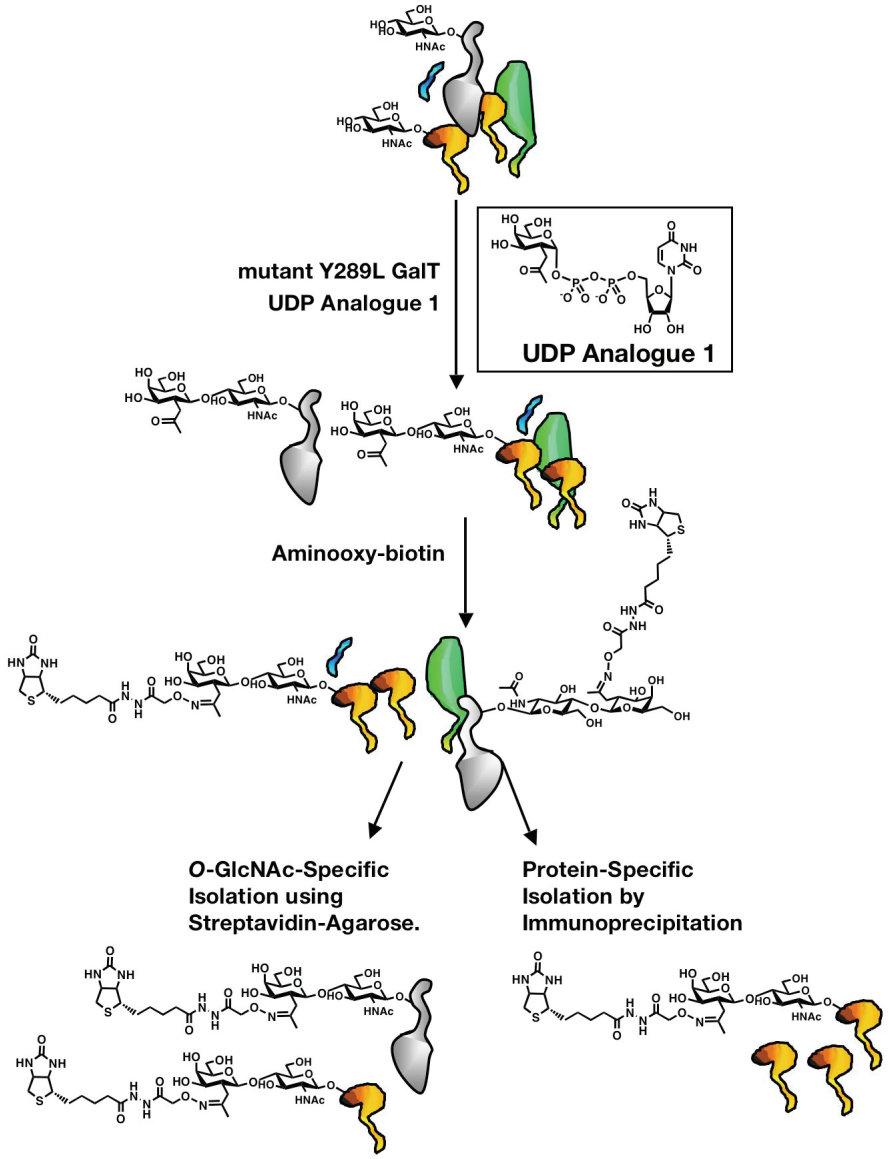
Chapter 5
CREB Glycosylation Moderates Phosphorylation-Dependent CREB Activity in Cultured Pancreatic Cell Lines.

Summary. *In vitro* studies of CREB glycosylation found that the transactivation potential of CREB is inhibited by glycosylation. Whether *O*-GlcNAc glycosylation is sufficiently dynamic to play a role in cellular activity has been addressed by several previous studies performed in pancreatic β-cell lines [1-6]. The pancreatic cells were chosen as a model system because of the high levels of OGT and *O*-GlcNAc glycosylation present in b-cells [5, 7]. Our work using cultured pancreatic cells has shown that CREB glycosylation is indeed dynamic and capable of effecting a decrease in CREB-dependent transcription.

CREB is *O*-GlcNAc Glycosylated in Pancreatic Islets and Cultured Pancreatic Cell Lines. *O*-GlcNAc proteins from primary rat islets were biotinylated using a chemoenzymatic approach, in which an engineered β-1,4-galactosyltransferase (Y289L GalT) enzyme was utilized to selectively tag *O*-GlcNAc proteins with a keto-galactose sugar (Scheme 5-1) [8, 9]. Once transferred, the ketone functionality on UDP analogue **1** was reacted with an amino-oxy biotin analogue, thereby permitting chemiluminescence detection of the *O*-GlcNAc modification following immunoprecipitation of CREB (Fig. 5-1A). Strong and selective biotinylation of CREB was observed (Fig. 5-1B), indicating that CREB is *O*-GlcNAc glycosylated in the pancreas.



Scheme 5-1. Method of ketone labeling for the measurement of CREB *O*-GlcNAc stoichiometry and relative changes in CREB glycosylation. Samples are treated with mutant Y289L GalT and UDP analogue 1 to attach the ketone functionalized galactose to the C4 hydroxyl of GlcNAc residues. Samples are then treated with an aminooxy biotin probe that attacks the ketone functionality to form the an imine bond with the galactose residue. *O*-GlcNAc glycosylated proteins can then be isolated using the biotin handle by streptavidin agarose chromatography, or specific proteins can be isolated by immunoprecipitation and possible *O*-GlcNAc glycosylation detected by streptavidin immunoblotting.

