Exploiting O-GlcNAc transferase promiscuity to dissect site-specific O-GlcNAcylation

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Abstract

Protein O-GlcNAcylation is an evolutionary conserved post-translational modification catalysed by the nucleocytoplasmic O-GlcNAc transferase (OGT) and reversed by O-GlcNAcase (OGA). How site-specific O-GlcNAcylation modulates a diverse range of cellular processes is largely unknown. A limiting factor in studying this is the lack of accessible techniques capable of producing homogeneously O-GlcNAcylated proteins, in high yield, for *in vitro* studies. Here, we exploit the tolerance of OGT for cysteine instead of serine, combined with a co-expressed OGA to achieve site-specific, highly homogeneous mono-glycosylation. Applying this to DDX3X, TAB1, and CK2α, we demonstrate that near-homogeneous mono-S-GlcNAcylation of these proteins promotes DDX3X and CK2α solubility and enables crystallisation of mono-S-GlcNAcylated TAB1. Taken together, this work provides a new approach for functional dissection of protein O-GlcNAcylation.

Introduction

O-GlcNAcylation, the decoration of serine and threonine residues on nucleocytoplasmic proteins with a single moiety of N-acetylglucosamine (GlcNAc) (Torres and Hart 1984, Holt and Hart 1986), has roles in the regulation of signal transduction (Pathak, Borodkin et al. 2012), transcription (Lewis, Burlingame et al. 2016), translation (Jang, Kim et al. 2015), and cellular viability among others (Shafi, Iyer et al. 2000, Zhu, Liu et al. 2015, Lee, Kim et al. 2020, Zhu, Cheng et al. 2020). Only two enzymes are known to be responsible for the dynamics of O-GlcNAcylation in eukaryotes, diverging from other PTMs such as phosphorylation which relies on hundreds of kinases and phosphatases (Manning, Whyte et al. 2002). A single O-GlcNAc transferase (OGT) attaches O-GlcNAc (Martinez-Fleites, He et al. 2010), and a single O-GlcNAc hydrolase (OGA) hydrolyses O-GlcNAc (Dong and Hart 1994). Unlike many other eukaryotic glycosyltransferases that have well-defined acceptor sequences and substrate selectivities, OGT is a more promiscuous enzyme, as the most preferred OGT recognition sequon - [TS][PT][VT]-**S/T**-[RLV][ASY] - is only found in a fraction of the reported O-GlcNAc sites in large scale O-GlcNAcomics studies (Pathak, Alonso et al. 2015, Woo, Lund et al. 2018). Indeed, mass spectrometry studies have demonstrated that OGT decorates whole regions of proteins such as the nucleoporins, HCF1, and ankyrin G in an apparently non-specific way (Trinidad, Barkan et al. 2012, Woo, Lund et al. 2018). Overall, O-GlcNAc is a widespread PTM, present on > 5,000 proteins, playing key roles in multiple cellular and physiological processes, although the site-specific functions of O-GlcNAc are poorly understood, with only a small proportion of O-GlcNAc sites functionally

interrogated to date (Nie, Ju et al. 2020, Zhu, Cheng et al. 2020, Wulff-Fuentes, Berendt et al. 2021, Zhu, Zhou et al. 2022).

A key limitation in dissecting the functional consequences of site-specific O-GlcNAcylation is the difficulty in producing sufficient quantities of stoichiometrically mono-O-GlcNAcylated proteins at specific sites of interest, for use in downstream experiments. Chemical methods, such as post-translational chemical mutagenesis using dehydroalanine (dha) as a reactive handle for subsequent attachment of PTM mimics, have been used previously to generate homogenous, mono-GlcNAcylated protein mimetics (Chalker, Lercher et al. 2012, Dadová, Galan et al. 2018). However intrinsic drawbacks in using dha-chemistry to produce protein species carrying PTMs include the uncontrolled production of a racemic mixture of D/L enantiomers at the Cα, which may explain reduced recognition of the modified protein by endogenous PTM processing enzymes. For example, acetylated histones are poorly recognised by histone deacetylases after dha-chemistry (Chalker, Lercher et al. 2012). Furthermore, dha-derived O-GlcNAcylated proteins have an extra methylene linker in the side chain that is absent in native proteins (Chalker, Lercher et al. 2012, Dadová, Galan et al. 2018). Other methods such as expressed protein ligation of semi-synthetic O-GlcNAcylated proteins (Muir, Sondhi et al. 1998), have been used to investigate the role of O-GlcNAc on α-synuclein (Marotta, Lin et al. 2015), and HSP27 (Balana, Levine et al. 2021). However, this requires a desulfurization step that converts all native cysteines to alanine (Balana, Levine et al. 2021), necessitating extensive control experiments to ensure that global desulfurization does not affect the protein folding or function.

The use of bacterial systems to co-express OGT with recombinant protein substrates has also been explored (Shen, Gloster et al. 2012, Gao, Shi et al. 2018, Li, Yang et al. 2023). This approach takes advantage of the lack of OGT in the *E. coli* genome and the absence of bacterial OGT substrates (Lubas, Frank et al. 1997). Therefore, only the desired substrate is GlcNAc modified in bacterial expression systems (Gao, Shi et al. 2018). However, while some substrates, like TAB1 or histone H2B, produce high yields of soluble O-GlcNAc modified protein (Shen, Gloster et al. 2012, Gao, Shi et al. 2018), others, like the crumbs cell polarity complex 1 (CRB1) or the Abelson tyrosine-Kinase 2 (ABL2-FABD), become insoluble following co-expression with OGT (Goodwin, Thomasson et al. 2013). Furthermore, it has been shown that over-expression of OGT and substrates in bacterial systems can lead to modification of off-target sites, which may not reflect the physiological O-GlcNAcylation patterns observed in eukaryotes (Shen, Gloster et al. 2012, Goodwin, Thomasson et al. 2013). The lack of straightforward and accessible methods for producing stoichiometric and homogeneous quantities of O-GlcNAcylated proteins at specific sites, in high yields, remains as one of the main technical hurdles in the O-GlcNAc field.

Recently, we reported a genetic recoding approach to dissect site-specific O-GlcNAcylation (Gorelik, Bartual et al. 2019). We demonstrated that mutation of endogenous O-GlcNAc sites to Cys leads to isosteric S-GlcNAcylation within an otherwise unaltered O-GlcNAcome (Gorelik, Bartual et al. 2019). *In cellulo* experiments in eukaryotic cells with the S-GlcNAc mimetic demonstrated that OGA is unable to hydrolyse S-GlcNAc, resulting in elevated GlcNAcylation stoichiometries on OGA^{S405C}, $TAB1^{S395C}$, and $FEN1^{S352C}$ (Gorelik, Bartual et al. 2019, Tian, Zhu et al. 2021). Here, we

exploit this approach in a recombinant system to produce highly homogenous, site-specifically and stoichiometrically mono-S-GlcNAcylated recombinant proteins. We demonstrate that bacterial co-expression of OGT with a highly active OGA from *Clostridium perfringens* and OGT substrates carrying cysteine mutants at desired O-GlcNAc sites produces mg quantities of near homogenous mono-S-GlcNAcylated protein. Applying this approach to TAB1, casein kinase 2α (CK2α), and DEAD box RNA helicase 3 X-linked (DDX3X), we demonstrate that this rescued the solubility of DDX3X and CK2α which otherwise precipitate when only co-expressed with OGT, indicating that this approach may be broadly applicable to a range of OGT substrates and target sites. Moreover, applying this approach enabled crystallisation of mono-S-GlcNAcylated TAB1, whereas poly-GlcNAcylated TAB1 precipitated as observed previously. Therefore, this method may facilitate interrogation of the effects of O-GlcNAcyation on protein structure and function.

Results and Discussion

Co-expression of OGT and TAB1 produces heterogeneous O-GlcNAcylation

We previously published stable complexes of OGT with peptide substrates by fusing a truncated version of OGT to peptides containing known GlcNAc sites (Rafie, Raimi et al. 2017). This method, initially intended for the structural determination of the canonical OGT recognition sequon, showed the presence of off-target GlcNAc modification (Fig. 2A) (Rafie, Raimi et al. 2017). To determine whether this off-target activity is limited to peptide substrates, we transformed BL21 (DE3) bacterial cells already containing an OGT expressing plasmid with a His-tagged human TAB1 construct (hTAB1). hTAB1 was purified to high yield and the extent of hTAB1 O-GlcNAcylation assessed by conjugating GlcNAc moieties to GalNAc azide (GalNAz), using the azide group as a handle to attach a polyethyleneglycol (PEG) mass tag (Darabedian, Thompson et al. 2018). Using this PEGylation technique, we observed a heterogenous GlcNAc profile for the recombinant hTAB1 with up to four different GlcNAc moieties per molecule of hTAB1 protein (Fig. 2B). However, it is widely accepted that hTAB1 contains a single functional GlcNAc site on Ser395, which has been validated both by mass spectrometry and functional assays (Pathak, Borodkin et al. 2012, Woo, Lund et al. 2018, Joiner, Hammel et al. 2021). Taken together, these data show that co-expression of OGT and TAB1 produces heterogeneous O-GlcNAcylation.

