Chapter 4

The roles of *O*-GlcNAc and CREB in the transcription of key neuronal gene networks

4.1 The histone and PTM codes

Transcription must integrate a variety of extracellular signals in order to faithfully execute distinct biological outcomes. A critical mechanism conferring biological fate specificity is through the post-translational modification (PTM) of transcriptional regulators such as histones. The 'histone code hypothesis' involves acetylation, methylation, phosphorylation, glycosylation, and many other modifications of histones at over a hundred sites.[1] Combinations of these modifications are integrated to modulate DNA accessibility and recruit transcriptional factors to control biological function.[1-5] In neurons, distinct epigenetic codes dictate everything from neurodevelopment and neuronal activity to neurodegenerative diseases.[6-8] Similarly, transcription factors (TFs) are modified by a variety of PTMs, which confer a PTM 'code'.[9]

In order to impart different neuronal outcomes, two potential mechanisms could be in play: either (1) certain subsets of modifications confer distinct biological consequences or (2) each individual modification contributes to activity through additive effects to influence net activity at particular sites.[4] While the individual contributions of each of these PTMs have been explored in isolation, their combinatorial integration to elicit specific transcriptional destinies are still poorly understood.[2]

4.2 The role of *O*-GlcNAc in the epigenetic code

The "epigenetic code" refers to the integration of the histone code and DNA methylation code in order to govern transcription and biological fates.[10] OGT has been shown to regulate chromatin remodeling (Figure 4.1). Chromatin, where genomic DNA envelopes histone octamers, can be made more (euchromatin) or less (heterochromatin) accessible to transcription through the dynamic PTM regulation of the histone code.[11]

Generally, acetylation of histone lysines is a dynamic modification that increases the accessibility of chromatin for transcriptional activation.[12] Methylation often at the same histone lysine residues is a longer-lived modification that is associated with heterochromatin and gene silencing with some exceptions (notably, H3K4 methylation is activating).[13] DNA methylation on cytosine residues at CpG dinucleotides is an important epigenetic regulation that impairs the binding of many TFs to DNA and recruits proteins including histone methyltransferases.[14,15] The net result of DNA methylation is to confer an epigenetic "memory" whereby critical genes are silenced during various cellular processes and in particular, during development.[16]

In 2009, it was discovered that super sex combs, an essential component of the polycomb repressive complex 2 (PRC2), is actually *O*-GlcNAc transferase in drosophila.[17] PRC2 mediates the silencing of chromatic regions through the methylation of histone H3 on lysine 27 and is necessary for the proper *O*-GlcNAcylation of proteins.[18,19] In addition, OGT *O*-GlcNAcylates and stabilizes EZH2 (enhancer of zeste 2 polycomb repressive complex 2 subunit), a component of the PRC2 complex.[20] A major component of polycomb repressive complex 1 (PRC1), RING1B or E3 ubiquitin ligase RING1, preferentially associates with neuronal genes or cell cycle genes when it is *O*-GlcNAc glycosylated or non-glycosylated respectively.[21] OGT further influences PRC1 activity by directly *O*-GlcNAcylating and protecting the PRC1 oncogene Bmi-1 (BMI1 proto-oncogene, polycomb ring finger) from proteosomal degradation.[22] In these ways, *O*-GlcNAc can alter the transcriptional regimes regulated by PRC1 influencing cellular pluripotency, differentiation, and proliferation.