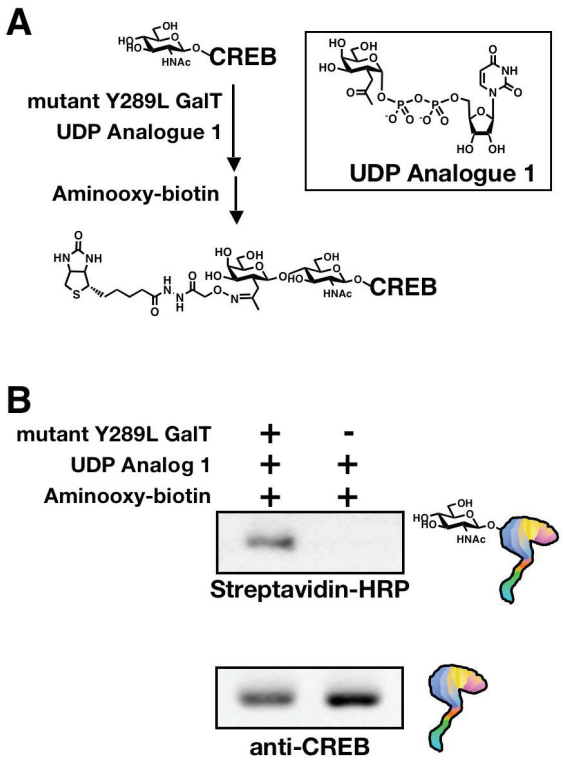


Figure 5-1. Identification of CREB *O*-GlcNAc glycosylation in pancreatic islets. **(A)** Method for selective labeling of O-GlcNAc glycosylated proteins . *O*-GlcNAc glycosylated proteins were labeled by incubating cell extracts with a non-natural UDP-galactose substrate containing a ketone functionality (UDP analogue 1) and an engineered β-1,4-galactosyltransferase enzyme (mutant Y289L GalT). Labeled proteins were then modified with an aminooxy biotin derivative (Dojindo). **(B)** CREB is *O*-GlcNAc glycosylated in primary rat islets. The islet cells were isolated from rat pancreas , and glycosylation levels were measured by selective biotinylation of *O*-GlcNAc glycosylated proteins as described in (A), and CREB was immunoprecipitated . Glycosylated CREB was detected using streptavidin conjugated to horseradish peroxidase.

It should be noted that the chemoenzymatic tagging method was essential, as conventional ^{3}H-labeling with GalT successfully detected the modification on CREB but was much less sensitive, requiring weeks for detection (data not shown). Furthermore, WGA lectin and anti-*O*-GlcNAc antibodies (RL2 (Abcam, Cambridge, MA) and CTD110.6 (Covance, Berkeley, CA) failed to detect the sugar modification on CREB because these methods are capable of detecting only a subset of *O*-GlcNAc proteins.

To probe the stoichiometry of CREB glycosylation *in vivo*, *O*-GlcNAc proteins from the pancreatic cell line HIT-T15 were biotinylated using the chemoenzymatic approach and captured by streptavidin affinity chromatography. A significant fraction of CREB was pulled down by streptavidin, and the stoichiometry of CREB glycosylation was estimated to be approximately 25%, based on the relative amounts of captured versus uncaptured protein (Fig. 5-2). Notably, the stoichiometry of CREB glycosylation (~25%) is comparable to

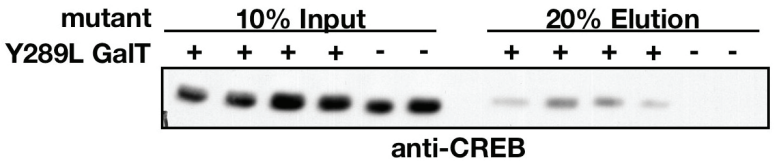


Figure 5-2. Stoichiometry of CREB *O*-GlcNAc Glycosylation in HIT-T15 Cells. Approximately a quarter of cellular CREB is *O*-GlcNAc glycosylated in HIT-T15 cells. *O*-GlcNAc glycosylated proteins were labeled as described in Figure 5-1A and isolated using streptavidin-bound sepharose (Pierce) and CREB content in the input and elution were measured by Western blotting.

that of CREB phosphorylation (~40%) upon stimulation of PC12 cells with forskolin (Fsk) [10]. These results demonstrate that a significant fraction of CREB is *O*-GlcNAc glycosylated *in vivo* and suggest an important role for the modification in β-cells.

We next attempted to ascertain whether glycosylation of CREB could be induced in response to cellular stimuli. If CREB glycosylation were to play a role in regulation of CREB activity, it would be necessary for cells to modulate glycosylation levels. Glucosamine (GlcN) treatment has been shown to cause a significant increase in the levels of *O*-GlcNAc glycosylation as a result of increased production of UDP *N*-acetylglucosamine (UDP-GlcNAc; [11, 12]), the substrate for OGT. The conversion of glucose to GlcN represents the rate-limiting step in the synthesis of UDP-GlcNAc, by bypassing this step with GlcN treatment, UDP-GlcNAc levels are rapidly elevated [13, 14]. Elevation of cellular *O*-GlcNAc glycosylation levels upon GlcN treatment was confirmed by immunoblotting (Fig. 5-3A). A maximal 39% increase was observed after 9 h of treatment. Complementary to the increase in glycosylation, a rapid decrease in OGT protein levels was observed, suggesting that a negative feedback loop may exist to prevent chronic OGT hyperactivity.