A bacterial system to produce recombinant mono-GlcNAcylated TAB1

Off-target O-GlcNAcylation (i.e., O-GlcNAcylation occurring at non-physiological O-GlcNAc sites) may alter protein activity, stability, or protein:protein interactions, complicating interpretation of downstream *in vitro* studies. Additionally, different O-GlcNAcylation sites may exert different regulatory effects on proteins, challenging interpretation of *in vitro* studies using heterogeneous poly-O-GlcNAc proteins. We therefore explored the possibility of permanently installing a non-cleavable, single GlcNAc moiety in OGT substrates using bacterial host expression systems ((Gorelik, Bartual et al. 2019) ; see Fig. 1B for an illustrated summary). As a first step we mutated the single reported functional TAB1 GlcNAc site Ser395 to cysteine ($TAB1^{S395C}$) and demonstrated that this can still be modified by co-expression with OGT (Fig. 2C). However, like the wild type TAB1 co-expressed with OGT (Fig. 2B), the resulting GlcNAcylated $TAB1^{S395C}$ contained 4 GlcNAc sites (Fig. 2D). We next took advantage of the inability of glycoside hydrolases to hydrolyse thioglycosides (De Leon, Levine et al. 2017), by co-expressing $TAB1^{S395C}$ with a customized plasmid harbouring both OGT and the highly active bacterial O-GlcNAcase *Cp*OGA (Rao, Dorfmueller et al. 2006), anticipating this would lead to retention of a single S-GlcNAc site, with removal of spurious O-GlcNAcylation. Indeed, co-expression with *Cp*OGA resulted in TAB1 carrying a single GlcNAc modification as assessed by the PEG mass tagging approach, although a faint band at ~80 kDa was observed following mass tagging with DBCO-mPEG (5 kDa), this additional band may represent non-specific thiol labelling by the DBCO probe as a faint band shift is also observed in – GalT1 (unlabelled) control) (Fig. 2D). Taken together, these data show that we have established a bacterial system to produce recombinant mono-GlcNAcylated TAB1.

Crystallization of mono-S-GlcNAcylated TAB1

Determining the atomic structure of any macromolecule requires a pure and homogeneous protein sample. Failure in obtaining complete PTM stoichiometry often leads to a decrease in the sample's homogeneity which significantly reduces the likelihood of success (McPherson and Gavira 2014). It is worth noting that there are currently no experimentally determined protein structures with structurally defined O-GlcNAc sites. Indeed, we have previously unsuccessfully attempted to crystallise GlcNAcylated TAB1. Failure to achieve this is probably linked to the TAB1 heterogeneity when co-expressed with OGT (Fig. 2B). We wondered if our mono-GlcNAcylated TAB1^{S395C} would improve the chances of crystallising GlcNAcylated TAB1. Under our experimental conditions, TAB1^{S395C} purified from the co-expressed system with OGT and *Cp*OGA produced well-defined hexagonal crystals while TAB1^{S395C} co-expressed only with OGT failed to crystallise and instead precipitated (Supplementary Fig. 1A). A non-modified TAB1^{S395C}, used as an experimental control, produced the same hexagonal-shaped crystals as the mono-GlcNAcylated TAB1^{S395C}, but with a much higher nucleation rate. Further analysis of the TAB1^{S395C} crystals' content by Western blot ruled out the possibility of the crystals being formed by non-modified protein (Supplementary Fig. 1B). However, the low-resolution diffraction datasets of the TAB^{S395C} crystals did not allow further structural determination . Additional analysis by differential scanning fluorimetry showed no differences in the thermal stability of S-GlcNAcylated TAB1^{S395C} versus un-modified TAB1^{S395C} (Supplementary Fig. 1C). Poly-GlcNAcylated TAB1, produced by co-expression with

OGT in *E. coli*, was protected against thermally-induced aggregation in a previous study (Yuzwa, Shan et al. 2012). Our inability to detect a similar thermoprotective effect of GlcNAc on mono-S-GlcNAcylated TAB1 likely stems from analysing TAB1 modified at a single physiological site, rather than poly-GlcNAcylated TAB1, highlighting the importance of studying site-specific effects *in vitro* using the S-GlcNAc approach when assigning functionality to O-GlcNAcylation events. Overall, to the best of our knowledge these data demonstrate the first successful crystallisation of a GlcNAcylated protein – TAB1 - facilitated by the approach of generating stoichiometrically mono-S-GlcNAcylated proteins.

Mono-S-GlcNAcylation of DDX3X and CK2α rescues protein solubility

For some OGT substrates, previous attempts to produce soluble recombinant GlcNAc-modified protein were unsuccessful, owing to the O-GlcNAcylated form of the protein being insoluble (Goodwin, Thomasson et al. 2013), possibly due to off-target GlcNAcylation that might either affect solubility or disrupt protein folding. We next explored whether restriction to homogeneous mono-S-GlcNAcylation of physiologically relevant sites would remove these issues. An example of this phenomenon is DEAD box RNA helicase 3 X-linked (DDX3X), an RNA helicase with diverse roles in transcriptional regulation and translation initiation (Lai, Chang et al. 2010, Chen, Yu et al. 2016, Cannizzaro, Bannister et al. 2018). A previous study linked DDX3X Ser584 O-GlcNAcylation to OGT intellectual disability aetiology (Mitchell, Czajewski et al. 2022). *In vitro* dissection of the role of O-GlcNAc on DDX3X is hampered by the aggregation of DDX3X following OGT co-expression, irrespective of whether full-length DDX3X or a

previously reported soluble truncate was used for expression tests (Fig 3A,B) (Floor, Condon et al. 2016). The DDX3X^{S584C} mutant was co-expressed with either OGT only, or with OGT and *Cp*OGA, alongside a DDX3X^{S584A} mutant as a negative control.. Whereas co-expression of DDX3X^{S584C} with OGT resulted in precipitation of the O-GlcNAcylated form of the protein, the non-modified protein remained soluble (Fig 3B). However, co-expression with OGT and *Cp*OGA resulted in purification of GlcNAcylated DDX3X^{S584C} in high yield (typical yield: ~ 1.25 mg DDX3X per L of *E. coli* culture used). Crucially, O-GlcNAc Western blots revealed GlcNAcylation of DDX3X^{S584C} after co-expression with OGT/*Cp*OGA, and this GlcNAc signal was absent if DDX3X^{S584C} was expressed on its own (Fig 3C). PEGylation of purified DDX3X^{S584C} after OGT/*Cp*OGA co-expression, with alkyne-PEG (10 kDa) revealed a single 10 kDa band shift, and an estimated stoichiometry of 95% (Fig 3D). The DDX3X^{S584A} negative control co-expressed with OGT/*Cp*OGA lacked an observable O/S-GlcNAc signal by Western blotting (Fig 3C). Furthermore, as mutagenesis of Ser584 has not previously been reported, we verified by DSF that mutagenesis of Ser584 to Cys does not affect the thermal stability and, by extension, the folding of DDX3X (Supplementary Fig. 2). Therefore, cysteine mutagenesis of Ser584 on DDX3X, paired with OGT/*Cp*OGA co-expression, enables expression and purification of near-homogenous mono-S-GlcNAcylated DDX3X at a targeted site, in high yield, for subsequent *in vitro* studies.

To assess the broader applicability of this approach, we next targeted the known GlcNAc site (Ser347) on casein kinase 2α (CK2α) (Tarrant, Rho et al. 2012). CK2α is dual specificity Ser/Thr kinase that forms part of the CK2 holoenzyme, along with CK2β (Niefind, Guerra et al. 2001), and plays essential roles in cell survival (Yamane and

Kinsella 2005), DNA replication (Feng, Lu et al. 2021), and development (Götz and Montenarh 2017). O-GlcNAcylation of a single site – S347 – destabilises CK2α (Tarrant, Rho et al. 2012), and modulates CK2 kinase activity (Schwein, Ge et al. 2022). In the same fashion as TAB1 and DDX3X, we co-expressed CK2α^{WT} and the cysteine mutant (CK2α^{S347C}) with OGT +/- *Cp*OGA. We observed that co-expression of both CK2α^{WT} and CK2α^{S347C} with OGT alone resulted in aggregation of >50% of the O/S-GlcNAcylated glycoform of the protein (Fig 4A, lanes 4-6, 10-12). This was reflected in the poor yields of soluble CK2α^{WT} and CK2α^{S347C} following co-expression with OGT, compared to the yields of CK2α^{WT} and CK2α^{S347C} expressed without OGT (Supplementary Fig. 3). However, co-expression of CK2α^{WT} and CK2α^{S347C} with both OGT and *Cp*OGA improved soluble protein yields, suggesting that hyper-O-GlcNAcylation of off-target CK2α O-GlcNAc sites causes protein aggregation in *E. coli*. We additionally observed by O-GlcNAc Western blot that CK2α^{WT} was not GlcNAcylated following co-expression with OGT/*Cp*OGA, whereas CK2α^{S347C} was (Fig 4A). Utilising the PEG mass tagging approach, we determined that CK2α^{S347C} was mono-S-GlcNAcylated at a stoichiometry of >95% (Fig 4B). Although a putative di-glycosylation signal can be observed at ~75 kDa, the presence of this band in the absence of the DBCO-mPEG probe suggests that it results from non-specific reactivity of the His-tag antibody with bacterial protein contaminants present in the commercially available GalT1 stock, a phenomenon observed previously (Darabedian, Thompson et al. 2018).Taken together these data show that the bacterial OGT/*Cp*OGA co-expression system combined with targeted Ser->Cys mutations leads to mono-S-GlcNAcylation of DDX3X and CK2α and increased solubility.

