Chapter 1
Introduction: CREB Regulation of Eukaryotic Gene Expression

The control of gene expression has evolved to respond to the environmental and intracellular cues that influence cell growth and survival. Key to this control is the ability of cells to affect the activity of transcription factors. Levels of control brought to bear on transcription factors include DNA-binding specificity, post-translational modifications, cis/trans DNA-binding elements and interaction with co-repressors, co-activators and other transcription factors (Fig. 1-1). These modes of regulation provide cells with the capacity to respond with exquisite speed and accuracy to differentiate between the myriad environmental, intercellular and intracellular cues in a context-dependent manner. In multicellular organisms, transactivation of the transcription factor CREB is required for cell survival in neurons and pancreatic β-cells [1-5], the development of cell-type specific functions such as control of glucose and lipid metabolism in hepatic cells [6, 7] and the consolidation of long-term memory [1, 8, 9]. The diverse array of stimuli and accompanying kinase cascades that lead to CREB activation made it an ideal subject for investigation as a target for *O*-GlcNAc glycosylation, a unique form of intracellular and dynamic form of glycosylation. Given its placement at the epicenter of many signaling pathways, we posited it likely that additional layers of control, such as undiscovered post-translational modifications, might exist to regulate CREB activity.

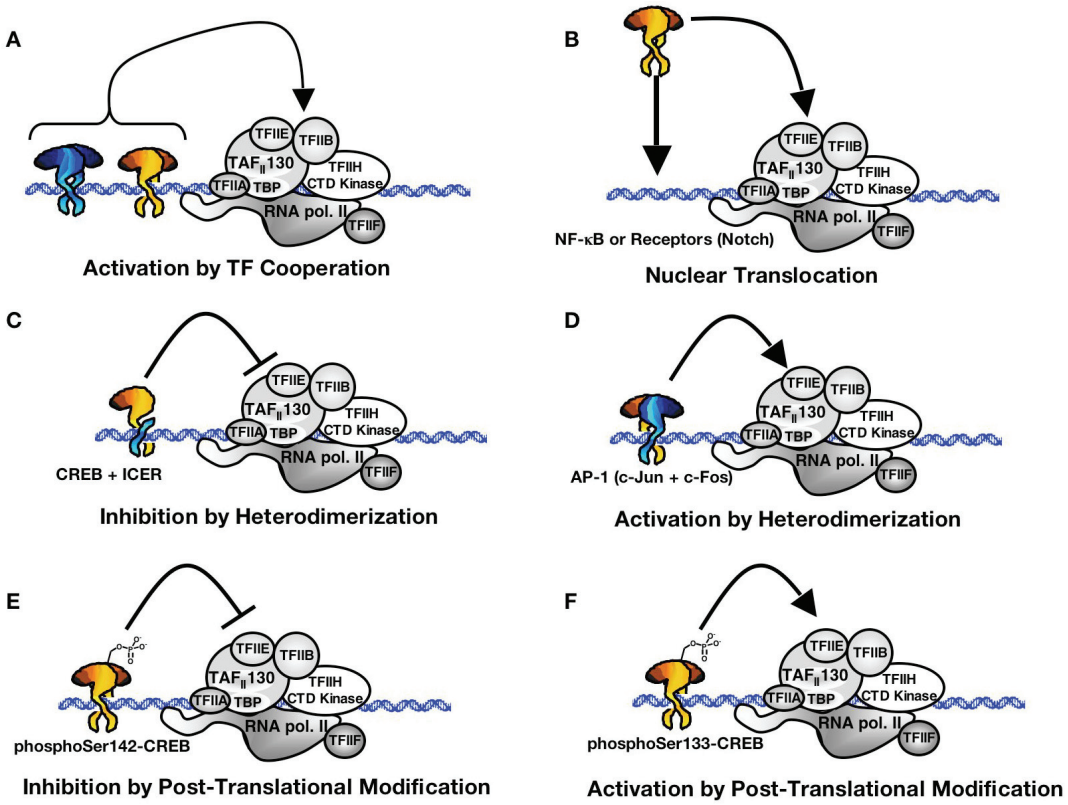

Figure 1-1. A number of modes of transactivation have evolved in eukaryotic cells to allow for acute regulation of gene expression. Examples include: **(A)** the cooperation of multiple transcription factors to recruit RNA polymerase II, as in the case of the regulation of the *c-Fos* gene; **(B)** regulated nuclear localization of transcription factors like the receptor Notch or the transcription factor NF-κB; **(C, D)** activation or inhibition by the interaction of transcription factors; **(E, F)** activation or inhibition by the post-translational modification of transcription factors.

