essential in metazoa [12,47,48]. However, it is still unclear how a single OGT enzyme recognizes its multitude of substrates. Previous work has proposed sequence specificity targeting −2 to +3 relative to the acceptor residue [33,49]. Previous work has also suggested the involvement of the TPR domain in substrate recognition by incrementally removing repeats from the TPR domain, resulting in a loss of activity on substrates even on a peptide level, although the molecular basis of this was as yet unclear [20–23,25–27]. Using the proteolytic OGT substrate HCF1, Lazarus et al. [24] revealed the involvement of multiple OGT residues on the concave surface of the TPR domain in binding side chains and backbone of the HCF1PRO repeat proteolytic substrate. Here, we used a fusion approach to trap OGT-substrate complexes to investigate the role of the TPRs in recognition of glycosylation substrates. We first demonstrated that this fusion approach recapitulates the published HCF1 PRO peptide binding mode and then used that to reveal how the C-terminus of the OGT glycosylation substrate TAB1 is recognized by the enzyme. The TAB1 C-terminus binds in an extended conformation in the TPR domain, making extensive contacts with the concave surface through regularly spaced asparagines in OGT. An OGT mutant lacking these asparagines was deficient in glycosylation of TAB1. Interestingly, the data show a complete loss of O-GlcNAcylation of a free TAB1 C-terminal peptide, whereas activity on a TAB1 protein is more modestly reduced. These findings, coupled with recently published work on an OGT substrate sequence preference [33], suggest that OGT may bind its substrates through a combination of mechanisms. It is interesting to note that many other OGT substrates (e.g. Casein kinase II and CRMP2; figure 1a) also possess similarly disordered material, figure S2b), on the TAB1 (7–409) protein (figure 3c), suggesting that while interactions of the TAB1 C-terminus with the OGT TPRs are important, further interactions with the globular pseudophosphatase domain of TAB1 may exist. Nevertheless, interactions with the OGT TPRs contribute to TAB1 O-GlcNAcylation.

Figure 3. Activity of wild-type and mutant OGT on peptide and protein substrates. (a) Western blot analysis of CpOGA treated purified TAB1:OGT fusion protein. Briefly, 0.5 μg of TAB1:OGT was incubated in the presence and absence of approximately 10 μg ml⁻¹ CpOGA for 30 min at 37°C. Reactions were stopped by addition of LDS-loading buffer and boiling at 95°C. (b) Graph showing the steady-state kinetics to determine the Kₘ of the TAB1 peptide (KKPVSVPYSSAQSTSKTSVTLSL). Briefly, 50 nM hOGT(WT/SN5A) was pre-incubated with varying concentrations of TAB1 peptide before starting the reaction by adding UDP-GlcNAc to a final concentration of 50 μM. The reaction was stopped before 10% of substrate was converted by addition of detection reagent in 50% MeOH. Kₘ(WT) = 42 ± 7 μM, Kₘ(SN5A) = N.D. (c) Western blot analysis of in vitro glycosylation reactions of TAB1 with OGT (WT/mutants). Briefly, 10 μM TAB1 protein was incubated with 50 nM hOGT in 100 μl 0.1 M Tris-HCl pH 7.4, 0.15 M NaCl, 0.5 mM TCEP buffer. Reactions were started by the addition of UDP-GlcNAc to a final concentration of 100 μM and incubated at 25°C. Samples were taken at indicated time points and reactions stopped by boiling for 5 min at 95°C in LDS-loading buffer.
regions C-terminal of the O-GlcNAcylatation site, suggesting that this may be a general mode of OGT substrate recognition. However, O-GlcNAc sites have also been reported to reside in/close to secondary structure motifs, as is the case for Histone H2B [50], p53 [51], the glucose-6-phosphate dehydrogenase G6PD [52] and SNAP-29 [53]. It is possible that a subset of substrates is O-GlcNAcylated in a co-translational fashion as proposed by recent work [54]. In this work, we have shown, using crystallography and site-directed mutagenesis, that the OGT substrate TABI binds the enzyme in the same way as the proteolytic substrate HCF1 [24] and that the five asparagine residues found on the concave surface of the TPR domain (Asn321, Asn322, Asn356, Asn390 and Asn424) are important for binding. Future studies could be directed at dissecting which other parts of OGT and/or substrate proteins contribute to substrate binding.