Discussion

O-GlcNAcylation modulates diverse cellular and developmental processes, from stem cell pluripotency through to bioenergetics (Jang, Kim et al. 2012, Zhu, Zhou et al. 2022), although the role of O-GlcNAc is only understood for a small fraction of the >5,000 O-GlcNAc proteins identified to date. Elucidating the site-specific effects of O-GlcNAc on protein function will be key to understanding the myriad roles O-GlcNAc plays in cellular processes. However, the study of the site-specific effects of GlcNAc on particular proteins has historically proven challenging. Progress in the O-GlcNAc field towards site-specific installation of O-GlcNAc or GlcNAc mimetics has lagged behind compared to other PTMs, such as phosphorylation, where Ser -> Asp/Glu mutagenesis has been routinely implemented to mimic and study specific phosphorylation events. The study, especially at the molecular level, of the effects of O-GlcNAcylation requires accessible and scalable tools for producing mg quantities of stoichiometrically GlcNAcylated proteins, modified at a single target site of interest.

Co-expression of OGT with protein substrates has previously been used to study the effects of O-GlcNAc on Tau and malate dehydrogenase 1, among other targets (Gao, Shi et al. 2018, Zhu, Zhou et al. 2022, Li, Yang et al. 2023). This method provides a considerable simplicity over the most demanding and comparatively expensive chemical alternatives only affordable by a handful of very specialised laboratories worldwide (Dadová, Galan et al. 2018, Balana, Levine et al. 2021). However, this approach is not without issues as several groups, including ourselves, have observed that co-expression with OGT often leads to off-target modifications which could lead to undesired effects. (Goodwin, Thomasson et al. 2013, Rafie, Raimi et al. 2017). This,

together with the possibility of the bacterial NagZ hydrolase acting as a putative GlcNAc hydrolase (Goodwin, Thomasson et al. 2013), potentially complicates data derived from proteins produced by co-expression with OGT.

We have previously demonstrated that OGT is capable of S-GlcNAcylating Cys thiol groups *in cellulo* after mutagenesis of Ser/Thr O-GlcNAc sites to Cys, while the endogenous OGA is unable to hydrolyse the S-GlcNAc moiety (Gorelik, Bartual et al. 2019). Therefore, to compensate for the spurious off-target O-GlcNAcylation observed in the traditional OGT co-expression approach, we mutated the Ser/Thr O-GlcNAc sites on several target proteins to Cys. Then, to remove any off-target O-GlcNAc sites, we co-expressed OGT and substrates with the highly efficient *Clostridium perfringens* OGA (Rao, Dorfmueller et al. 2006). As we observed in all our tested proteins, this approach leaves a single S-GlcNAc moiety attached to the OGT substrate and leads to the observed site-specific elevation of GlcNAcylation stoichiometries. The ability to study the effects of individual GlcNAcylation events may be useful in instances where multiple O-GlcNAcylation sites on the same protein exert different effects, e.g. on protein activity and localisation. For example, O-GlcNAcylation of SP1 at Ser491 blocks its interaction with transcription initiation factor (TAF) 110D (Roos, Su et al. 1997), whereas modification of S613, Thr421, Ser699, and Ser703 may regulate SP1 localisation in response to insulin signalling (Majumdar, Harrington et al. 2006). Therefore, studies of multi-site O-GlcNAcylation, which require differentiating the effects of different O-GlcNAcylation sites on protein function, may be facilitated by the approach we describe here.

Interestingly, we observed that co-expression of DDX3X or CK2α with OGT resulted in precipitation of the O-GlcNAcylated glycoform of the protein. A similar phenomenon has been reported previously for CRB1 and ABL2, where the O-GlcNAcylated glycoforms of both proteins were exclusively found in the insoluble fraction – as we observed for full-length DDX3X co-expressed with OGT (Goodwin, Thomasson et al. 2013). This may stem from co-translational O-GlcNAcylation of OGT substrates (Zhu, Liu et al. 2015), which could interfere with the protein surface electrostatic properties or prevent proper folding. Additionally, NagZ in *E. coli* may reduce stoichiometry by stripping GlcNAc, an issue which cannot be overcome by treating *E. coli* with the OGA inhibitor PUGNAc (Haltiwanger, Grove et al. 1998, Goodwin, Thomasson et al. 2013). Indeed, a recent study utilising OGT co-expression with malate dehydrogenase 1 yielded only 31% stoichiometry, which may at least partially be attributable to NagZ activity (Zhu, Zhou et al. 2022). These issues previously limited the utility of *E. coli* for producing large quantities of GlcNAcylated protein for study. However, the ability to recombinantly produce hydrolase-resistant mono-S-GlcNAcylated TAB1, DDX3X and CK2α using our approach suggests that these limitations can now be overcome.

The ability to purify mg quantities of recombinant mono-S-GlcNAcylated proteins opens many avenues for future research. For example, similar to the effect observed in a TAB1 peptide-OGT fusion construct (Rafie, Raimi et al. 2017) and replicated again here with the isolated TAB1 protein, the spurious GlcNAcylation of TAB1 by OGT produces a complex, heterogeneously GlcNAcylated, sample. Furthermore, it is possible that the very same effect could be one of the reasons behind the lack of an O-GlcNAcylated protein in structural databases. However, as the samples produced by

our approach are near-stoichiometrically mono-S-GlcNAcylated, these samples are highly homogenous and thus more amenable to crystallography as demonstrated by the successful crystallisation of mono-S-GlcNAcylated TAB1^{S395C} reported here. Obtaining the atomic-resolution structure of GlcNAcylated proteins is of the utmost importance as these may provide insights into the effects of GlcNAcylation on protein folding, protein-protein interactions, or protein conformation, the latter permitting study of structure-activity relationships. This is particularly relevant considering recent studies demonstrating, by thermal profiling, that O-GlcNAc can act as both a stabiliser and destabiliser of different proteins, a phenomenon that could be explained by GlcNAc-dependent conformational changes in the protein (King, Serrano-Negrón et al. 2022).

Substitution of Ser/Thr for Cys can be achieved by conventional molecular cloning techniques, and production of mono-S-GlcNAcylated proteins requires a basic set-up for *E. coli* culture and protein purification that is widely available across many research groups. Due to the relatively recent discovery that OGT tolerates Cys to produce S-GlcNAc, this approach needs further validation on a greater number of OGT substrates, especially if used in *in vivo* biological systems. Nevertheless S-GlcNAc so far presents potentially the most straightforward and effective approach to study the effects of protein mono-GlcNAcylation both *in vivo* and especially in *in vitro* experimental setups.

Materials & Methods

Cloning of constructs for E. coli expression

Full-length human DDX3X was obtained by One-step RT-PCR from human RNA using the Takara Primescript High fidelity RT-PCR kit. This was cloned as a *Bam*HI-*Not*I fragment into a modified version of pGEX6P1. This vector had an added 6xHis sequence between the start codon and the GST tag. The region of DDX3X 132-607 was ordered as a codon-optimised geneblock from Integrated DNA Technologies including an N-terminal *Bam*HI site, a C-terminal stop codon and a *Not*I site. This fragment was digested and cloned into a *Bam*HI-*Not*I digested and dephosphorylated vector pHEX6P1 (this proprietary vector is based on pGEX6P1 with a 6xHis tag in place of the GST tag). The CK2α (1-361) and TAB1 (7-410) inserts were cloned in the same way into the same vector. All inserts were confirmed by DNA sequencing. The mutations in DDX3X (Ser584Ala and Ser584Cys), CK2α (Ser347Cys) and TAB1 (Ser395Cys) were carried out by site directed mutagenesis and verified by DNA sequencing.

