Supplementary Table S1: Data collection and refinement statistics for *Cp*OGA^{D298N} - O-GlcNAcylated dHCF peptide complex

	*Cp*OGA^{D298N} - dHCF O-GlcNAc peptide (Ac-VPS(O-GlcNAc)TMSAN-NH_2)
Data collection	
Beamline, wavelength	ID23-1 (ESRF), 0.8729
Space group	$P6_1$
Cell dimensions (Å)	*a*=*b*=118.6, *c*=148.4
Resolution (Å)	34.90 - 2.60 (2.72 - 2.60)
R_{merge} (within I+/I-)	0.134 (0.733)
I/σI	7.3 (1.5)
Completeness (%)	99.4 (99.0)
Redundancy	3.3 (3.8 - 3.3)
CC-half	0.983 (0.980 – 0.601)
Refinement	
Unique reflections	36087
R_{work}, R_{free}	0.1705/ 0.2201
No. atoms:	
Protein	4576
Cadmium	15
Glycopeptide	39
Water	82
B-factors:	
Protein	291.36
Glycopeptide	341.61
R.m.s. deviations:	
Bond lengths (Å)	0.0061
Bond angles (°)	1.0067
Ramachandran plot:	
in preferred regions (%)	96.72
in allowed regions (%)	2.76
outliers (%)	0.52
PDB ID	4ZXL

Figure S1 (Related to Figure 1): HCF-1 O-GlcNAc site mapping. O-GlcNAcylated peptide was site mapped through mass spectrometry and ETD MS/MS fragmentation to identify the O-GlcNAc site. ETD-MS/MS spectrum of HCF-1 [^{614}VPSTMSANVVLSSSSSTLR^{632} + GlcNAc]$^{+}$ = 2142.13 *m/z*, produced an intense ion at *m/z* 715.47 corresponding to the tricharged precursor. The spectrum shows the c^{+1} ions (orange), the z^{+1} ions (green) and the y^{+1} ions (blue). The O-GlcNAcylation at T617 is identified by the detection of its c^{+1} ion at *m/z* 605.86, z^{+1} at *m/z* 1539.77 and y^{+1} at *m/z* 1858.91.

Figure S2 (Related to Figure 1): Surface Plasmon Resonance binding assay

a) Sensorgram for binding of O-GlcNAcylated hOGA peptide to *Cp*OGA^{D298N}. The peptide was injected in duplicates at various concentrations (2-500 μM and 0.04-10 μM respectively). RU: relative units.

b) Sensorgram for binding of O-GlcNAcylated TAB1 peptide to *Cp*OGA^{D298N}. The peptide was injected in duplicates at various concentrations (2-500 μM and 0.08-20 μM respectively). RU: relative units.

Figure S3 (Related to Figures 2 and 3):

a) Far Westerns with GST-*Cp*OGA^{WT}, GST-*Cp*OGA^{D401A} and GST-*Cp*OGA$^{D298N, D401A}$ were performed on either unmodified or *in vitro* O-GlcNAcylated TAB1 (labels adjacent to the blots). Pre-treatment as indicated above the blots were performed to demonstrate the activity of all the *Cp*OGA constructs.

b) HEK293 lysates deglycosylated with *Cp*OGA^{WT} or without were subjected to GST Far Western or immunoblot with anti-GST antibody or IR-labelled anti-sheep secondary antibody.

c) HEK293 lysates without or with *Cp*OGA^{WT} pre-treatment were subjected to Concanavalin A (ConA) Eastern blot.

d) Identical duplicate HEK293 lysates were subjected to Far Westerns with GST-*Cp*OGA^{D298N}, GST-*Cp*OGA^{WT}, GST-*Cp*OGA^{D401A} and GST-*Cp*OGA$^{D298N, D401A}$.

e) 0-16 h *Drosophila* embryonic lysates without or with PNGase F pre-treatment were subjected to ConA Eastern blot.

Figure S4:

a) 0-16 h *Drosophila* embryonic lysates without or with PNGase F/*Cp*OGA^{WT} pre-treatment were subjected to GST-*Cp*OGA^{D298N} Far Western (first left panel), immunoblotted with anti-O-GlcNAc antibody, RL2 (second panel from left) or anti-O-GlcNAc antibody, CTD110.6 (third panel from left).

The right panel is a high contrast rendering of the CTD110.6 immunoblot to demonstrate lack of specific signal. The lysates were separated on a 6% SDS PAGE gel.

b) 0-16 h *Drosophila* embryonic lysates without or with PNGase F/*Cp*OGA^{WT} pre-treatment were subjected to in the presence (+) or absence (-) of GalTI. The lysates were then separated on a 6% SDS PAGE gel and blotted with Streptavidin conjugated to infrared 680 dye.