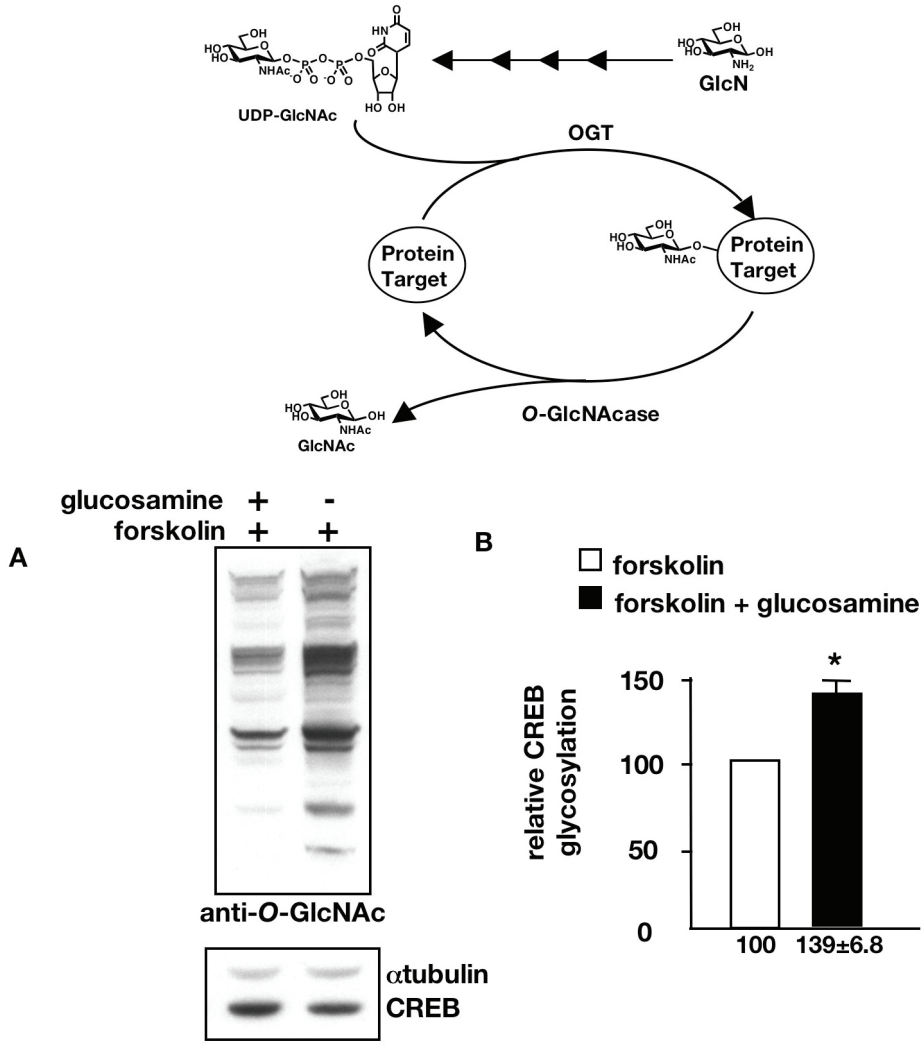


Figure 5-3. Elevation of *O*-GlcNAc Glycosylation and CREB Glycosylation by Glucosamine Treatment. Stimulation of the hexosamine biosynthesis pathway using GlcN enhances CREB glycosylation levels. HIT-T15 cells were incubated with GlcN (10 mM, 9 h) for 3 h prior to the addition of Fsk (10 μM, 6 h). **(A)** Cellular *O*-GlcNAc levels are increased by 39%±4.5% by GlcN treatment, while CREB levels are unaffected**.** Cellular *O*-GlcNAc glycosylation and CREB levels were measured by immunoblotting using an anti-*O*-GlcNAc (CTD110.6, Covance, Berkeley, CA; 1:5000) and anti-CREB (Upstate, Charlottesville, VA; 1:1000) and normalized to αtubulin levels measured by immunoblotting using an anti-tubulin antibody (Sigma-Aldrich, St. Louis, MO; 1:5000). **(B)** CREB glycosylation levels were measured as described in Figure 5-1A. Values were quantified using NIH Image 1.62 software and represent the mean ± SEM. (Student's T-test, * $P < 0.01$; n = 3)

Glycosylation of CREB increased by 39 ± 7% upon treatment of pancreatic HIT-T15 cells with GlcN and Fsk, a stimulator of the cAMP pathway, relative to Fsk alone (Fig. 5-3B). The increase in CREB glycosylation measured by labeling was nearly identical to the increase observed in cellular glycosylation.

In contrast, GlcN had no observable effect on the Fsk-induced phosphorylation of CREB at Ser133 (Fig. 5-4A). Comparison of *O*-GlcNAc levels from identical samples showed that Fsk had no effect on cellular *O*-GlcNAc glycosylation levels (Fig. 5-4B). Also of note were differences in the level and duration of changes in CREB phosphorylation and cellular *O*-GlcNAc glycosylation. Within 30 min of Fsk administration, CREB phosphorylation had reached a maximal 3-fold enhancement over basal levels, and had returned to basal levels by 6 h post-treatment. The effect of GlcN on *O*-GlcNAc glycosylation was much weaker by comparison, resulting only in a ~1.4-fold increase 2.5 h after addition of GlcN. However, glycosylation persisted at this ~1.4-fold increase for nearly 4-5 h. GlcN–enhanced flux through the hexosamine biosynthesis pathway has a relatively weak effect on CREB glycosylation when compared to the 3- to 5-fold increase in CREB phosphorylation caused by activation of PKA [10]. It is possible that, like CREB phosphorylation (which is increased robustly by PKA and mildly by MAPKs) there may exist mechanisms for the robust and mild activation of CREB glycosylation [15]. It is also possible that the extended duration of the GlcN-induced glycosylation is sufficient to achieve a significant effect on CREB transactivation, without robust activation. The low level of CREB glycosylation may also reflect a targeted glycosylation of a limited subset of CREB molecules that are bound to a specific set of genes targeted by OGT-bearing regulators complexes.

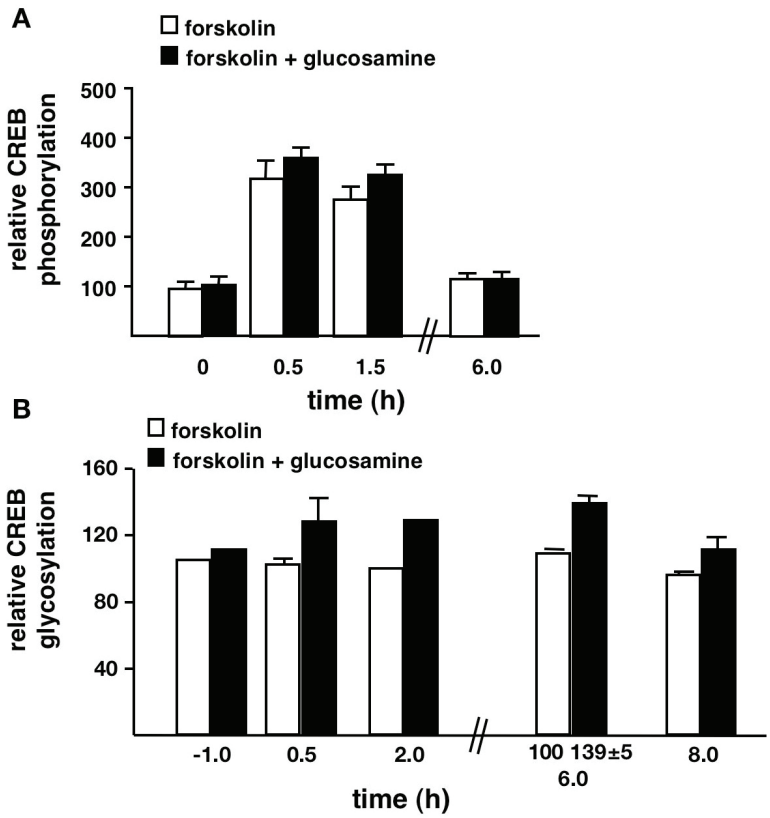


Figure 5-4. Glucosamine treatment increases cellular *O*-GlcNAc levels, but has no effect on the level of CREB phosphorylation. **(A)** GlcN does not affect the levels of CREB phosphorylation at Ser133. Cells were treated as described in Figure. 5-3, except that cells were harvested in boiling 1% SDS containing protease (5 μg/ml pepstatin, 5 μg/ml chymostatin, 20 μg/ml leupeptin, 20 μg/ml aprotinin, 20 μg/ml antipain, 0.2mM PMSF) and phosphatase (20 mM NaF, 1 mM Na_3VO_4, 50 μM Na_2MoO_4) inhibitors at the indicated times, with the addition of Fsk at time = 0 h. Cell lysates were analyzed by immunoblotting with an anti-phospho-CREB antibody (Cell Signaling; 1:1,000). **(B)** Increases in glycosylation were observed by immunoblotting using an anti-*O*-GlcNAc antibody (CTD110.6, Covance, Berkeley, CA). Values were quantified using NIH Image 1.62 software and represent the mean ± SEM. (n = 4).