4. Material and methods

4.1. Construct design/cloning

A codon-optimized version of hOGT 313–1031, based on the boundaries described in [31], was ordered from GenScript and cloned as a BamHI–NotI restriction fragment into a modified version of pGEX6P1 containing a 6His tag instead of a GST tag. PCR primers (6H_HCFI_GGG_hOGT_fwd GTATTCACTGCAATCCACCACTACAgccggccgagacacagcag, 6H_HCFI_GGG_hOGT_rev CAGGTTGTTCAAGGGATACAG CATGGGTacccgacctgtggcttgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtg
by adding UDP-GlcNAc to a final concentration of 100 nM and incubating the reaction mixtures at 25°C. Ten microlitres of sample mixtures was taken at indicated times and mixed with 4x LDS sample loading buffer to a final volume of 50 µl and boiled at 95°C for 5 min. Proteins were resolved using precast SDS-PAGE gels (NuPAGE 4–12% Bis-Tris gels, Invitrogen) and blotted onto nitrocellulose membranes (GE Healthcare). The primary antibodies were used at the following concentrations: Anti-TAB1-O-GlcNAc (1:1000 [45]), anti-TAB1 (1:1000, Division of Signal Transduction and Translation, University of Dundee) and anti-OGT (1:2000, DM17, Sigma-Aldrich, Cat#: O6264). Li-Cor secondary antibodies (IRDye 680 Donkey anti-rabbit and IRDye 800 Donkey anti-rabbit, anti-sheep) were used at dilutions of 1:10 000. Blots were imaged using the Li-Cor Odyssey infrared imaging system (Li-Cor, Lincoln, NE). Quantification of the O-GlcNAc specific signal (gTAB1) was performed using imageStudioLite (Li-Cor) and normalized to total OGT (tOGT) and total TAB1 (tTAB1) signal. Data were plotted with GraphPad PrisM 7.

4.5. Steady-state kinetics

hOGT activity was determined in reactions containing 50 nM of either WT or 5N5A Hs-hOGT (312–1031), 50 mM Tris-HCl pH 7.4, 0.1 mg ml⁻¹ BSA, 10 µM sodium dithionate and varying concentrations of the TAB1 peptide KKPVSVPYSSAQSTSKTSVTLS or at a fixed concentration of 10 µM of the TAB1 peptide KKPVSVPYSSAQSTSKTSVTLS, in a total volume of 100 µl. Reaction mixtures were preincubated for 15 min before initiating the reaction by adding UDP-GlcNAc to a final concentration of 50 nM. Reactions were incubated for 30 min at 21°C before addition of 200 µl of 75 µM pyrocatechol violet/15 µM fluorophore, DP-sensitive xanthene-based Zn(II) compound [33,62,63], in 25 mM HEPES pH 7.4, 10 mM NaCl, 50% (v/v) MeOH. UDP formation was detected on a Gemini EM fluorescent Microplate reader (Molecular Devices) using excitation and emission wavelengths of 485 nm and 530 nm, respectively. Turnover did not exceed 10% for either substrate. Data are presented as average of three measurements, with error bars showing s.e.m. Data were analysed with GraphPad PrisM 7.

4.6. Western blot analysis of purified TAB1:OGT

Samples of purified TAB1:OGT fusion protein were incubated for 30 min at 37°C in the presence and absence of approximately 10 µg ml⁻¹ CpOGA, a promiscuous bacterial O-GlcNAc hydrolase [64]. Samples were supplemented with 4x LDS-loading buffer and boiled for 5 min at 95°C. A total of 0.5 µg of each untreated and treated TAB1:OGT fusion protein were subjected to SDS-PAGE analysis and transferred onto a nitrocellulose membrane (GE Healthcare), using a wet-transfer system (Invitrogen). The membrane was blocked in 5% BSA for 30 min at 21°C before incubating with anti-O-GlcNAc AB (RL2, 1:1000, Abcam, catalogue no. ab27399) and anti-OGT AB (1:2000, Abcam, catalogue no. 177941). Li-Cor secondary antibodies IRDye 680 Donkey anti-mouse (anti-O-GlcNAc) and IRDye 800 Donkey anti-rabbit (anti-OGT) were used at dilutions of 1:10 000. Blots were imaged using the Li-Cor Odyssey infrared imaging system (Li-Cor, Lincoln, NE).

5. Accession codes

X-ray diffraction data and refined structures have been deposited in the Protein Data Bank under accession codes 5LWV (HCF1PRO:OGT) and 5LVV (TAB1:OGT).

Data accessibility. This article has no additional data.

Authors’ contributions. D.M.F.v.A. conceived the study; O.R., K.R., A.T.F. and V.K. performed experiments; V.S.B. performed peptide synthesis; O.R., K.R., V.K. and D.M.F.v.A. analysed and interpreted the data; K.R. and D.M.F.v.A. wrote the manuscript with input from all authors.

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