Figure S1

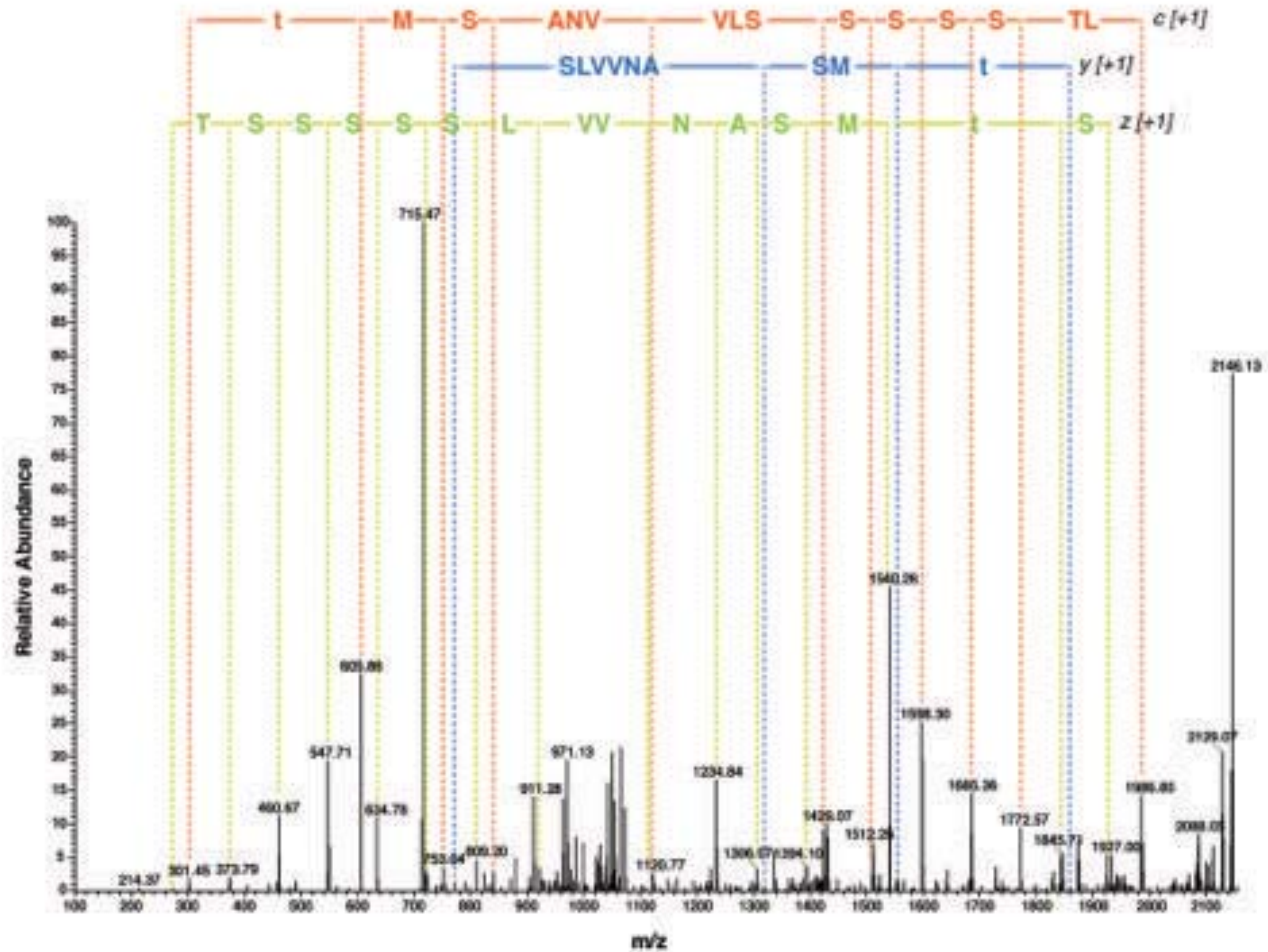
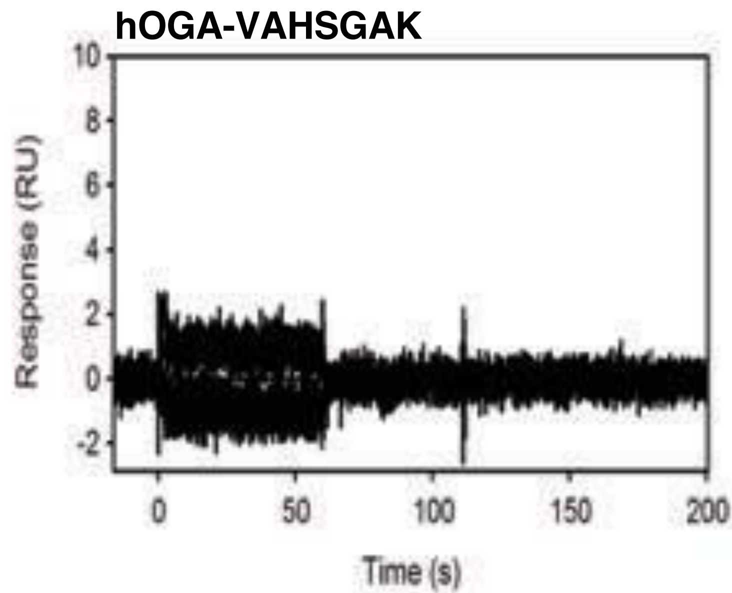
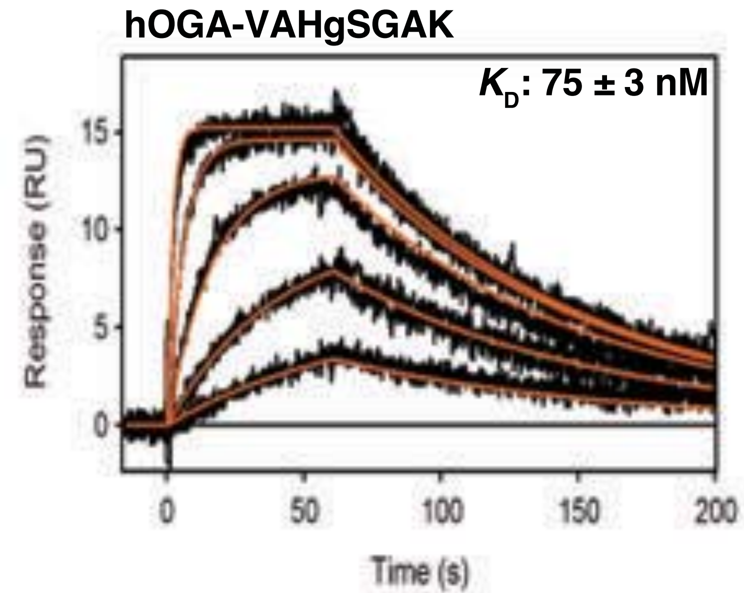