In addition, OGT is also a major component of the SET1/COMPASS histone H3K4 methylation complex, whose activity is important for elevating transcription.[23] Part of the SET1/COMPASS (histone-lysine *N*-methyltransferase Set1/complex proteins associated with Set1) complex, the heavily *O*-GlcNAc glycosylated host-cell factor 1 (HCF1) is activated upon proteolytic cleavage, which is catalyzed by OGT itself.[24] Another component of the SET1/COMPASS complex, MLL5 (histone-lysine N-methyltransferase 2E (mixed lineage leukemia 5)), is glycosylated at S435 and T440, which prevents its proteosomal degradation.[25] Additionally, OGT interacts with and mediates the activity of the mSin3A/HDAC1 (SIN3 transcriptional regulator family member A/histone deacetylase 1) complex, which mediates transcriptional repression by deacetylating histones.[26] *O*-GlcNAc glycosylation of HDAC1 regulates its enzymatic activity; indeed, ablation of the *O*-GlcNAc sites on HDAC1 leads to a slight reduction in HDAC1 activity.[27] CARM1 (coactivator-associated arginine methyltransferase 1) and OGT interact to regulate mitosis through the methylation of histone 3 R2, R17, and R38.[28,29] Finally, studies have shown that OGT forms a complex with NSL3 (nonspecific lethal protein 3), a component of the histone acetyltransferase complex that glycosylates histone 4 at K5, K8, and K16.[30] The glycosylation of NSL3 stabilizes the protein and increases the methylation of histone 4. The interdependence of OGT and these transcriptional complexes results in a complex interplay that define the cellular fate.

OGT further regulates transcriptional activity by directly modifying histones.[31-39] Initially, Hart and colleagues identified *O*-GlcNAc glycosylation sites on Thr101 of H2A, Ser36 of H2B, Ser47 of H4, and H3.[40] However, some studies have found that detection of *O*-GlcNAc on certain site is sensitive to specific growth or stimulation conditions (like

cell cycle state), enrichment, mass spectrometry, and western blotting conditions.[33,36,39] Furthermore, the conservation of site-specific histone *O*-GlcNAcylation can vary by across species with one study demonstrating that Ser40 *O*-GlcNAcylation on H2A evolved with viviparity.[36]

Despite the difficulty and variability in detection of histone *O*-GlcNAcylation, researchers have begun to dissect the functions of specific histone glycosylation events. For example, one study found that histone H2B *O*-GlcNAcylation at Ser112 facilitates the ubiquitination of histone H2B at Lys120, which both lead to active transcription.[39] The phosphorylation of OGT by AMPK (5'adenosine monophosphate-activated protein kinase) downstream of glucose stimulation leads to impaired chromatin association and a reduction in *O*-GlcNAc and monoubiquitination of H2B.[31] *O*-GlcNAc glycosylation of H2A at threonine 101 perturbs the dimerization of H2A and H2B resulting in euchromatin and enhanced transcription.[41] *O*-GlcNAc glycosylation of H3S10 is dependent on cell cycle and directly precludes with phosphorylation at the same site.[35] Furthermore, glycosylation on the same histone at T32 prevents the cell cycle-dependent phosphorylation events at S10 and S28 (and T32 through direct competition).[42] *O*-GlcNAcylation of H2AX S139 occurs in response to DNA damage, competes directly with S139 phosphorylation, and most importantly, demarcates the DNA damage region preventing its suffusion.[43] Moreover, OGT plays a critical role in cellular senescence through the modification of HIRA (histone cell cycle regulator) and Ubn1 (ubinuclein 1), consituents of the HIRA chaperone complex necessary for installation of histone variant 3.3.[44] *O*-GlcNAcylation of HIRA and Ubn1 is necessary for the appropriate formation and timing of heterochromatic, senescence-related nucleosome assembly.[44] Either

through direct modification or through the targeting of its interacting partners, OGT dynamically determines the structure of chromatin and ultimately the transcriptional availability of genes.