ATF/CREB Family of bZIP Transcription Factors. Transcription factors of the basic leucine zipper (bZIP) super family are conserved from *S. cerevisiae* to mammals. A number of bZIP transcription factors are critical to cellular function, including c-Fos, c-Jun (which together are known as AP-1), C/EBPβ and CREB. Distinguished by the conserved DNA-binding motif, a key representative of the bZIP transcription factors is the Activating Transcription Factor (ATF)/CREB family.

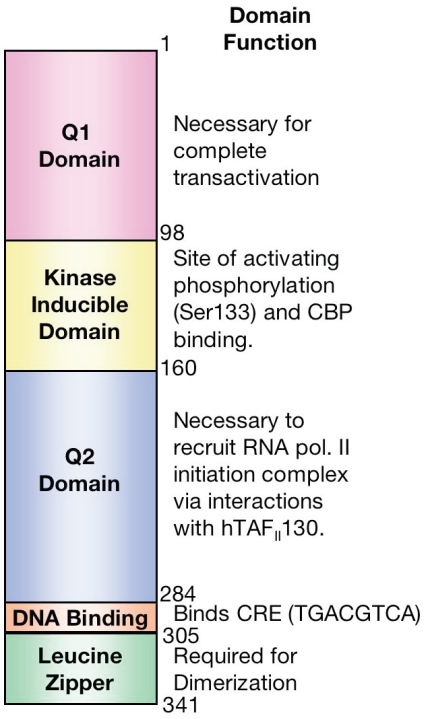

Figure 1- 2. cAMP Response Element Binding Protein (CREB) domains and functionality. CREB is commonly divided into five distinct domains based on primary sequence characteristics and homology with related transcription factor. The Q1 domain, aa1-98, has no particular function assigned to it, but is required for complete wild-type transactivation. The Kinase Inducible Domain (KID, aa99-165) contains Ser133, a site of phosphorylation for multiple kinases that is required for CREB transactivation in cells. The Q2 domain (aa166-284) is required for transactivation through direct interactions with the RNA polymerase II initiation complex. The DNA binding domain (aa285-305) associates with the CRE palindrome (TGACGTCA) through a unique Mg^{2+}-dependent mechanism. The leucine zipper (aa306-341) is required for dimerization of CREB.

Functional Domains of CREB.

Intensive study has shown that CREB function can be separated into a number of distinct domains that affect CREB activity through specific constitutive and inducible protein interactions [10]. These domains include a basic DNA binding domain, a leucine zipper dimerization domain and three transactivation domains: the glutamine-rich domain 1 (Q1 domain), kinase inducible domain (KID) and glutamine-rich domain 2 (Q2 domain) (Fig. 1-2).

Leucine Zipper. The leucine zipper domain (aa305-341) is nearly completely conserved across the three major members of the ATF/CREB family: ATF-1, CREB and CREM. This distinct homology is reflected in the ability of CREB to dimerize with both ATF-1 and CREM, as well as other bZIP family members, including c-Jun and C/EBPβ [11-13]. While the majority of CREB within the cell is believed to bind DNA as a homodimer, dimerization flexibility may confer an additional level of

regulation. More recently, the leucine zipper of the CREB has been shown to anchor binding of the CREB cofactor transducer of regulated CREB (TORC), which can facilitate and enhance CREB activity, lending credence to the possibility that heterodimerization may contribute to regulation of CREB [14].

Basic DNA Binding Domain. The basic DNA binding domain (aa284-305) of the CREB/ATF family members binds to the cAMP-response element (CRE), TGACGTCA, with a K_D of 1 nM. Few of the identified CREB-dependent genes contain a full-CRE within their promoter. The half-CRE, CGTCA, which CREB binds with 5 nM affinity, is more prevalent. Interestingly, the binding of CREB to full-CRE sites is Mg^{2+}-dependent, based on a central DNA contact that is mediated by a Lys304-Mg^{2+}-DNA contact. However, to date no regulatory function has been ascribed to this Mg^{2+} dependence. Recent studies have shown by deletion analysis that DNA binding, originally thought to be constitutive, is sensitive to deletion of specific regions of the Q2 domain [15]. The basis for the relationship between the DNA binding and Q2 domain identified by Mayr, Guzman and Montminy, and whether it contributes to regulation of CREB activity are still unknown [15].