Expression and purification of OGT substrates

A list of the proteins used in this work, with associated tags and construct boundaries, can be found in Supplementary Table 1. Briefly, each protein construct was transformed in either BL21 DE3 pLysS (NEB) harbouring a truncated OGT version with an intact catalytic core (OGT^{SW}) (Lazarus, Nam et al. 2011) or transformed in BL21 DE3 pLysS cells already containing a co-expression plasmid which simultaneously expresses

tagless OGT^{SW} and tagless *Cp*OGA. Each protein was expressed by inoculating 5 mL of overnight culture into 1 L of LB broth supplemented with ampicillin and kanamycin (total volume: 6 L). At an OD of 0.6, cultures were induced with the addition of 250 µM IPGT (Formedium) and grown at 16-18 4 °C overnight (except for CK2α, see below). Cultures were then recovered by centrifugation (4,000 x g, 4 °C, 40 minutes in a Beckman Avanti JXN-26 (rotor: JLA 8.1)) and pellets resuspended in approximately 30 mL of lysis buffer (20 mM HEPES, pH 7.5, 250 mM NaCl, 30 mM imidazole, 0.5 mM TCEP, 1 mM benzamidine, 0.2 mM PMSF, 5 µM leupeptin). Samples were subsequently stored at -20 °C until further use.

For the expression of CK2α, a previously described protocol (Olsen, Boldyreff et al. 2006) was used. Briefly, *E. coli* expressing the plasmid encoding CK2α were grown to an OD 0.5, followed by induction with 300 µM IPTG for 2 h (37 °C). Cells were harvested and lysed as described above in lysis buffer (20 mM Tris-HCl, pH 7.4, 200 mM NaCl, 0.5 mM TCEP, supplemented with 1 mM benzamidine, 0.2 mM PMSF, 5 µM leupeptin). All downstream purification steps were identical to those detailed above.

Frozen samples were defrosted and supplemented with ~0.1 mg/mL lysozyme, and ~0.1 mg/mL bovine DNase (Sigma Aldrich). Sample lysis was achieved with a French press pressure cell homogeniser adjusted to 1500 psi (Thermo Fisher Scientific). Then, lysates were centrifuged at 60,000 x g (4 °C) for 45 minutes (Beckman Avanti JXN-26 (rotor: JA 25.5)) and immediately supplemented with 10 mL pre-washed Ni^{2+}-NTA agarose (QIAGEN) followed by incubation for 2 h at 4 °C with agitation in a roller. Beads containing the protein of interest were washed twice with lysis buffer, twice with a buffer supplemented with 20 mM imidazole, to remove non-specific binders, and proteins

subsequently eluted with 250 mM imidazole. Due to the nature of DDX3X as a transcription factor, one additional step of washing with 1M NaCl to remove bound nucleic acids followed by a wash with lysis buffer was introduced for this protein prior of the wash with 20 mM imidazole (Floor, Condon et al. 2016). Eluted proteins were supplemented with 150 µL 4 mg/mL recombinant PreScission protease (Leong 1999) and simultaneously dialysed overnight to cleave the 6xHis tag and remove the excess of imidazole. After dialysis, the soluble protein was incubated with fresh Ni^{2+}-NTA beads, and eluent containing the tagless protein of interest was then concentrated to 5 mL and loaded on a HiLoad™ 16/600 superdex™ 200 pg size exclusion column (GE) equilibrated with lysis buffer. Fractions containing the protein of interest at the expected Mw were pooled, buffer exchanged into 20 mM HEPES, pH 7.5, 250 mM NaCl, 0.5 mM TCEP, concentrated to 5 - 10 mg/mL and flash frozen in liquid nitrogen prior to storage at -80 ° C until further use. In the case of DDX3X, as previously reported, the buffer was exchanged to 20 mM HEPES, pH 7.5, 250 mM NaCl, 500 mM NaCl, 10% (v/v) glycerol, 0.5 mM TCEP prior to storage (Floor, Condon et al. 2016).

GalNAc-azide labelling and CuAAC PEGylation of O-GlcNAc sites

20 µg of purified protein was chloroform/methanol precipitated by sequential addition of 600 µL methanol, 150 µL chloroform, and 400 µL water, followed by centrifugation. After removal of the upper (aqueous) phase, 450 µL MeOH was added, and protein pelleted by centrifugation. Proteins were re-suspended in 20 µL of 20 mM HEPES, pH 7.9, 1% (w/v) SDS, and azide-labelled using the Click-iT™ O-GlcNAc Enzymatic Labelling System (Thermo Fisher Scientific). Briefly, 24.5 µL miliQ water, 40 µL labelling buffer,

5.5 µL 100 mM $MnCl_2$, 10 µL 2 mM UDP-GalNAz, and 4 µL galactosyltransferase T1 Y289L were added to the 20 µL resuspension. The azide-labelling reaction was incubated at 4 °C overnight with rotation. After overnight labelling, cysteine residues were alkylated via incubation with 22.5 mM iodoacetamide for 30 minutes, followed by chloroform/methanol precipitation and resuspension in 20 µL 25 mM Tris-HCl, pH 8.0, 1% (w/v) SDS. CuAAC reactions were performed using the Click-IT Protein Reagent Buffer kit. Briefly, 20 µL of Click-IT reaction buffer, supplemented with 3.3 mM MeO-PEG-alkyne 10 kDa (final concentration), was added to the resuspended protein, followed by addition of 2 µL of 40 mM $CuSO_4$, 2 µL Click-IT reaction buffer additive 1 and 4 µL Click-IT reaction buffer additive 2. CuAAC PEGylations were incubated for 2 h at RT (1300 rpm). After a final round of chloroform/ methanol precipitation, PEGylated proteins were resuspended in 45 µL 1 x LDS loading buffer for Western blotting.

SPAAC PEGylation of GalNAc-azide labelled O-GlcNAc sites

Purified proteins were GalNAc-azide labelled as described above. For SPAAC PEGylation of azide-labelled proteins, after alkylation with 22.5 mM iodoacetamide, proteins were chloroform/ methanol precipitated and resuspended in 90 µL Tris-HCl, pH 8.0, 1% (w/v) SDS. 10 µL of 10 mM DBCO-mPEG was added to the 90 µL resuspension and incubated at 98 °C (5 minutes, 900 rpm). After reaction completion, PEGylated glycoproteins were chloroform/ methanol precipitated, resuspended in 45 µL 1 x LDS and analysed by Western blot.

Western blotting

250 to 500 ng of purified recombinant protein, or 500 ng of PEGylated recombinant protein, was loaded onto a NuPAGE Bis-Tris (8% polyacrylamide) gel. After SDS-PAGE and transfer to nitrocellulose membrane, proteins were blotted using one of the following antibodies, with their respective dilutions: rabbit α-DDX3X (1:1,000 ; Proteintech), mouse α-His-tag (1:1,000 ; Proteintech), α-O-GlcNAc (RL2 ; 1:1,000; Novus Biologicals); α-O-GlcNAc (CTD110.6 ; Cell Signalling Technology), rabbit α-Tab1 (1:1,000 ; Cell Signalling Technology), rabbit α-OGT (1:2,000 ; Sigma Aldrich).

S-GlcNAc Tab1^{S395C} crystallisation

Recombinant TAB1^{S395C} protein was crystallised as previously described in (Conner, Kular et al. 2006). Briefly, TAB1^{S395C} protein (10mg/mL) was mixed with the mother liquor in a 1:1 drop ratio. An identical procedure was followed for TAB1^{S395C} co-expressed with OGT and TAB1^{S395C} co-expressed with OGT and *Cp*OGA. As previously reported, hexagonal shaped crystals were observed after 3 to 5 days of incubation (Conner, Kular et al. 2006). To investigate the S-GlcNAcylation of TAB1^{S395C} crystals by Western blot, crystals were washed extensively in mother liquor prior to dissolution in lysis buffer and boiling in 1 X SDS (final concentration: 0.5 mg/mL).

Differential scanning fluorimetry

Protein denaturing profiles were recorded with a BioRAD CFX Opus 96 Real-time PCR configured for detecting FRET signal. Briefly, 1 mg/mL of recombinant protein was mixed with SYPRO Orange at a 5X final concentration (Invitrogen) reactions were supplemented with 50 mM HEPES, 150 mM NaCl pH 7.5 up to a final volume of 20 µL. All experiments were recorded in sextuplicate, and GraphPad Prism was used to plot and further analysed the data.

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Author contributions

D.M.F.v.A, S.G.B, and C.W.M conceived the study. A.T.F cloned all constructs used in this study. S.G.B and C.W.M performed recombinant expression of protein constructs and DSF experiments. C.W.M carried out PEGylations and stoichiometry measurements. S.G.B performed crystallisation and structural analysis. The manuscript was written by D.M.F.v.A, S.G.B, and C.W.M.