In addition to OGT's aforementioned role in chromatin structure, deletion of *O*-GlcNAcase globally perturbs chromatin structure, chromatin *O*-GlcNAcylation, and gene expression.[45,46] Interestingly, OGA also contains a pseudo-histone acetyltransferase (HAT) domain that contains high homology with other HATs, but lacks the requisite acetyl-coenzyme A (acetyl-CoA) binding residues.[47] The function of the pseudo-HAT domain of OGA remains to be determined especially since it appears to be unnecessary for the proper *O*-GlcNAcase enzymatic activity.[47,48] Moreover, *O*-GlcNAcylation has been shown to mediate the selectivity of the nuclear pore and be necessary in order maintain the nuclear pore integrity through its modification and stabilization of nucleoporins, the major structural elements that comprise the nuclear pore.[49] Together, OGA and OGT act in concert to regulate global gene transcription, chromatin structure, and nuclear pore stability and selectivity.

In addition, OGT has been shown to be a major regulator of DNA methylation as well. As discussed earlier, methylation of DNA at 5-methylcytosine (5mC) in CpG islands or gene bodies leads to gene silencing.[50] The Tet (ten-eleven translocation methylcytosine dioxygenase) family catalyzes the conversion of 5mC to 5-hydroxymethylcytosine (5hmC) and then to 5-formylcytosine (5fC) and 5-carboxylcytosine (5caC), which are required for base excision repair-mediated removal of 5-methylcytosine.[50,51] The Tet protein family is made up of Tet1, Tet2, and Tet3, which have been shown to be involved in neuronal differentiation, activity, memory

formation, and neurodegeneration.[52-56] OGT and the Tet proteins interact and enhance each others activity across the genome.[23,57-62] In particular, the *O*-GlcNAcylation of Tet1 prevents the proteosomal degradation of Tet1.[62] In addition, the interaction of OGT with Tet3 increases its stability and DNA association.[63] Through regulation of Tet activity and localization, OGT regulates transcriptional activity.