Glutamine-Rich Domain 2/Constitutive Activity Domain (Q2 Domain). The Q2 domain (aa160-283), neighboring the DNA binding domain of CREB, was first identified as a domain required for recruitment of the RNA polymerase II initiation complex by CREB [2]. The Q2 domain alone is sufficient to facilitate transcription *in vitro* as a result of direct contacts between the Q2 domain and the TFIID component

TATA Binding Protein-Associated Factor 130 II ($TAF_{II}130$) [16-20]. However, Asahara and coworkers subsequently found that while the Q2 domain is sufficient for transcription of "naked" DNA, transcription of chromatin DNA requires the histone acetyltransferase activity of CREB-binding protein (CBP), which itself is recruited by the kinase inducible domain [21].

Kinase Inducible Domain (KID). The KID domain (aa98-159), couched between the two glutamine-rich domains, undergoes a conformational change that, when phosphorylated at Ser133 (pS133), results in the recruitment of CBP [22, 23]. Two gain-of-function mutants of CREB confirmed the role of the KID domain in the sequestration of CBP. Tyr134Phe mutation results in prolonged pS133 and concomitant extended association with CBP [24]. A more extensive conversion of ^{129}RRPSYR^{135} to the CBP-binding motif of the sterol-responsive element binding protein (SREBP), DIEDML, resulted in a constitutively active CREB mutant [25].

Glutamine-Rich Domain 1 (Q1 Domain). The Q1 domain (aa1-97) of CREB, while necessary to elicit maximal transactivation by CREB, has no identified role in the recruitment of either coactivators such as CBP or the RNA polymerase II intitiation complex [2].

Role of CREB in Cellular Processes. Since identification of CREB as a kinase-inducible transcription factor, CREB activity has been linked to a number of cellular

processes, with most efforts focusing on the role of CREB in the brain [26]. I will highlight its role in three biological processes.

Learning and Memory. One of the first links made between CREB activity and cell-specific function was in the development of learning and memory. Seminal work done by Kandel and coworkers in the sea slug, *Aplysia*, found that primitive forms of motor neuron memory were dependent on CREB activity [27]. CREB mediates the development of memories by initiating transcription events required for the strengthening of synaptic connections between neurons known as either long-term facilitation (LTF) or long-term potentiation [28, 29]. Later studies showed that similar processes were involved in the function of the mammalian brain. Disruption of CREB activity in specific regions of the brain (amygdala, hippocampus, cortex and suprachiasmatic nucleus) through expression of dominant negative inhibitors or inducible deletion of CREB using the Cre-recombinase system, led to deficits in various forms of learning and memory [8, 30-34]. Over-expression of CREB in the amygdala enhanced fear conditioned memory consolidation [34]. In a complementary study, suppression of inducible Ser133Ala-mutant CREB within the hippocampus, cortex and amygdala of mice led to a loss of a conditioned fear response [32]. In the forebrain, disruption of CREB activity in the hippocampus and barrel cortex resulted in a loss of protein expression-dependent LTP [30, 31], that was complemented by a loss of spatial memory [8]. The effects of these animal studies are complicated by the fact that CREB not only regulates synaptic plasticity but is integral to cell survival, and abnormal cell death may contribute to cognitive losses [1, 35].

Stimuli that lead to CREB Phosphorylation	Associated pS133 Kinases
Growth Factors	
EGF	PKA and MAPK
FGF	MAPK
GIP	PKA
IGF-1	PKA and ERK1/2
Steroid Hormone Signaling	
Estrogens	PKA, MAPK and PI3K
Peptide Signaling	
GLP-1	PKA, ERK1/2
Insulin	ERK, p38, PI3K
Thyroid-Stimulating Hormone	PKA
Neurosignaling	
Dopamine receptor	PKA, MAPK, PKC, CaMK
$GABA_A$ receptor	PKA and MAPK
Neurotrophins	PKC, ERK, PI3K, CaMKIV
Cytokines	
Interleukins	PKA, ERK, p38, PKC, RTK
TGF-b	
Oxidative Stress	
H_2O_2	MAPK
Enviromental Stress Factors	
Fasting/refeeding	PKA
Pain	PKA
Stress	MAPK
Ion channel/ Intracellular Signaling	
L-type Ca^{2+} channel activator	CaMK
KATP channel blockers	
Membrane Depolarization	PKA, ERK1/2, CaMK
High Ca^{2+} concentration	

Table 1-1. A summary of the general classes of stimuli that lead to CREB phosphorylation at Ser133. Representative stimuli for each class were taken from a list compiled in Johannessen, Delghandi and Moens [47].