Abbreviations

O-GlcNAc Transferase (OGT), O-GlcNAc Hydrolase (OGA), N-acetyl glucosamine (GlcNAc), TGFβ activated kinase 1 (TAB1), Casein Kinase 2α (CK2α), DEAD box RNA Helicase 3, X-linked (DDX3X), Host Cell Factor 1 (HCF1), Post-Translational Modification (PTM), dehydroalanine (dha), Crumbs Cell Polarity Complex 1 (CRB1), Abelson Tyrosine-Kinase 2 (ABL2), *Clostridium perfringens* OGA (*Cp*OGA), Flap Structure-Specific Endonclease 1 (FEN1), Dibenzocycloocytne Polyethylene Glycol (DBCO-PEG), Methoxy Alkyne Polyethylene Glycol (MeO-alkyne PEG), Strain

Promoted Azide Alkyne Cycloaddition (SPAAC), Copper Catalysed Azide Alkyne Cycloaddition (CuAAC).

Data availability statement

The data underlying this article will be shared upon reasonable request to the corresponding author. E-mail : daan@mbg.au.dk

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FIGURE LEGENDS

Figure 1. Illustrated summary of the dual cysteine mutagenesis/ *in cellulo* O-GlcNAc stripping approach to producing highly homogenous mono-S-GlcNAcylated proteins

A) Previously, OGT substrates of interest were co-expressed with OGT in *E. coli*. This process introduces multiple O-GlcNAc sites and produces a heterogenous sample of variable yield and purity.
B) The method reported in this study, mutating the O-GlcNAc site of interest from Ser/Thr to Cys followed by *in cellulo* stripping of O-GlcNAc sites by *Clostridium perfringens* OGA, is illustrated.

Figure 2. Tab1^{S395C} co-expression with OGT/ *Cp*OGA removes off-target O-GlcNAcylation sites and yields a near stoichiometrically modified S-GlcNAc protein

a) Crystal structure of a TAB1 peptide, including the canonical Ser395 O-GlcNAc site, fused to OGT shows off-target GlcNAcylation (PDB: 5LVV)
b) 1 µg of purified Tab1 co-expressed with OGT was azide labelled with GalT1 and conjugated to either DBCO-PEG (2 kDa) or DBCO-PEG (5 kDa) via strain promoted azide-alkyne cycloaddition. Mass shifts compatible with the presence of several GlcNAc moieties were detected by Western blot against TAB1.
c) Confirmation of TAB1^{S395C} GlcNAcylation by Western blot using anti-O-GlcNAc CTD 110.6 antibody (red channel). Lane A contains TAB1^{S395C}, lane B contains TAB1^{S395C} co-expressed with OGT and lane C contains TAB1^{S395C} co-expressed with OGT and *Cp*OGA. The identity of TAB1 was confirmed by using an anti-TAB1 antibody (green channel).
d) 1 µg of purified Tab1^{S395C} was azide labelled with GalT1 and conjugated to either DBCO-PEG (2 kDa) or DBCO-PEG (5 kDa) via strain promoted azide-alkyne cycloaddition. PEGylated glycoproteins were detected via Western blotting for Tab1.

Figure 3. DDX3X^{S584C} is near homogenously mono-S-GlcNAcylated at C584 following OGT/*Cp*OGA co-expression

a) GST-His$_6$-DDX3X^{WT} was co-expressed with OGT (see Materials & Methods section for more details) in *E. coli* and purification attempted via Ni^{2+} affinity chromatography. Aliquots of the annotated fractions were blotted for DDX3X and O-GlcNAc. Note the absence of an O-GlcNAc DDX3X signal in the soluble fraction.
b) His$_6$-DDX3X$^{132-607}$ Ser584Cys was co-expressed with OGT (see Materials & Methods section for more details) in *E. coli*. After purification of O-GlcNAcylated DDX3X (lane 5), the His-tag was cleaved during overnight dialysis in imidazole-free lysis buffer with PreScission protease and tagless DDX3X eluted from Ni^{2+} resin. Fractions from each purification step were blotted for OGT, DDX3X, and O-GlcNAc. Note the loss of DDX3X O-GlcNAc signal after tag cleavage.
c) O-GlcNAc and DDX3X Western blots for DDX3X^{S584C} and DDX3X^{S584A} after co-expression with OGT and *Cp*OGA, and DDX3X^{S584C} after expression without either OGT or *Cp*OGA (non-O-GlcNAcylated control).
d) Copper catalysed azide alkyne cycloaddition of DDX3X^{S584C}, after co-expression with OGT and *Cp*OGA, to alkyne-PEG (10 KDa). A separate labelling reaction without GalT1 was carried out as a negative control.

Figure 4. S-GlcNAcylation of CK2α^{S347C} after OGT/*Cp*OGA co-expression yields stoichiometrically mono-S-GlcNAcylated CK2α and rescues protein solubility

a) *E. coli* were transformed with plasmids encoding either CK2α only, CK2α with OGT, or CK2α with OGT/*Cp*OGA. After induction with IPTG, soluble (S) and insoluble fractions (P) of *E. coli* lysate, and the elution from Ni^{2+} beads following Ni^{2+}-affinity chromatography (E), were analysed by blotting for the N-terminal His tag and O-GlcNAc.
b) O/S-GlcNAc sites on purified CK2α^{WT}, expressed in the presence or absence or OGT/*Cp*OGA, and CK2α^{S347C}, expressed with OGT/*Cp*OGA, were azide-labelled via GalT1 and conjugated to DBCO-mPEG (5 kDa) via strain promoted azide-alkyne cycloaddition. Total CK2α (both labelled and unlabelled) was detected by blotting for the N-terminal His tag. CK2^{S347C} was reacted with DBCO-mPEG (5 kDa) in the absence of GalT1 azide labelling to control for non-specific labelling of cysteine thiols by the DBCO probe.

Supplementary Figure S1: Mono S-GlcNAcylated TAB1 crystallisation and thermal stability

a) Images of the crystal trials of TAB1^{S395C} drops, from left to right: TAB1^{S395C}, TAB1^{S395C} co-expressed with OGT and finally, TAB1^{S395C} co-expressed with OGT plus *Cp*OGA.
b) TAB1^{S395C} and TAB1^{S395C} co-expressed with OGT plus *Cp*OGA crystals were fished, extensively washed in mother liquor and analysed for the presence of S-GlcNAc by Western blot.
c) DSF profile of TAB1^{S395C} and TAB1^{S395C} co-expressed with OGT plus *Cp*OGA. Each point in the graph represents the average of 6 replicates. Standard deviations are represented.

Supplementary Figure S2. The thermal stability of DDX3X^{WT} and both un-modified and S-GlcNAcylated DDX3X^{S584C}.

The thermal stability of 1 mg/ mL of DDX3X was analysed by differential scanning fluorimetry, as described in the Materials and Methods. Fluorescence signals are shown relative to the maximal fluorescence signal detected for each replicate (n = 6). Due to the small error between technical replicates, error bars (standard deviation) are not visible.

Supplementary Figure S3. Comparison of soluble CK2α levels following co-expression with OGT with and without *Cp*OGA

Soluble (S) and pellet (P) fractions of IPTG-induced *E. coli* lysate, along with aliquots of flow-through (F), imidazole elutions (E), and Ni^{2+} beads post-elution (B), were analysed by SDS-PAGE and Coomassie staining to compare relative yields of CK2α in the presence or absence of OGT and *Cp*OGA.

TABLES

Supplementary Table 1. Protein constructs used in this study.

Uniprot IDs of proteins are provided, along with construct boundaries. The affinity tag used for each construct is also provided. All tagged constructs are linked to their respective affinity tags via a PreScission protease cleavage site

Protein name (uniprot ID)	Construct boundaries	Affinity tag
Full length DDX3X (O00571)	1-662	MBP (N-terminal)
Truncated DDX3X (O00571)	132-607	His_6 (N-terminal)
CK2α (P68400)	1-361	His_6 (N-terminal)
Tab1 (Q15750)	7-410	His_6 (N-terminal)
OGT (O15294)	323-1041	Tagless
*Cp*OGA (Q0TR53)	31-618	Tagless