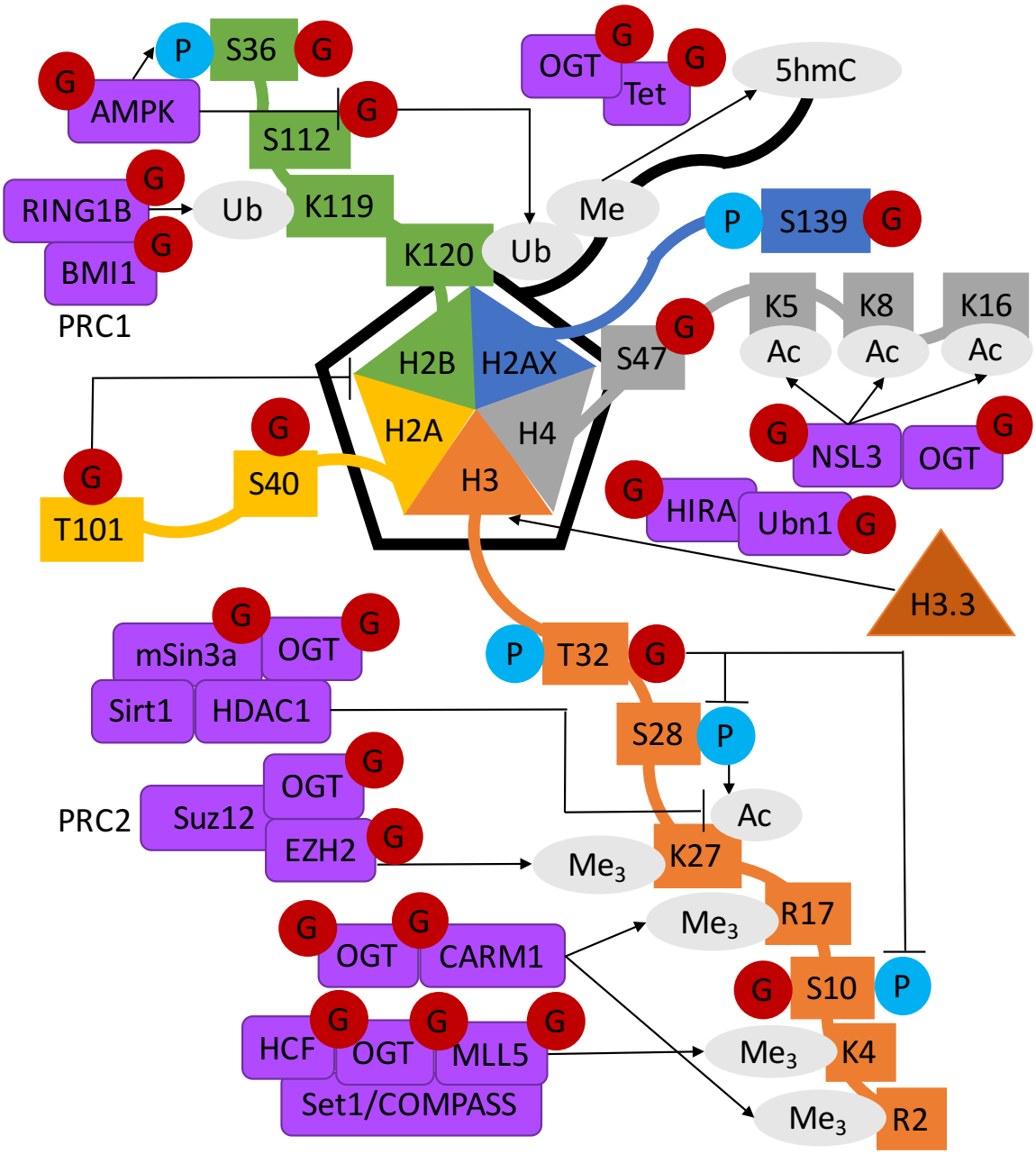

Figure 4.1 The histone and epigenetic codes are heavily regulated by OGT. **H2A** (in yellow) has been shown to be glycosylated at S40, which is associated with vivaparity. Glycosylation at H2A T101 disrupts the association of H2A and H2B leading to euchromatin and transcriptional activation. **H2B** (in green) is glycosylated at S36, which directly competes with AMPK-catalyzed phosphorylation at the same site. AMPK, which itself is activated through glycosylation, phosphorylates OGT, which reduces the glycosylation of H2B at another site, S112. H2B glycosylation at S112 enhances ubiquitination at K120, which is associated with transcriptional activation. **H2AX** (in blue) is glycosylated at S139 in response to DNA damage, which abrogates phosphorylation at the same site and delimits the DNA damage remodeling boundaries. **H4** (in grey) is glycosylated at S47, but its function is currently unknown. **H3** (in orange) is glycosylated at T32 and T10, which directly competes with cell-cycle dependent phosphorylation at the same sites. In addition, H3 T32 glycosylation indirectly reduces the phosphorylation at S28.

In addition to directly modifying histones, OGT interacts and influences the activity of several histone and DNA modifying complexes. OGT interacts with the polycomb repressor complex 2 (PRC2) and modifies and stabilizes the EZH2 protein, which directly methylates H3K27 leading to gene silencing. In addition, OGT glycosylates, interacts with, and stabilizes HCF and MLL5, which are members of the Set1/COMPASS H3K4 methylation complex. Methylation via the Set1/COMPASS complex at H3K4 leads to enhanced transcriptional activity. The H2B K119 residue is ubiquitinated by the RING1B protein, the active enzyme in the PRC1. This ubiquitination event is associated with gene silencing. The *O*-GlcNAc glycosylation of RING1B causes RING1B to associate with neuronal genes while unglycosylated RING1B is associated with cell cycle genes. Another component of the PRC1, BMI1, is glycosylated and stabilized by OGT. OGT also enhances the activity of NSL3, the histone acetyltransferase responsible for acetylating histone 4 and therefore increasing chromatin accessibility. *O*-GlcNAcylation of the histone chaperone complex proteins, HIRA and Ubn1, is necessary for the proper installation of the senescence-related histone 3.3 variant. OGT directly glycosylates and stabilizes Tet proteins, which catalyze the removal of DNA methylation. The removal of CpG methylation leads to transcriptional activation.