Cell Growth/Survival. CREB activity has been linked with both cell growth and survival in both neurons and endocrine β-cells of the pancreas [36-44]. Transgenic mice over-expressing dominant negative forms of CREB in β-cells exhibit increased levels of apoptosis and reduced β-cell mass [43]. Similarly, brain-specific deletion of *CREB* or both *CREB* and *CREM* genes leads to increased apoptotic cell death of cortical, striatal and hippocampal neurons, as well as neurons of the dorsal root ganglia [36, 39, 44]. In both neurons and β-cells CREB activity can be induced by insulin-like growth factor-1 (IGF-1) and glucagon-like peptide-1 (GLP-1), as well as neurotrophins (NGF and BDNF) and NMDA (*N*-methyl-D-aspartate) in neurons (Table 1-1) [40, 43, 45-47].

A number of insults that led to ischemia also activate CREB in neurons (Table 1-1) [1, 47-49]. These stimuli led to the activation of PKA, PI3K/Akt, MEK/ERK and CaMKII/IV pathways and induce expression of genes that directly antagonize apoptotic

pathways, such as Bcl-2 family proteins, and genes supporting pro-survival signaling, such as insulin receptor substrate 2 (IRS2) [43, 48, 50].

Metabolism. The maintenance of proper glucose and free-fatty acid (FFA) levels are critical to cell survival in multicellular organisms and CREB plays an important role in their regulation [7, 51]. Both glucose and FFA are produced in the liver by activation of gluconeogenic and lipogenic gene programs.

CREB contributes to the control of gluconeogenesis by activating the expression of the transcriptional coactivator PGC-1. PGC-1, in concert with CREB, activates transcription of genes in the gluconeogenic program, such as the rate-limiting enzyme phosphenolpyruvate carboxykinase (PEPCK) [7]. The role of CREB in regulating gluconeogenesis suggested that dysfunction in CREB signaling may contribute to the development of hyperglycemia via excess gluconeogenesis in diabetes.

Mice expressing a dominant negative form of CREB produced high levels of triglycerides, indicating that CREB may negatively regulate the gluconeogenic program. CREB was found to prevent lipogenic gene expression by inhibiting expression of the nuclear hormone receptor PPAR-γ, itself a transcription factor. This inhibition was achieved indirectly through activation of the corepressor Hairy Enhancer of Split (HES-1) [6]. Similar to the failure of CREB to correctly regulate gluconeogenesis, a failure of CREB to maintain homeostatic lipogenesis could contribute to diabetes by potentiating hepatic and pancreatic β-cell death via increased levels of circulating FFAs.

Activation of CREB. The CBP:pS133 paradigm developed over the past 20 years for the activation of CREB-dependent gene expression is depicted by the two-state model shown in Figure 1-3. CREB is thought to bind constitutively to CRE sites. Upon stimulation of Ser133 phosphorylation, CBP is recruited. CBP histone-acetyl transferase activity relaxes the local chromatin, allowing the latent affinity of the Q2 domain for $TAF_{II}130$ to stimulate the deposition of the RNA polymerase II initiation complex and start transcription [2, 26]. A number of kinase signaling cascades converge on Ser133; these include PKA, PKB, PKC, the mitogen-activated protein kinases (MAPKs): p38, ERK 1/2 and Rsk, and Ca^{2+}/calmodulin-dependent kinases II and IV (CamKII/IV) (Table 1-1) [47]. Differences in kinase kinetics can result in unique patterns of gene expression. For example, PKA induction can result in a brief, but large, increase in CREB phosphorylation while MAPKs produce a sustained and gradual increase in CREB phosphorylation [2, 52-54]. Expression of the inducible cAMP early repressor (ICER) form of the ATF/CREB family member CREM, as a result of specific kinase-induced CREB activity, has been identified as one potential means of differentiating stimuli [53].