FIG. 1

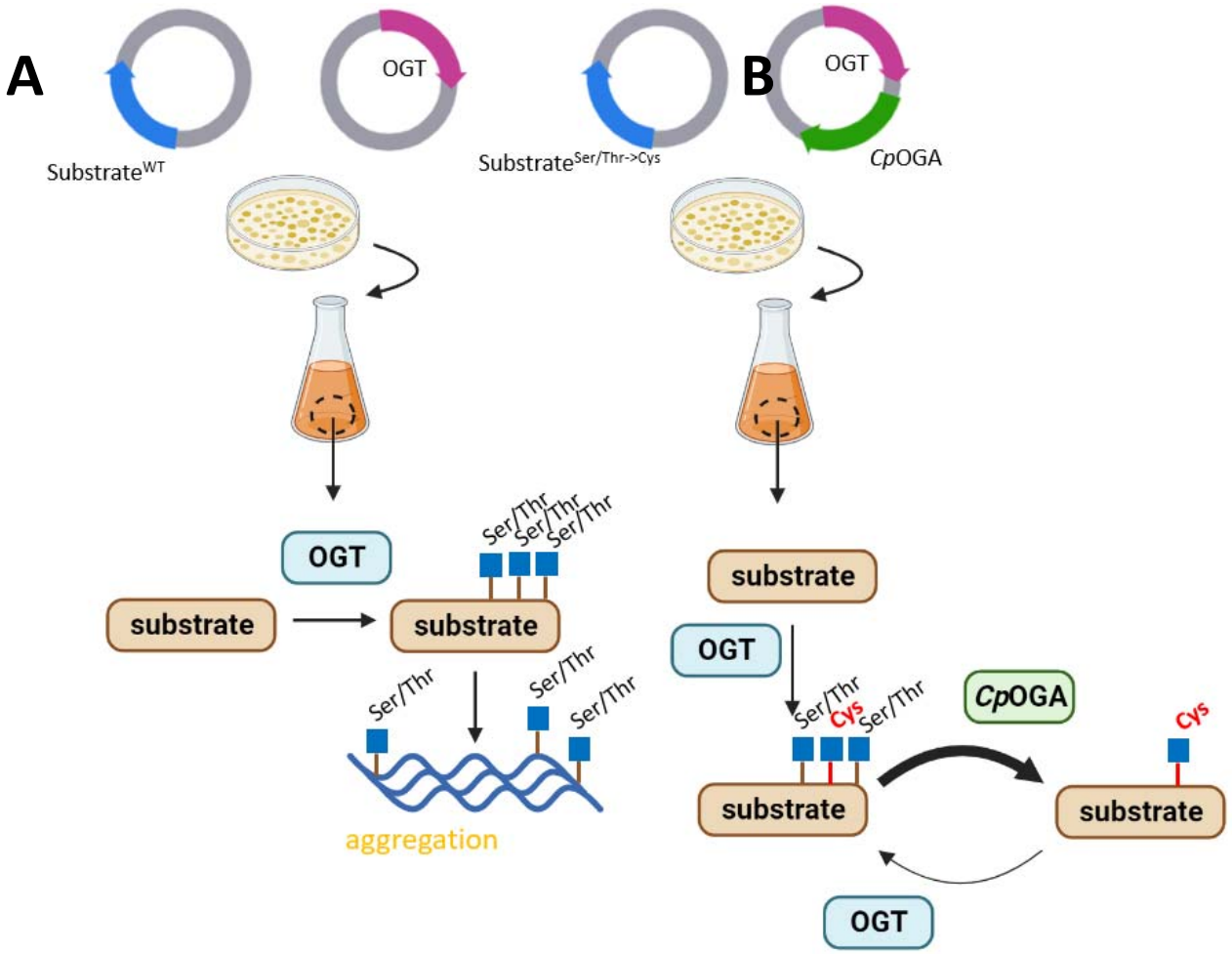

A
OGT
Substrate^WT
B
OGT
Substrate^Ser/Thr->Cys
CpOGA
OGT
substrate
substrate
Ser/Thr
Ser/Thr
Ser/Thr
Ser/Thr
Ser/Thr
Ser/Thr
aggregation
substrate
OGT
Ser/Thr
Cys
Ser/Thr
CpOGA
substrate
Cys
substrate
OGT

FIG. 2

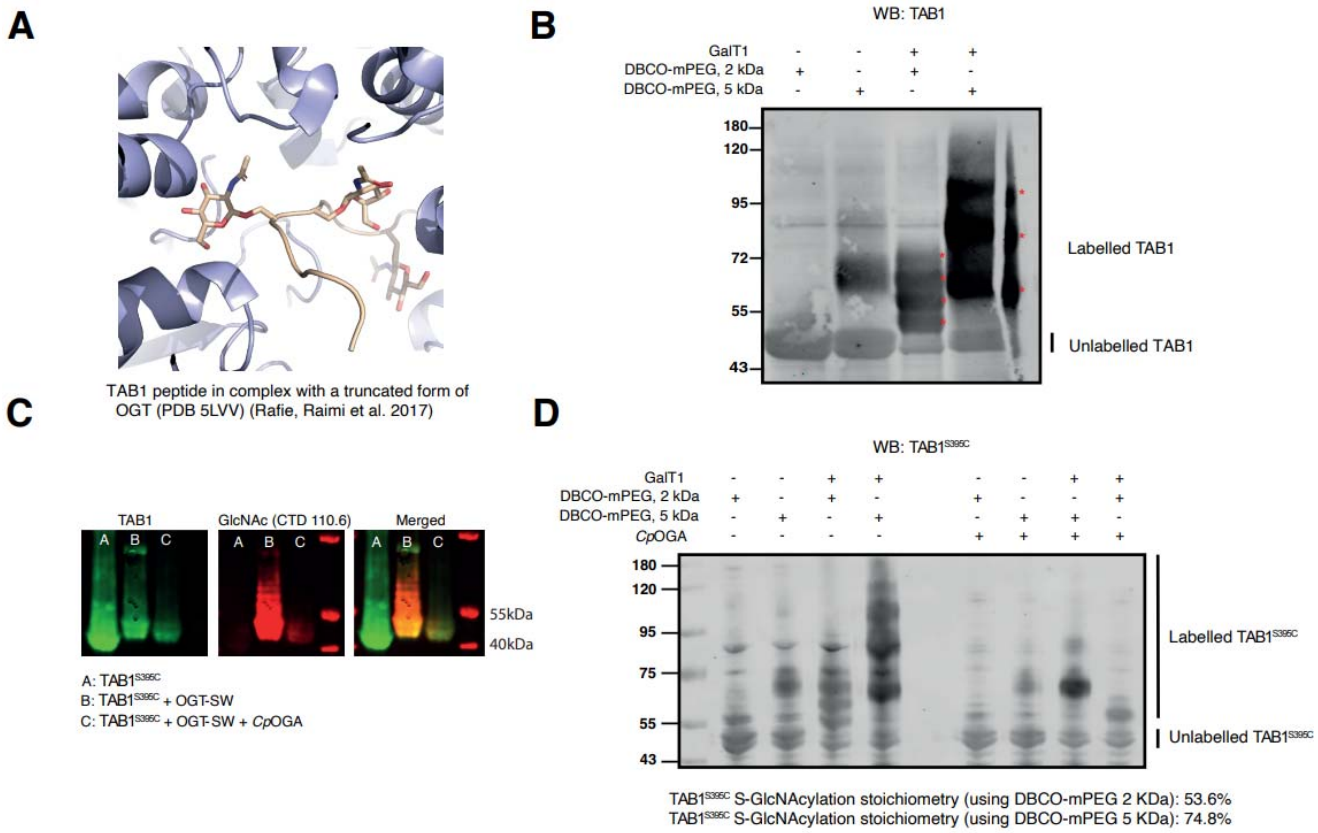

A
TAB1 peptide in complex with a truncated form of OGT (PDB 5LVV) (Rafie, Raimi et al. 2017)
B
WB: TAB1
GalT1
DBCO-mPEG, 2 kDa
DBCO-mPEG, 5 kDa
180
120
95
72
55
43
Labelled TAB1
Unlabelled TAB1
C
TAB1
GlcNAc (CTD 110.6)
Merged
A B C
55kDa
40kDa
A: TAB1S395C
B: TAB1S395C + OGT-SW
C: TAB1S395C + OGT-SW + CpOGA
D
WB: TAB1S395C
GalT1
DBCO-mPEG, 2 kDa
DBCO-mPEG, 5 kDa
CpOGA
180
120
95
75
55
43
Labelled TAB1S395C
Unlabelled TAB1S395C
TAB1S395C S-GlcNAcylation stoichiometry (using DBCO-mPEG 2 KDa): 53.6%
TAB1S395C S-GlcNAcylation stoichiometry (using DBCO-mPEG 5 KDa): 74.8%