The dual administration of Fsk and GlcN is capable of increasing CREB glycosylation and phosphorylation in parallel. To determine whether phosphorylation and glycosylation could occur at the same time, HIT-T15 cells treated with Fsk and GlcN were labeled with UDP analogue **1** and *O*-GlcNAc glycosylated proteins were isolated using streptavidin sepharose. The presence of pSer133-CREB on *O*-GlcNAc glycosylated CREB was identified by pS133-CREB immunoblotting of the eluents from the streptavidin isolation (Fig. 5-5). CREB glycosylation levels were increased by 25% ± 1% in the HIT-T15 pancreatic cell line by inhibiting *O*-GlcNAcase using the small molecule inhibitor, PUGNAc (*O*-(2-acetamido-2-deoxy-D-glucopyranosylidene)amino-*N*-phenylcarbamate) (Fig. 5-6) [16]. Together, these results indicate that *O*-GlcNAc glycosylation of CREB in pancreatic β-cells is an inducible and reversible modification.

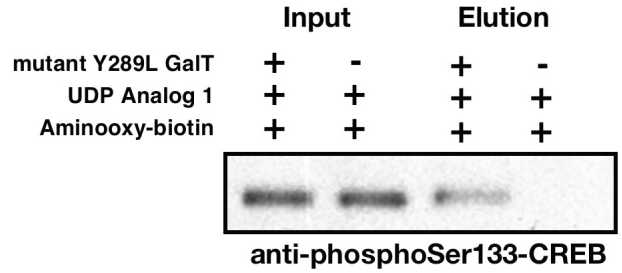


Figure 5-5. CREB Glycosylation and Ser133 Phosphorylation are not mutually exclusive. *O*-GlcNAc glycosylated proteins were labeled as described in Figure 5-1A and isolated using streptavidin-bound sepharose (Pierce, Rockford, IL), and phosphoSer133-CREB content in the input and elution were measured by immunoblotting with an anti-phospho-CREB antibody (Cell Signaling, Danvers, MA; 1:1,000).

Unlike other proteins that are modified by both *O*-GlcNAc glycosylation and phosphorylation (such as the C-terminal domain of RNA polymerase II) glycosylation within Q2 domain of CREB and phosphorylation of Ser133 are not mutually exclusive. The presence of both glycosylation and phosphorylation, along with the identification of *O*-GlcNAc glycosylation on CBP, raise the possibility that *O*-GlcNAc glycosylation may also disrupt the CREB:CBP interaction [9].

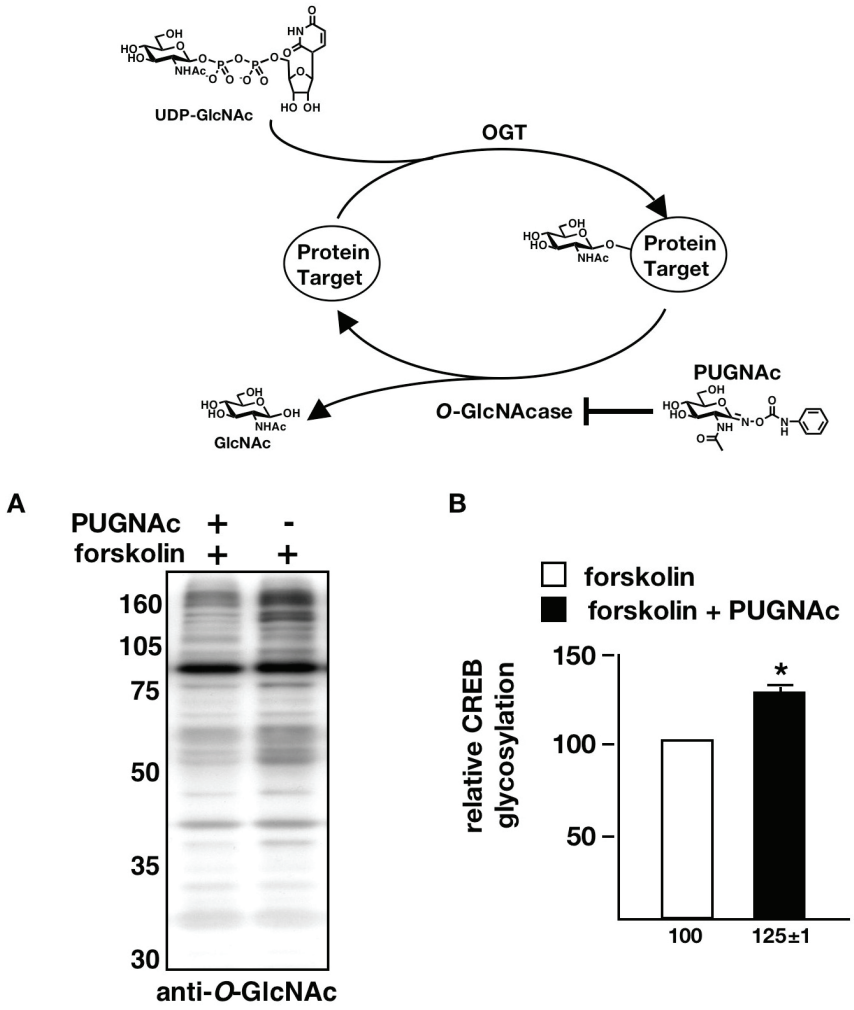


Figure 5-6. The *O*-GlcNAcase inhibitor PUGNAc (*O*-(2-acetamido-2-deoxy-D-glucopyranosylidene)amino-*N*-phenylcarbamate) stimulates CREB glycosylation levels in pancreatic cells. **(A)** HIT-T15 cells were incubated with PUGNAc (Toronto Research Chemicals, 100 μM) for 5 h prior to the addition of Fsk (10 μM, 4 h) and cellular glycosylation levels were measured by the ketone-labeling approach as described in Figure 5-3A. **(B)** CREB glycosylation levels were measured as described in Figure 5-1A. Values were quantified using NIH Image 1.52 software and represent the mean ± SEM (ANOVA, * $P < 0.0005$; n = 3).