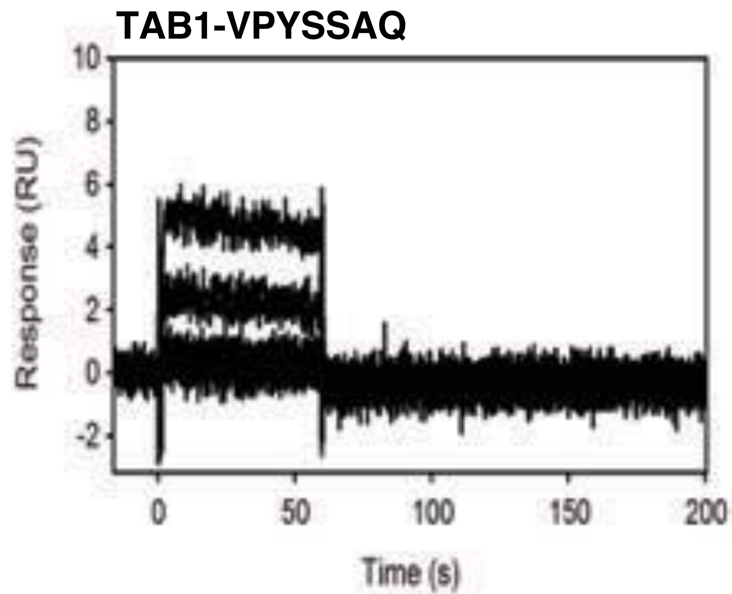
Figure S2

a





b



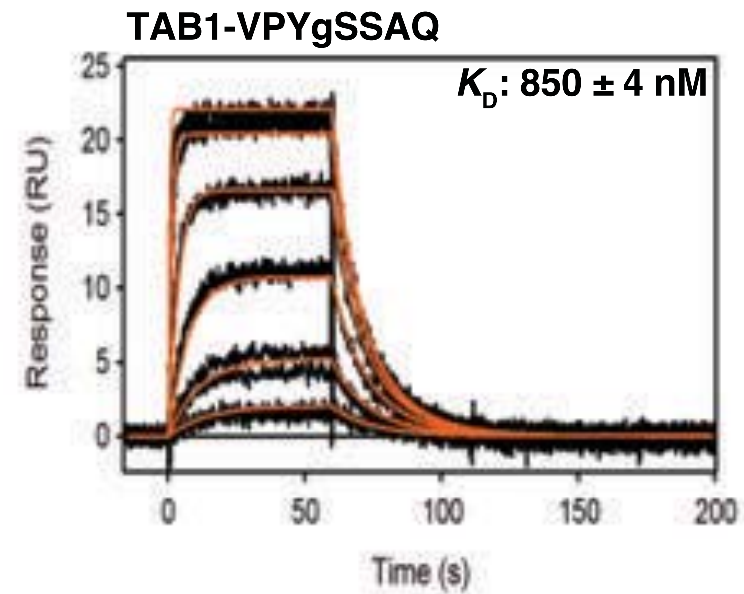