Abbreviations and legend: G (red) = *O*-GlcNAc; P (turquoise) = phosphate; Me/Me3 (pale grey) = methyl/trimethyl group; 5hmC (pale grey) = 5'-hydroxy methylcytosine; Ac (pale grey) = acetyl group; Ub (pale grey) = ubiquitin; OGT = *O*-GlcNAc transferase; H# = histone #; PRC = polycomb repressive complex; AMPK = 5'adenosine monophosphate-activated protein kinase; Tet = ten-eleven translocation methylcytosine dioxygenase; RING1B = E3 ubiquitin-protein ligase RING1; BMI1 = BMI1 Proto-Oncogene, Polycomb Ring Finger; CARM1 = coactivator-associated arginine methyltransferase 1; Set1 = histone-lysine N-methyltransferase Set1; COMPASS = complex proteins associated with Set1; MLL5 = histone-lysine N-methyltransferase 2E (mixed lineage leukemia 5); HCF = host cell factor; EZH2 = enhancer of zeste 2 polycomb repressive complex 2 subunit; Suz12 = SUZ12 polycomb repressive complex 2 subunit; HIRA = histone cell cycle regulator; Ubn1 = ubinuclein 1; mSin3a = histonedeacetylase complex subunit Sin3a; Sirt1 = sirtuin 1; HDAC1 = histone deacetylase 1; NSL3 = nonspecific lethal protein 3

4.3 The role of *O*-GlcNAc in the PTM code

Aside from influencing chromatin structure, *O*-GlcNAcylation plays an essential and specific role in transcriptional regulation.[64,65] The major component of the transcriptional machinery, RNA polymerase II (pol II), is heavily modified by O-GlcNAc, which is associated with pre-initiation complexes while loss of O-GlcNAcylation on pol II is linked to elongation.[66,67] *O*-GlcNAcase (OGA), the enzyme responsible for removing *O*-GlcNAc, is an pol II elongation factor itself.[68] In these ways,

O-GlcNAcylation and the enzymes that cycle it are essential regulators of basal transcription.

OGT and OGA dynamically cycle *O*-GlcNAc glycosylation on almost all transcription factors in eukaryotes.[34] *O*-GlcNAc glycosylation and phosphorylation have been shown to coordinate cellular function and transcriptional activity in tandem.[69] As discussed in Chapter 1, *O*-GlcNAc glycosylation and phosphorylation compete for the same serine and threonine residues leading to a "yin-yang" competitive relationship.[69] A classic example of this "yin-yang" relationship occurs on the TF c-myc (MYC proto-oncogene, bHLH (basic helix-loop-helix) TF); specifically, c-myc is rapidly degraded upon phosphorylation at Thr58, but the *O*-GlcNAc glycosylation at the same site increases the stability of c-myc.[70,71] In addition to competing for the same residues, a recent paper showed that phosphorylation at neighboring residues can directly prevent *O*-GlcNAcylation especially on the conserved (pSp/T)P(V/A/T)(gS/gT).[72] Together, *O*-GlcNAc and phosphorylation are integrated into a larger PTM Switchboard that controls the activity and specificity of transcription factors.[73]

Moreover, the *O*-GlcNAc modification of NF-κB (nuclear factor kappa-light-chain enhancer of activated B cells) RelA/p65 protein at Thr352 leads to disruption of its interaction with the protein IκBα, which normally sequesters NF-κB from the nucleus and prevents its transcriptional activation.[74] *O*-GlcNAc modification of RelA at another site, Thr305, is required for p300 to acetylate and fully activate RelA.[75] Thus, *O*-GlcNAcylation of RelA at Thr352 and Thr305 is necessary for the full activation of NF-κB transcription. Furthermore, our lab along with the Baltimore lab showed that *O*-GlcNAcylation at Ser350 of another NF-κB protein, c-Rel, enhances the expression of