CREB Glycosylation Is Dynamic and Inhibits CREB Activity in Cultured Pancreatic Cell Lines. Having shown that CREB glycosylation levels can be dynamically modulated by specific stimuli, we investigated the effects of glycosylation on the transcriptional activity of CREB. HIT-T15 cells were transfected with a CRE-

luciferase reporter gene and treated with GlcN in the presence or absence of Fsk. As expected, luciferase activity was increased by approximately 9-fold upon stimulation of the cAMP pathway by Fsk. Treatment of the cells with GlcN inhibited Fsk-induced CREB activity in a time-dependent manner, reducing maximal CREB transactivation by 39% ± 10% (Fig. 5-7).

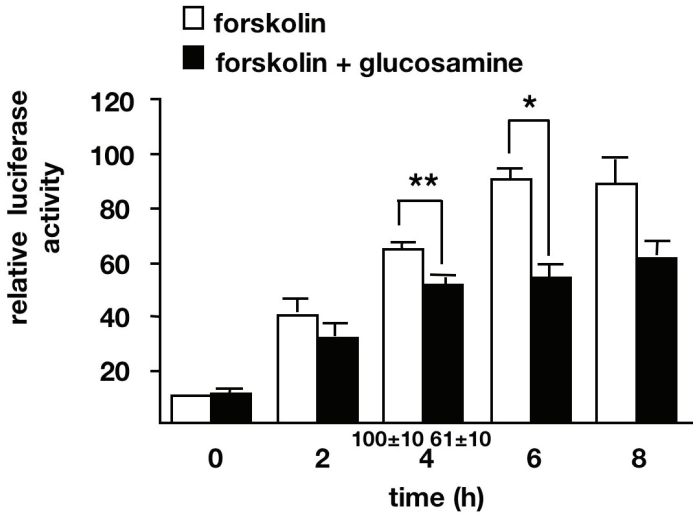


Figure 5-7. Glucosamine treatment reduces CREB activity. GlcN inhibits CREB activity in pancreatic β-cells in a time-dependent manner. HIT-T15 cells were transfected with a CRE-luciferase reporter and β-galactosidase expression plasmid 24 h prior to analysis. Cells were incubated in the presence or absence of GlcN (10 mM) 3 h prior to the addition of Fsk. Fsk (10 μM) was added to the medium for the times indicated and cells were harvested, lysed and luciferase and β-galactosidase activities were quantified. Values were normalized for transfection efficiency using a β-galactosidase assay and represent the mean ± SEM (Student's T-test,* $P < 0.0001$; n = 7 and **P = 0.05; n = 3).

The relative initial rate of CRE-driven luciferase production was slowed ~20% by GlcN treatment. This variation may be the result of reduced recruitment of $TAF_{II}130$ observed *in vitro*. More striking is the observation that with GlcN treatment, CRE-dependent luciferase production reaches a plateau 4 h after Fsk administration, whereas Fsk treatment plateaus at 6 to 8 h. The observed leveling off in luciferase expression after Fsk treatment parallels a decline in CREB phosphorylation (Fig. 5-4A). The correlation of the two observations suggests that the reduction in the pS133 may be responsible for

the plateau in luciferase expression at 6 to 8 h. In cells treated with Fsk and GlcN, the decline in luciferase production precedes the decrease in CREB phosphorylation by ~2 h (Fig. 5-7 vs. Fig. 5-4A). This suggests that, rather than changes in pS133, increases in *O*-GlcNAc glycosylation may be responsible for the decline in luciferase expression and the reduced total level of CRE-dependent luciferase expression.

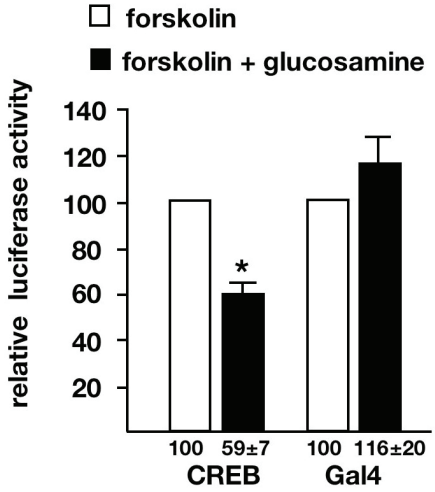


Figure 5-8. Glucosamine treatment reduces CREB activity and not the activity of the general RNA polymerase II complex. HIT-T15 cells were transfected with a β-galactosidase expression plasmid and either a CRE-luciferase reporter gene or a UAS-luciferase reporter gene and GAL4 expression vector 24 h before drug treatment. Cells were incubated in the presence or absence of GlcN (10mM) 3 h prior to the addition of Fsk (10 μM, 6 h). Cells were harvested, lysed in a non-denaturing buffer and luciferase activity was measured. Values were normalized for transfection efficiency using a β-galactosidase assay and represent the mean ± standard deviation. (Student's T-test, * $P <$ 0.002; n = 4).

Interestingly, GlcN alone had no observed effect on CREB activity. This could be explained by a couple of scenarios: (1) OGT is recruited specifically to activated promoters to moderate inducible CREB activity, or (2) the inhibition of $TAF_{II}130$ binding to CREB alone by *O*-GlcNAc glycosylation is insufficient to prevent transcription, and additional inhibitory effects on CBP recruitment are involved in inhibition of CREB transactivation.

To confirm that GlcN was not exerting an inhibitory effect through an alternative mechanism, such as hyper-glycosylation of RNA polymerase II, we examined the activity of Gal4, a yeast transcription factor that does not undergo *O*-GlcNAc glycosylation. Cells were transfected with a Gal4 expression vector and Gal4-dependent luciferase reporter (UAS-luciferase) prior to treatment with

GlcN and Fsk. No change in Gal4 activity was observed (Fig. 5-8), suggesting that the inhibitory effects of GlcN on CREB-dependent transcription are due to glycosylation of CREB. This was somewhat surprising, given that the C-terminal domain of RNA polymerase II is O-GlcNAc glycosylated and glycosylation attenuates elongation *in vitro* [17, 18].

Further supporting an inhibitory role for CREB glycosylation, CRE-dependent transcription was also reduced by treatment of HIT-T15 cells with PUGNAc (Fig. 5-9). A more modest reduction in CREB transactivation was observed upon treatment with PUGNAc relative to GlcN, consistent with the lower induction of CREB glycosylation by PUGNAc. The loss of CRE-driven luciferase production, with PUGNAc treatment mirrored that of Fsk treatment alone, but with a reduced level of total luciferase expression.