FIG. 3

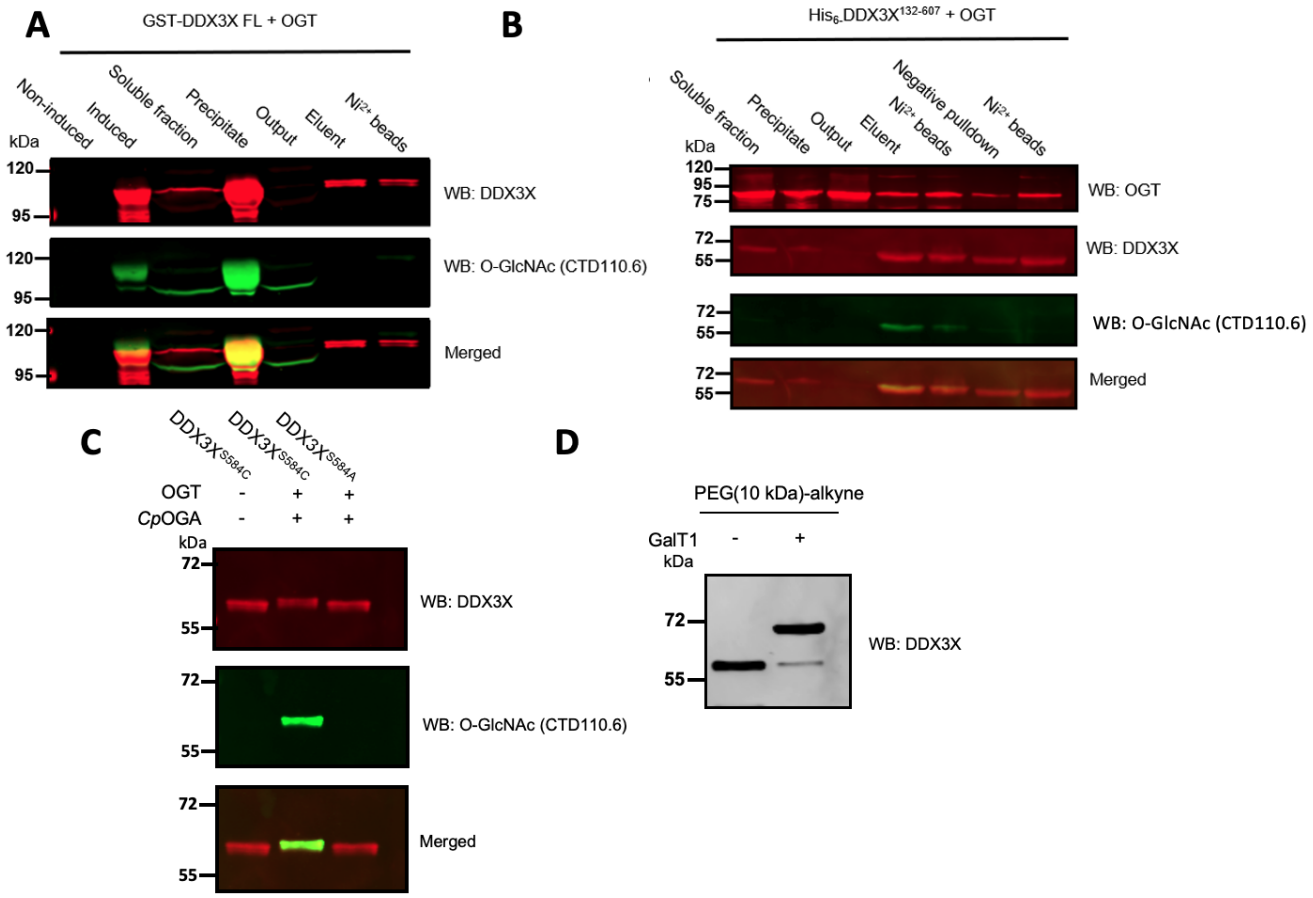
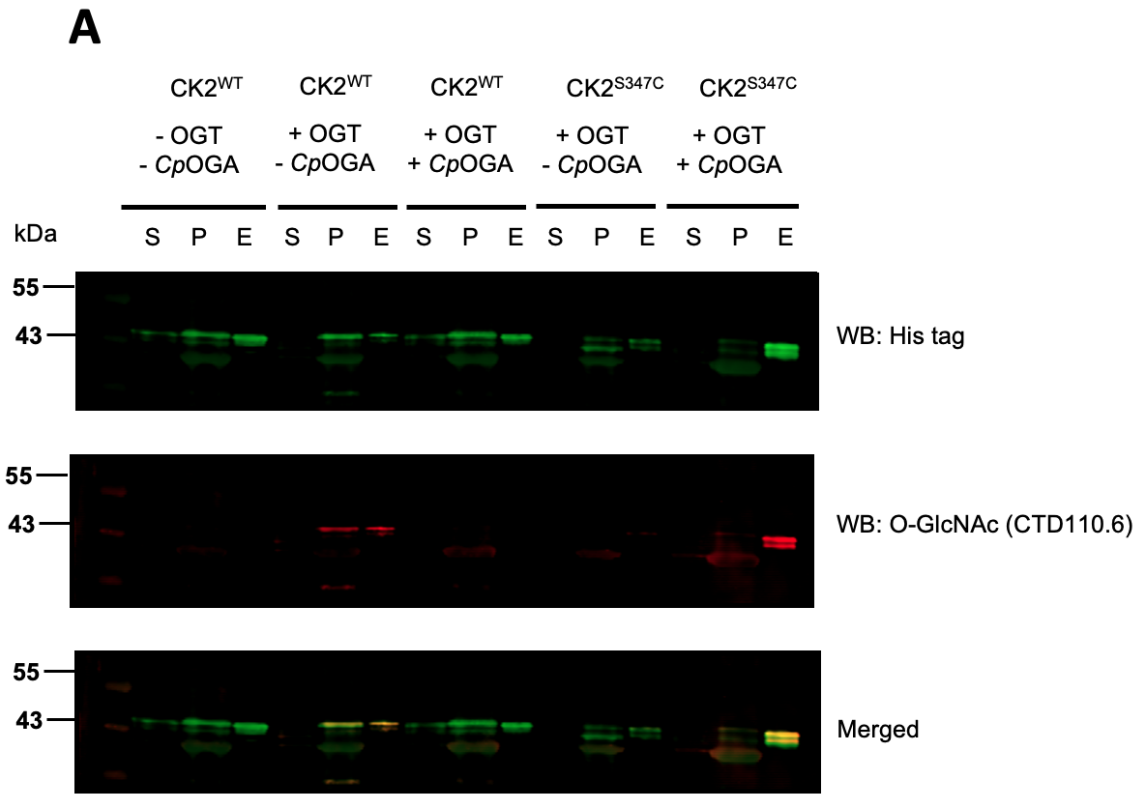
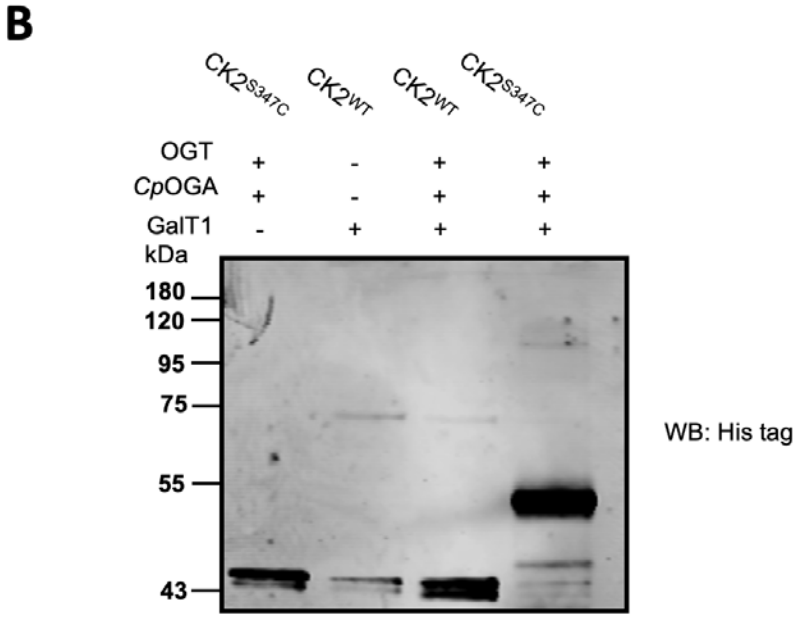
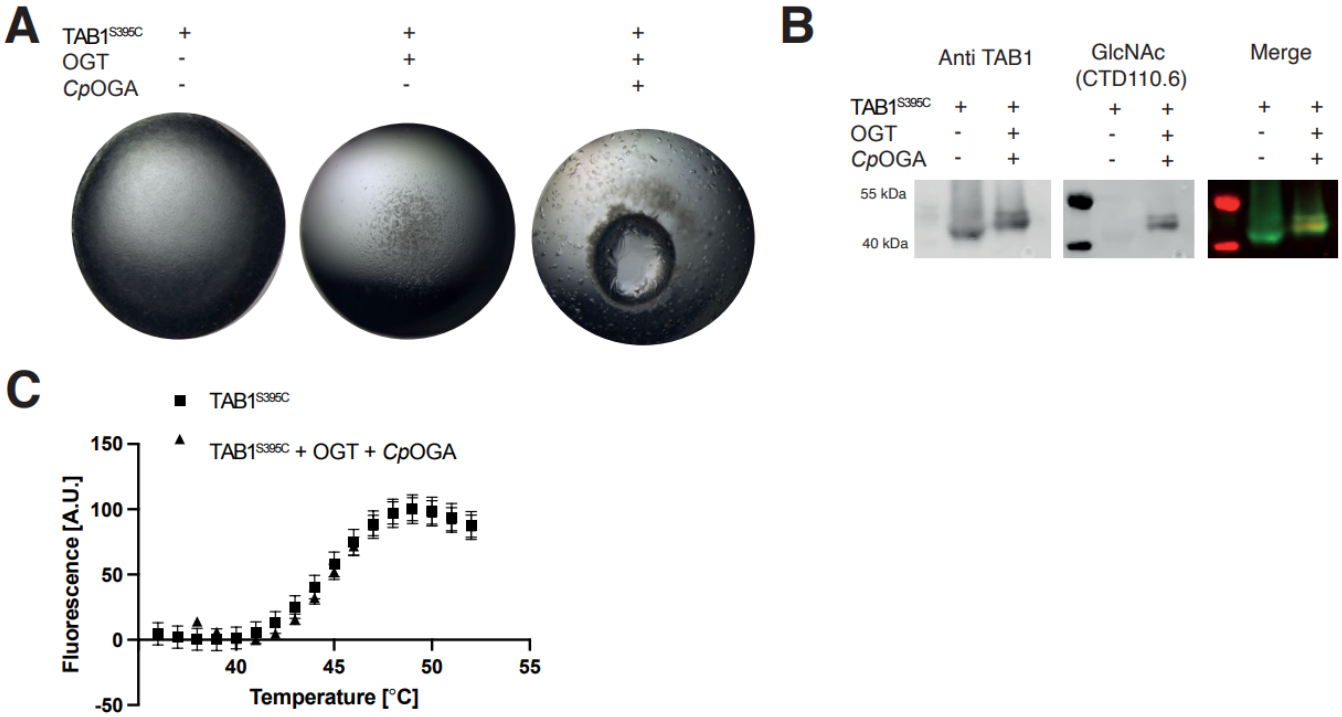