Figure S3

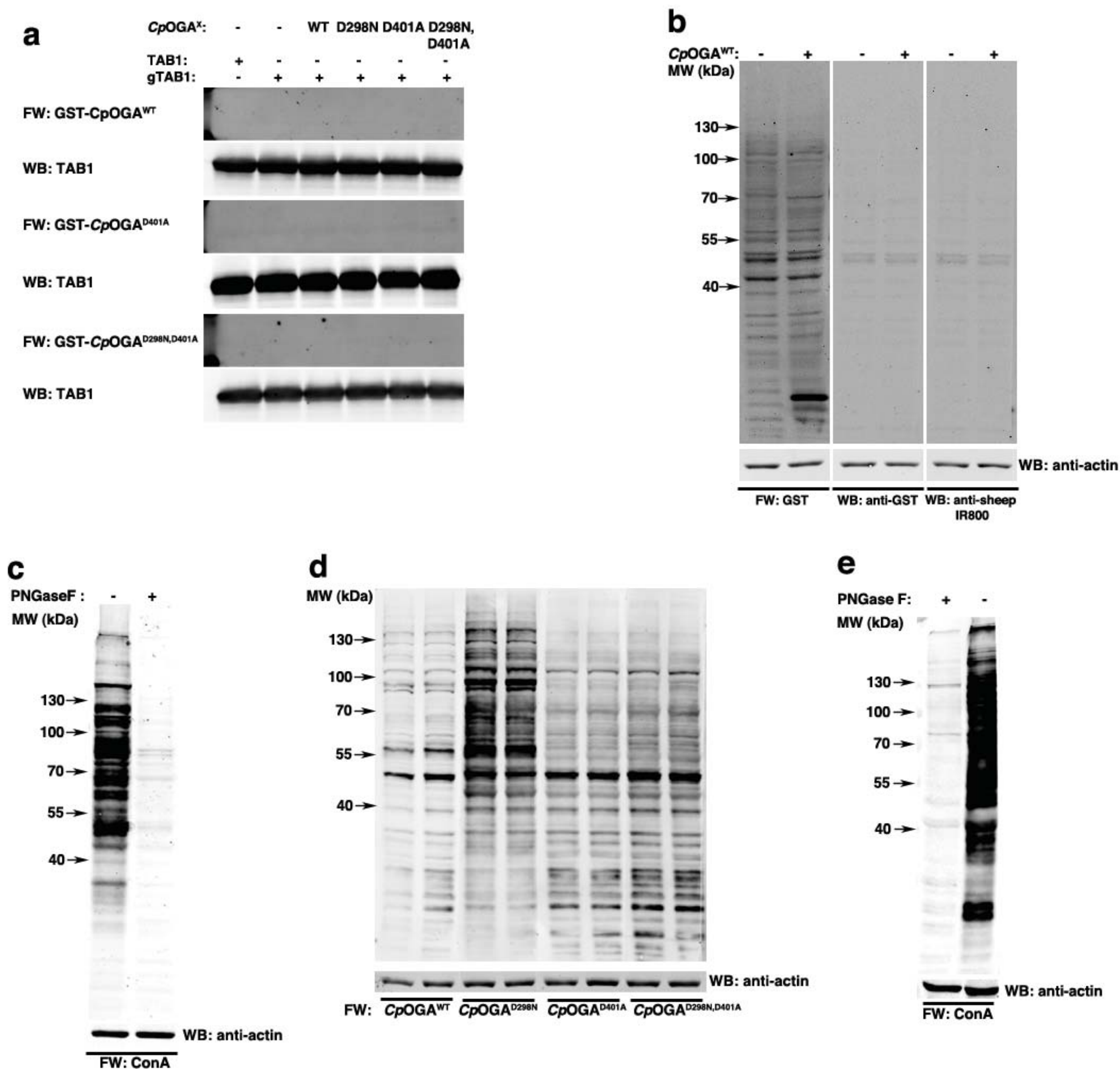


Figure S4