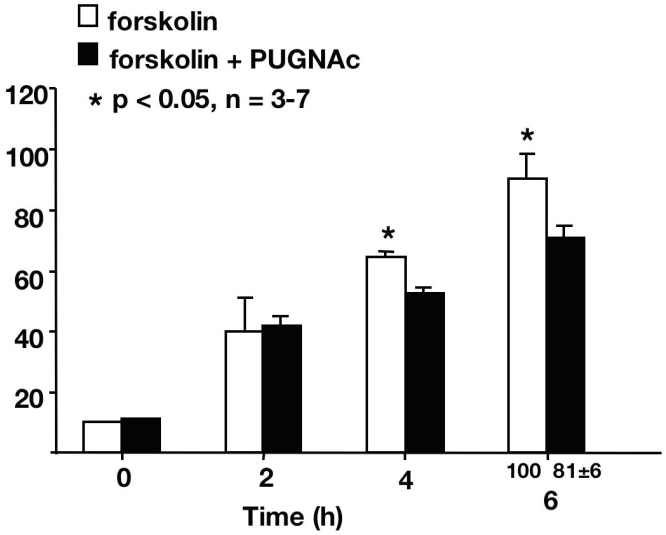


Figure 5-9. The *O*-GlcNAcase inhibitor PUGNAc reduces CREB transactivation. HIT-T15 cells were transfected with a CRE-luciferase reporter and β-galactosidase expression plasmid 24 h prior to analysis. Cells were treated as described in Figure 5-6. Values were normalized for transfection efficiency using a β-galactosidase assay and represent the mean ± SEM (Student's T-test, * $P <$ 0.03; n = 3).

CREB Glycosylation Is Reduced by Alanine Mutation of the Major Q2 Domain Sites of Glycosylation. We previously identified two major sites of *O*-GlcNAc glycosylation within the Q2 domain of CREB, between amino acids 253 and 268 (Fig. 16) [19]. Mutation of ^{259}TST^{261} to ^{259}AAA^{261} (T259A/S260A/T261A, AAA-CREB) significantly reduced the level of CREB glycosylation (Fig. 3-15). This AAA-mutant CREB was used to analyze the effect(s) of significantly reducing the potential for CREB glycosylation.

Removal of the Sites of CREB Glycosylation Relieves Glucosamine-Induced Inhibition of CREB Activity. We next tested the effects of the triple mutation on CREB-mediated transcription in HIT cells. To compare the ability of wild-type and mutant CREB to activate transcription and distinguish the effects of exogenously introduced CREB from those of endogenous CREB, we fused the Q2 transactivation domain (aa165-282) or the Q1, kinase-inducible and Q2 domains (aa1-282) to the DNA binding domain of Gal4. Plasmids that drive the expression of wild-type or AAA mutant Gal4-CREB were transfected into cells with a luciferase reporter, and the ability of the Gal4-CREB proteins to activate reporter gene expression was measured and normalized to transfection efficiency using a β-galactosidase expression vector. The glycosylation-deficient AAA mutants displayed a 2-fold increase in CREB activity relative to wild-type Gal4-CREB (Fig. 5-10A and B), further supporting the hypothesis that glycosylation of CREB exerts a repressive effect on CREB-dependent transcription in pancreatic β-cells. Additional detection of chimera expression by immunoblotting with an anti-KID domain antibody confirms the gain-of-function of the AAA chimera.

