**SUPPORTING INFORMATION**

# The conserved threonine-rich region of the HCF-$1_{PRO}$ repeat activates promiscuous OGT:UDP-GlcNAc glycosylation and proteolysis activities

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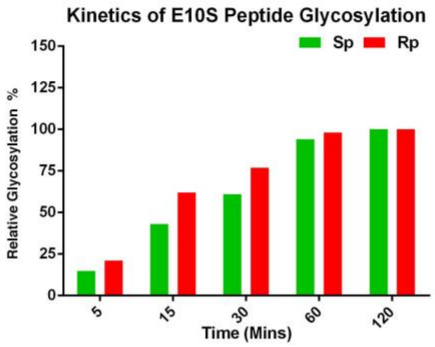
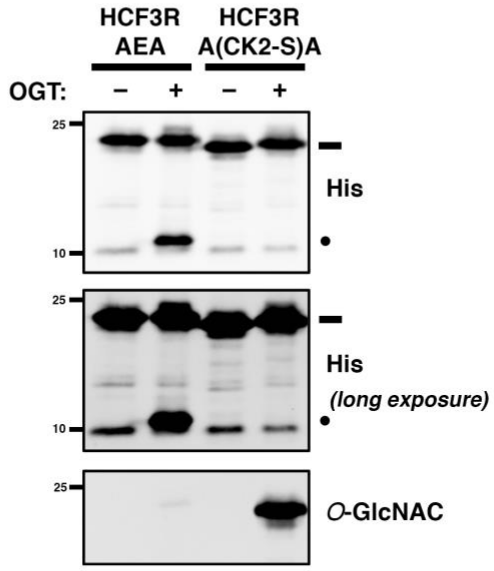
## Supplementary Figure 1




**Supplementary Figure 1. Kinetics of E10S peptide glycosylation by $S_p$- and $R_p$-αS-UDP-GlcNAc.**

In vitro peptide glycosylation assay was performed using HCF-$1_{PRO}$-repeat 2 E10S peptide. by incubating with WT OGT and either $S_p$-αS-UDP-GlcNAc (green bar) or $R_p$-αS-UDP-GlcNAc (red bar) as indicated. Samples were withdrawn at indicated time intervals and peptide glycosylation was detected using LC-MS as described in materials and methods. *O*-GlcNAcylation of the E10S peptide at different time points was normalized to the *O*-GlcNAcylation levels at 120 minute time point.

**Supplementary Figure 2**

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**<u>Supplementary Figure 2.</u> OGT mediated cleavage and glycosylation of HCF3R substrates.**

HCF3R-AEA or chimeric HCF3R-A(CK2-S)A protein were incubated with or without OGT for 6 hr. HCF3R protein cleavage and glycosylation was assayed by SDS-PAGE and immunoblotting using an anti-His antibody (for cleavage) and RL2 anti-*O*-GlcNAc antibody (glycosylation). Anti-OGT was used to detect levels of OGT protein. Chimeric HCF3R-A(CK2-S)A protein did not undergo any cleavage (lack of cleavage band in long exposure) but was heavily glycosylated as compared to native HCF3R-AEA substrate.
*( ▬, un-cleaved HCF3R substrate; ●, cleaved product.)*

## Supplementary Table 1

### Glycosylation assay peptides

| Peptide Name | Peptide Sequence | Purity |
|---|---|---|
| **CK2** (17-mer) | KKKYPGGSTPV**S**SANMM | 97% |
| **E10S** (26-mer) | VRVCSNPPC**S**THETGTTNTATTATSN | 96% |
| **Ch10** (28-mer) | KKKYPGGSTPV**S**THETGTTNTATTATSN | 98% |
| **Ch13** (28-mer) | KKKYPGGSTPV**S**SANTGTTNTATTATSN | 96% |
| **Ch13 T14A** (28-mer) | KKKYPGGSTPV**S**SANAGTTNTATTATSN | 99% |
| **Ch13 T14Y** (28-mer) | KKKYPGGSTPV**S**SANYGTTNTATTATSN | 95% |
| **Ch13 T(17-22)A** (28-mer) | KKKYPGGSTPV**S**SANTGTANAAAAATSN | 99% |

### Cleavage assay peptides

| Peptide Name | Peptide Sequence | Purity |
|---|---|---|
| **HCF-SHORT** (32-mer) | YVRVCSNPPC**E**THQTGTTNTATTATSNMAGQH –NH2 | 98% |
| **CK2(S10) – Thr-rich$^+$** (32-mer) | KKKPGGSTPV**S**SANTGTTNTATTATSNMAGQH –NH2 | 98% |
| **CK2(E10) – Thr-rich$^+$** (32-mer) | KKKPGGSTPV**E**SANTGTTNTATTATSNMAGQH –NH2 | 99% |
| **CK2(E10) – Thr-rich$^-$**(32-mer) | KKKPGGSTPV**E**SANTGTANAAAAATSNMAGQH –NH2 | 99% |
| **CK2(E9S10)– Thr-rich$^+$** (33-mer) | KKKPGGSTPVE**S**SANTGTTNTATTATSNMAGQH –NH2 | 98% |

**Supplementary Table 2**

**HCF3R-AEA**

PGTVTLVCSNPPC**A**THETGTTNTATTTVVANLGGHPQPTQVQFVCDRQEAAASLVTST

VGQQNGSVVRVCSNPPC**E**THETGTTNTATTATSNMAGQHGCSNPPC**A**THETGTTNTA
TTAMSSVGAN

**HCF3R-ASA**

PGTVTLVCSNPPC**A**THETGTTNTATTTVVANLGGHPQPTQVQFVCDRQEAAASLVTST

VGQQNGSVVRVCSNPPC**S**THETGTTNTATTATSNMAGQHGCSNPPC**A**THETGTTNTA
TTAMSSVGAN

**HCF3R-A(CK2-S)A**

PGTVTLVCSNPPC**A**THETGTTNTATTTVVANLGGHPQPTQVQFVCDRQEAAASLVTST

VGQQNGSVVRPGGSTPV**S**SANTGTTNTATTATSNMAGQHGCSNPPC**A**THETGTTNTA
TTAMSSVGAN

**HCF3R-A(CK2-E)A**

PGTVTLVCSNPPC**A**THETGTTNTATTTVVANLGGHPQPTQVQFVCDRQEAAASLVTST

VGQQNGSVVRPGGSTPV**E**SANTGTTNTATTATSNMAGQHGCSNPPC**A**THETGTTNTA
TTAMSSVGAN