A
GST-DDX3X FL + OGT
Non-induced
Induced
Soluble fraction
Precipitate
Output
Eluent
Ni2+ beads
kDa
120
95
WB: DDX3X
WB: O-GlcNAc (CTD110.6)
Merged
B
His6-DDX3X132-607 + OGT
Soluble fraction
Precipitate
Output
Eluent
Ni2+ beads
Negative pulldown
Ni2+ beads
kDa
120
95
75
72
55
WB: OGT
WB: DDX3X
WB: O-GlcNAc (CTD110.6)
Merged
C
DDX3XS584C
DDX3XS584C
DDX3XS584A
OGT - + +
CpOGA - + +
kDa
72
55
WB: DDX3X
WB: O-GlcNAc (CTD110.6)
Merged
D
PEG(10 kDa)-alkyne
GalT1 - +
kDa
72
55
WB: DDX3X

FIG. 4

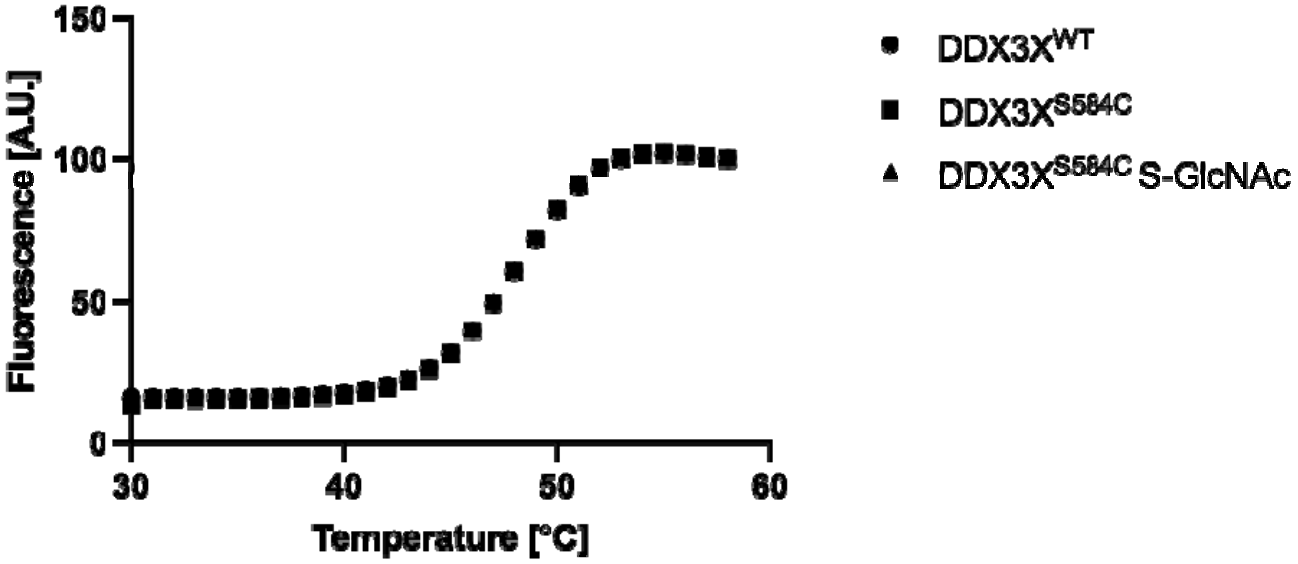
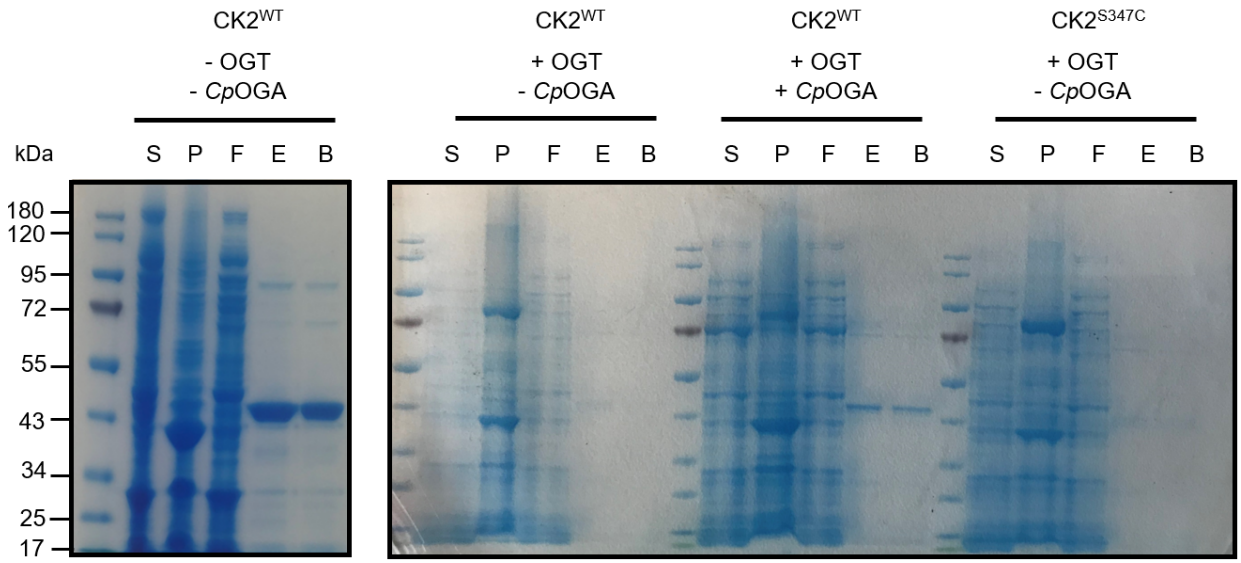
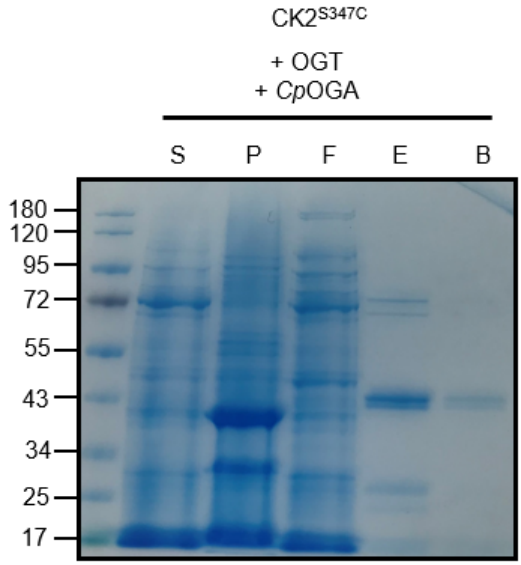

A
CK2WT
- OGT
- CpOGA
CK2WT
+ OGT
- CpOGA
CK2WT
+ OGT
+ CpOGA
CK2S347C
+ OGT
- CpOGA
CK2S347C
+ OGT
+ CpOGA
kDa
S P E S P E S P E S P E S P E
55
43
WB: His tag
55
43
WB: O-GlcNAc (CTD110.6)
55
43
Merged


B
CK2S347C
CK2WT
CK2WT
CK2S347C
OGT + - + +
CpOGA + - + +
GalT1 - + + +
kDa
180
120
95
75
55
43
WB: His tag

SUPPLEMENTAL FIG. 1



SUPPLEMENTAL FIG. 2



SUPPLEMENTAL FIG. 3