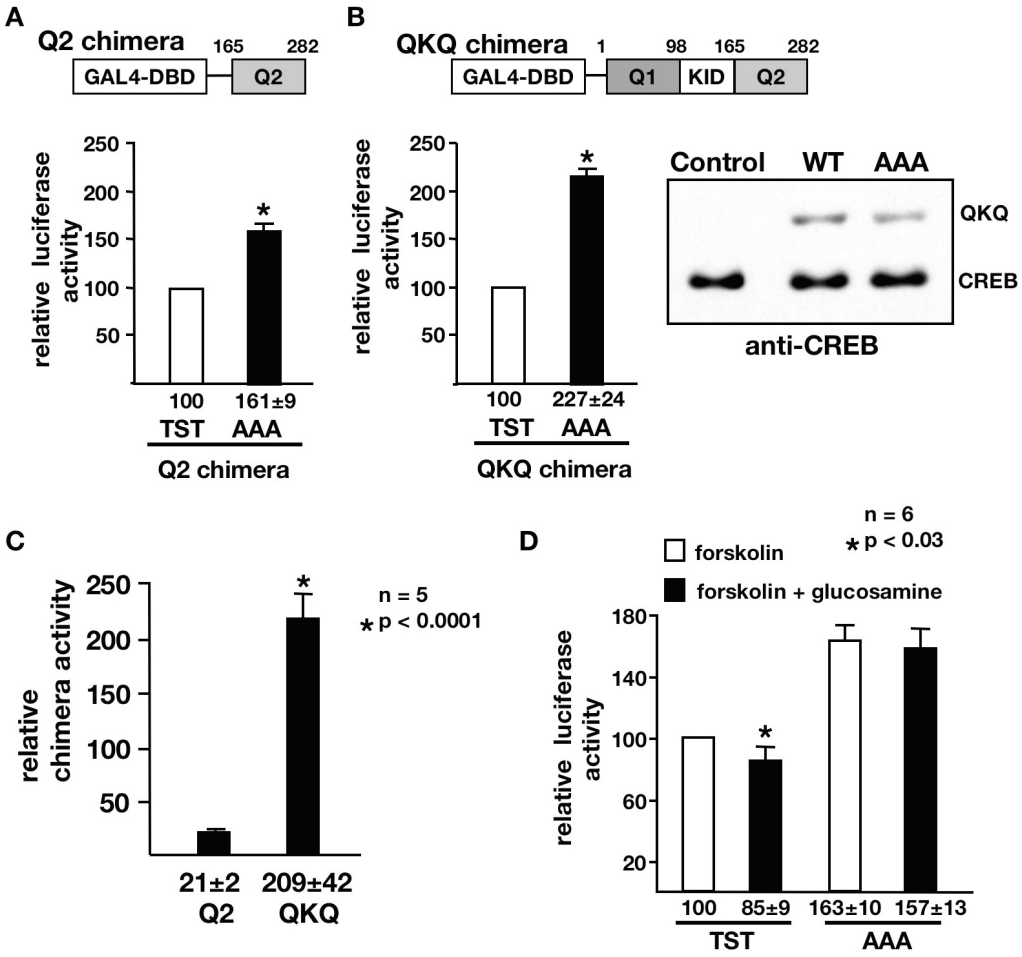


Figure 5-10. Triple alanine Gal4-CREB chimeras display a gain-of-function. **(A,B)** Wild-type and AAA mutant chimeras were generated in which the DNA-binding domain of Gal4 was fused to specific CREB domains as shown. HIT-T15 cells were transfected with expression plasmids containing a UAS-luciferase reporter, β-galactosidase, and the wild-type (TST) or AAA chimeras. Luciferase activity was determined after 24 h (Student's T-test, * $P < 0.01$; n = 3 and 9 for the Q2 and QKQ chimeras, respectively). **(B)** Immunoblotting using an anti-CREB-KID domain antibody (Cell Signaling) shows equivalent expression of wt and AAA chimeras. **(C)** Comparison of the relative activity of Q2 and QKQ chimeras shows order of magnitude change in the activity of the two chimeras (Student's T-test, * $P < 0.0001$; n = 5). **(D)** Glucosamine treatment reduces QKQ(wt) chimera activity and that of the QKQ(AAA) chimera. Cells were incubated in the presence or absence of GlcN (10mM) 3 h prior to the addition of Fsk (10 μM, 6 h). Values were normalized for transfection efficiency using a β-galactosidase assay and represent the mean ± one standard deviation (Student's T-test, * $P < 0.03$; n = 6). Values were normalized for transfection efficiency using a β-galactosidase assay and represent the mean ± SEM.

Direct comparison of the activity of Q2 and QKQ domain chimeras confirms previous reports that, while the Q2 domain is capable of initiating transcription, the activity of the full transactivation domain of CREB is roughly 10-fold more effective at inducing expression (Fig. 5-10C). Fsk activation of QKQ-chimera proved to be highly variable and resulted in a 2- to 17-fold increase in luciferase production when using the UAS-luciferase reporter. However, normalization of the data set to wild-type QKQ chimera activity revealed that, independent of the level of Fsk-induced transactivation GlcN causes a reduction in wild-type activity, while having no effect on the AAA mutant. The reduced magnitude of GlcN's effect on the chimera, when compared to its effect on endogenous CREB, may be a result of the inverted orientation of the CREB transactivation domains with respect to the DNA binding domain (Fig. 5-10D).

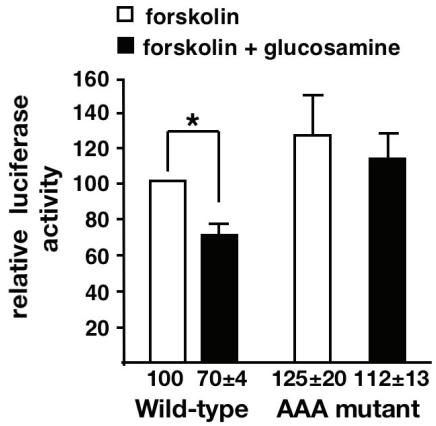


Figure 5-11. Transient expression of AAA mutant CREB reduces glucosamine-induced inhibition of CREB transactivation. HIT-T15 cells were transfected with expression plasmids containing a CRE-luciferase reporter, β-galactosidase, and either full-length wt or AAA CREB 24 h prior to drug treatment. Cells were treated as described in Figure 5-3. Values were normalized for transfection efficiency using a β-galactosidase assay and represent the mean ± SEM (Student's T-test, * $P < 0.04$; n = 5).

In addition to the analysis of the Gal4-CREB chimera, HIT-T15 cells were transfected with full length wild-type or AAA mutant CREB to supplement endogenous stocks of the transcription factor. Cells transfected with AAA mutant CREB gained resistance to GlcN-induced repression of CREB transactivation that was not afforded to cells transfected with wild-type CREB (Fig. 5-11). These results support the hypothesis that glycosylation of the Q2 domain of CREB

interferes with phosphoSer133-dependent transactivation in β-cells. Analysis of CRE-dependent luciferase activity suggests that CREB glycosylation and phosphorylation can act upon a single CREB molecule together or independently in a version of the Yin-Yang hypothesis of antagonistic glycosylation and phosphorylation (Fig. 5-12) [20-22]. The presence or absence of CREB glycosylation can allow for tighter regulation of CREB activity, in effect providing for two levels of induced CREB transactivation. Implicit in this model is the possibility that individual genes may be regulated by either phosphorylation and glycosylation or by phosphorylation alone, depending on possible regulation of OGT localization.