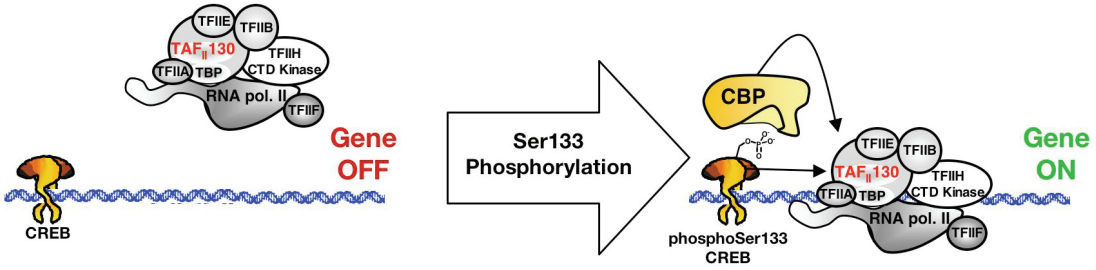

Figure 1- 3. The CBP/p300:phosphoSer133-CREB paradigm of CREB interaction. Phosphorylation of Ser133 leads to recruitment of the histone acetyltransferase CREB-Binding Protein (CBP) or its paralog p300. The HAT in turn relaxes the local chromatin and assists in the deposition of RNA polymerase II initiation complex.

Proteins that interact with CREB	
Protein	CREB Domain
C/EBPβ HDAC1 $TF_{II}A$	**Q1**
CBP p300 FHL/ACT Tip60	**KID**
v-Abl **$hTAF_{II}130/135$**	**Q2**
CREB BRCA1 CREM/ICER DNA Topoisomerase 1 HNFb Jun Family members pX p53 Tax **TORC** YY-1 ZPK	**bZIP**

Table 1-2. CREB-Interacting Proteins. A number of proteins have been shown to interact with CREB. Adapted from Johannessen, Delghandi and Moens [47].

In vivo phosphorylation of Ser133 is necessary for CREB-dependent gene expression, but in a number of instances phosphorylation of Ser133 alone is insufficient to mediate CREB activation [52, 55, 56]. For instance, different expression patterns of the CREB-dependent genes c-*fos*, *BDNF* and *tyrosine hydroylase* have been observed in neurons suggesting that there is more to CREB regulation than simply pS133 [1, 57, 58]. These results led to the hypothesis that the CBP:pS133 paradigm was only a general mechanism that is elaborated by additional mechanisms to connect stimuli and CREB activation to the desired CREB-dependent gene expression.

Elaborating the Regulation of CREB Transactivation. A growing list of CREB-associated proteins and CREB-PTMs have added to the evident complexity of CREB regulation (Tables 1-2 and 1-3) [47]. These interactions and modifications can both enhance and inhibit activation of CREB (Fig. 1-4).

Transducers of regulated CREB make up a new family of CREB-specific coactivators that associate with the bZIP domain of CREB [14]. TORCs enhance CREB activity through constitutive recruitment of RNA polymerase II. TORCs themselves are

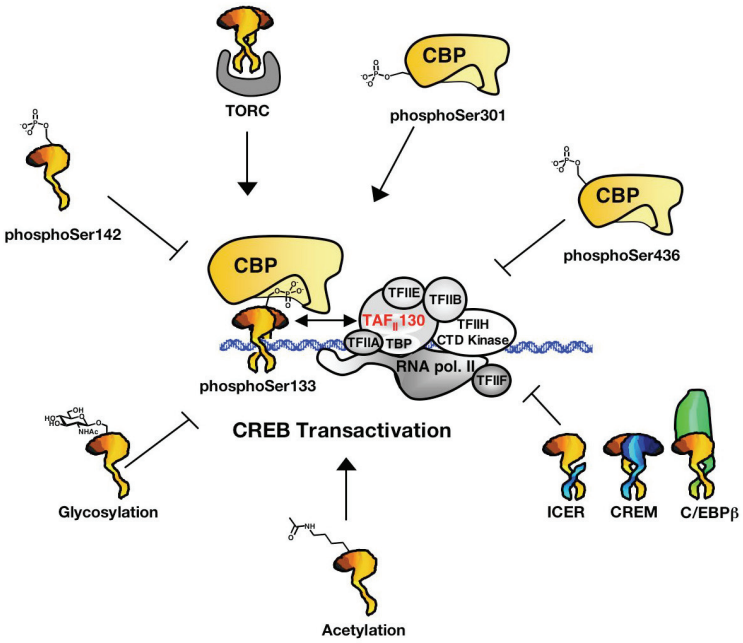

Figure 1-4. Cell-specific regulation of CREB transactivation, additions to the CBP/p300 paradigm. A number of alternative mechanisms for the regulation of CREB have been identified recently. These include: phosphorylation of Ser142, which disrupts recruitment of CBP; phosphorylation of Ser436 of CBP, which inhibits its activity and phosphorylation of Ser301 of CBP, which enhances its activity; glycosylation (detailed in this thesis) of the Q2 domain, which disrupts interaction with hTAF$_{II}$130; acetylation of the Q1 and KID domains that inhibits dephosphorylation of Ser133 to potentiation transactivation; heterodimerization of CREB with the co-repressor forms of the cAMP-Response Element Modulator (CREM and ICER) or other bZIP transcription factors; interaction the CREB dimer with the coactivator TORC.

regulated by a phosphorylation-dependent interaction with 14-3-3 scaffolding proteins, which sequester TORCs to the cytosol. Ca^{2+}-dependent dephosphorylation releases TORCs from the 14-3-3 complexes to translocate to the nucleus and support CREB activity. Ca^{2+}-dependent regulation of TORCs provides a mechanism for cells to distinguish between cAMP and Ca^{2+} signaling [59, 60]. The addition of TORC regulation to the basic pS133:CBP paradigm has been implicated in hepatic gluconeogenesis and possibly in pancreatic β-cells [60-62].

Regulation of CBP by dynamic post-translational modifications has also been implicated as an additional means of modifying the pS133:CBP paradigm. CaMKIV phosphorylation of Ser301 enhances CBP activity *in vitro* and in neurons, independent of its association with CREB [63]. Ser301 phosphorylation allows a range of pS133-CREB-dependent activity similar to the effect of the TORCs by acting as a pS133 plus Ca^{2+} coincidence detector. Additionally, PI3K phosphorylation of Ser436 (pS436) was identified in the HepG2 hepatic cell line [64]. pS436 was required for CBP binding to the transcription factors Pit-1 and AP-1 [65]. Conversely, pS436 inhibited CREB:CBP activity in hepatic cells. Transgenic mice expressing a liver-specific S436A-CBP mutant displayed increased gluconeogenesis, suggesting that, along with regulation by pS133 and TORCs, phosphorylation of CBP also regulates CREB-

CREB Post-Translational Modifications (PTMs)

PTM	Site/Domain	Function
Acetylation	Lys91	Sustains pSer133
Acetylation	Lys96	Sustains pSer133
Phosphorylation	Ser133	Activation
Acetylation	Lys136	Sustains pSer133
Phosphorylation	Ser142	Repression
~13x*O* -GlcNAc	Q1/Q2	Repression
	Thr259	Repression
2x*O* -GlcNAc	Ser260	Repression
	Thr261	Repression
SUMO-ylation	Lys285	NLS
SUMO-ylation	Lys304	NLS

Cofactor PTMs

CREB Binding Protein (CBP)

PTM	Site/Domain	Function
Phosphorylation	Ser301	Activation
Phosphorylation	Ser436	Repression
O -GlcNAc	No Data	No Data
Methylation	Arg714, 742, 768	
SUMO-ylation	Lys999	Repression
SUMO-ylation	Lys1034	Repression
SUMO-ylation	Lys1057	Repression

Transducers of Regulated CREB (TORC)

PTM	Site/Domain	Function
Phosphorylation	Ser171	Occlusion of NLS

Table 1-3. Post-Translational Modifications of CREB. The past years have seen a large expansion in the number of identified PTMs that modifiy CREB and its associated cofactors, TORC and CREB-binding protein (CBP).

dependent gene expression [66].

Other post-translational modifications of CREB have also been implicated in the modification of pS133:CBP paradigm (Table 1-3). The first CREB modification other than pS133 identified was Ser142 phosphorylation (pS142). Dual phosphorylation of Ser133 and Ser142 by CamKII blocks transactivation through inhibition of CBP association [67]. This modification is involved in the regulation of mammalian circadian rhythms in the suprachiasmatic nucleus, in which pS142 is induced by light to attenuate CREB activity during the day [68, 69]. More recently, CBP was found to acetylate CREB. Acetylation had the effect of inhibiting dephosphorylation of CREB, and as a result, is expected to prolong CREB activity [70].

These discoveries, taken together, are beginning to reveal a model of CREB regulation in which a combination of protein interactions and modifications act in concert to distinguish the multiple stimuli that act on CREB activity. To this model we add the unique modification of CREB with *O*-GlcNAc glycosylation. This modification acts on CREB, independent of pS133, to inhibit CREB:hTAF$_{II}$130 interaction and inhibit CREB activity in pancreatic β-cells. The effect of this modification is to moderate CREB activity. The nature of *O*-GlcNAc glycosylation, identification, characterization and the functional implications of CREB glycosylation will be addressed in this thesis.

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