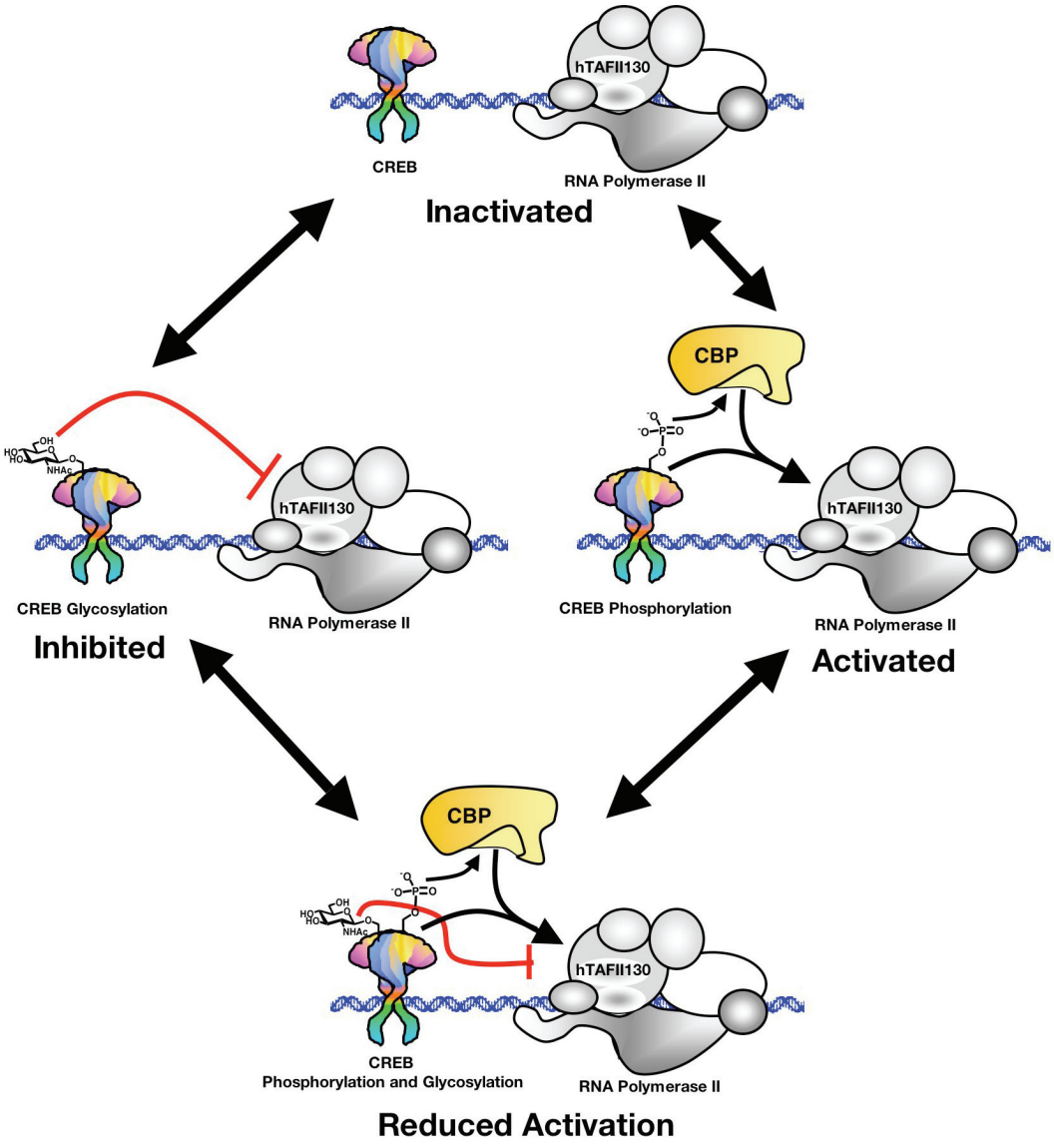


Figure 5-12. *O*-GlcNAc glycosylation allows an additional level of control over CREB activity by regulating the magnitude of phosphoSer133-dependent CREB activity. Studies in cultured cells have shown that CREB glycosylation has little or no effect on basal CREB activity, but reduces the level of induced activity by ~40%.

Bibliography

1. Gao, Y., et al., *Dynamic O-Glycosylation of Nuclear and Cytosolic Proteins* The Journal of Biological Chemistry, 2001. **276**(13): p. 9838-9845.

2. Akimoto, Y., et al., *Increased O-GlcNAc transferase in pancreas of rats with streptozotocin-induced diabetes.* Diabetologia, 2000. **43**: p. 1239-1247.

3. Roos, M.D., et al., *Streptozotocin, an analog of N-acetylglucosamine, blocks the removal of O-GlcNAc from intracellular proteins.* Proceedings of the Association of American Physicians, 1998. **110**(5): p. 422-432.

4. Konrad, R.J. and J. Kudlow, *The role of O-GlcNAc protein glycosylation in beta-cell dysfunction.* International Journal of Molecular Medicine, 2002. **10**: p. 535-539.

5. Konrad, R.J., et al., *The potential mechanism of the diabetogenic action of streptozotocin: inhibition of pancreatic beta-cell O-GlcNAc-selective N-acetyl-beta-D-glucosaminidase.* Biochemistry Journal, 2001. **356**: p. 31-41.

6. Akimoto, Y., et al., *Elevated post-translational modification of proteins by O-linked N-Acetylglucosamine in various tissues of diabetic Goto-Kakizaki rats accompanied by diabetic complications.* Acta Histochemica Cytochemistry, 2005. **38**(2): p. 131-142.

7. Hanover, J.A., et al., *Elevated O-Linked N-Acetylglucosamine Metabolism in Pancreatic beta-cells.* Archives of Biochemistry and Biophysics, 1999. **362**(1): p. 38-45.

8. Khidekel, N., et al., *A Chemoenzymatic Approach toward the Rapid and Sensitive Detection of O-GlcNAc Posttranslational Modifications.* Journal of the American Chemical Society, 2003. **125**(52): p. 16162-16163.

9. Tai, H.-C., et al., *Parallel Identification of O-GlcNAc-Modified Proteins from Cell Lysates.* Journal of the American Chemical Society, 2004. **126**(34): p. 10500-10501.

10. Hagiwara, M., et al., *Coupling of Hormonal Stimulation and Transcription via the Cyclic AMPR-Responsive Factor CREB is Rate Limited by Nuclear Entry of Protein Kinase A.* Molecular and Cellular Biology, 1993. **13**(8): p. 4852-4859.

11. Marshall, S., O. Nadeau, and K. Yamasaki, *Dynamic actions of glucose and glucosamine on hexosamine biosynthesis in isolated adipocytes.* The Journal of Biological Chemistry, 2004. **279**(34): p. 35313-35319.

12. Nakumura, M., et al., *Excessive Hexosamines Block the Neuroprotective Effect of Insulin and Induce Apoptosis in Retinal Neurons.* The Journal of Biological Chemistry, 2001. **246**(47): p. 43748-43755.

13. McKnight, G.L., et al., *Molecular cloning, cDNA sequence, and bacterial expression of human glutamine: Fructose-6-phosphate amidotransferase.* The Journal of Biological Chemistry, 1992. **267**: p. 25208-25212.

14. Oki, T., et al., *cDNA cloning and mapping of a novel subtype of glutamine: Fructose-6-phosphate amidotransferase (GFAT2) in human and mouse.* Genomics, 1999. **57**: p. 224-234.

15. Mayr, B. and M. Montminy, *Transcriptional Regulation by the Phosphorylation-Dependent Factor CREB.* Nature Reviews, 2001. **2**: p. 599-609.

16. Iyer, S.P.N. and G.W. Hart, *Dynamic Nuclear and Cytoplasmic Glycosylation: Enzymes of O-GlcNAc Cycling.* Biochemistry, 2003. **42**(9): p. 2493-2499.

17. Kelly, W.G., M.E. Dahmus, and G.W. Hart, *RNA polymerase II is a glycoprotein. Modification of the COOH-terminal domain by O-GlcNAc.* The Journal of Biological Chemistry, 1993. **268**(14): p. 10416-10424.

18. Bensaude, O., et al., *Regulated phosphorylation of the RNA polymerase II C-terminal domain (CTD).* Biochemistry and Cell Biology, 1999. **77**: p. 249-255.

19. Lamarre-Vincent, N. and L. Hsieh-Wilson, *Dynamic glycosylation of the transcription factor CREB: a potential role in gene regulation.* Journal of the American Chemical Society, 2003. **125**: p. 6612-6613.

20. Comer, F.I. and G.W. Hart, *O-glycosylation of nuclear and cytosolic proteins.* The Journal of Biological Chemistry, 2000. **275**(38): p. 29179-29182.

21. Slawson, C. and G.W. Hart, *Dynamic interplay between O-GlcNAc and O-phosphate: the sweet side of protein regulation.* Current opinion in Structural Biology, 2003. **13**: p. 631-636.

22. Zachara, N.E. and G.W. Hart, *The Emerging Significance of O-GlcNAc in Cellular Regulation.* Chemical Reviews, 2002. **102**: p. 431-438.