toll-like receptor-dependent cytokine genes, but had no impact on TNFα-regulated gene expression.[76] Therefore, *O*-GlcNAc regulates both (1) global NF- κB activity by increasing its nuclear availability and activating acetylation and (2) stimulation-specific NF-κB gene networks important for immune and stress response. Global gene expression studies are required to determine effects of glycosylation on all NF-κB-regulated processes including cell survival and synaptic plasticity.[77,78] In summary, *O*-GlcNAc and its cycling enzymes cooperate to encode complex histone and PTM codes that are decoded to alter TF global and gene-specific activity.

4.4 CREB is a key regulator of critical gene networks in neurons

The TF, CREB, or cyclic adenosine monophosphate (cAMP) response element (CRE)-binding protein is a key regulator of a variety of different gene networks in neurons. CREB homo- and heterodimers bind to the full CRE consensus sequence (TGACGTCA) with high affinity and to half CRE sites (TGACG/CGTCA) with lower affinity.[79] CpG methylation of the CRE sites disrupt CREB binding to its CRE site.[80] CREB is an intrinsically disordered protein that contains an N-terminal glutamine rich domain (Q1, residues 1-86), a short α peptide (alternatively spliced in α/δ isoforms, 87-101), a kinase-inducible domain (KID, residues 102-165), another glutamine rich domain (Q2, residues 166-282), and a C-terminal DNA-binding and dimerization domain (basic region/leucine zipper domain (bZIP), residues 283-341) (Figure 4.2).[81-84] While full length CREB is intrinsically disordered, researchers have obtained a crystal structure of the bZIP domain in complex with DNA and an NMR structure of the KID domain interacting with a coactivator, CBP (CREB binding protein).[81,85] A canonical nuclear localization sequence (NLS) resides in the bZIP domain.[86] The Q2 domain constitutively

interacts with the TATA-binding protein-associated factor, $TAF_{II}130$, and is required for transcriptional activation of CREB.[87,88] Indeed, certain isoforms of the CREB homologue, cAMP response element modulator (CREM), lacks the Q2 domain and therefore acts as a transcriptional repressor at CRE sites.[87] In addition to this basic structure of CREB, there are several different isoforms expressed including α (341 residues.), β (287 residues), and δ (327 residues, lacks the α peptide) isoforms.[89]

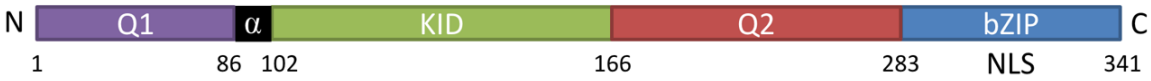

Figure 4.2 Structure of CREB. CREB contains 4 major domains: an N-terminal glutamine-rich Q1 domain (residues 1-86), kinase-inducible domain (KID) (residues 102-165), another glutamine-rich Q2 domain (residues 166-282), and a C-terminal bZIP domain (residues 283-341). The nuclear localization sequence (NLS) resides, DNA-binding, and dimerization capacity resides in the bZIP domain.

CREB has been shown to be important in memory formation from aplysia to drosophila to humans.[90] Nobel laureate Eric Kandel was the first to show that cAMP plays a role in learning and memory in aplysia.[91] Eventually, cAMP was shown to activate of protein kinase A (PKA), which in turn phosphorylated and activated the TF CREB at serine 133.[92,93] Researchers later discovered that many other stimuli and signaling pathways converge to activate and phosphorylate CREB at serine 133 including growth factors, neuronal membrane depolarization, calcium signaling, hormones, and stress (Figure 4.3).[94] Upon activation, CREB mediates the protein-synthesis independent transcription of immediate early genes (IEGs) such as *Fos* (c-Fos), *Jun* (c-Jun), and early growth response gene 1 (*Egr1*) within minutes of activation.[84] These IEGs are themselves TFs that lead to a downstream cascade of transcriptional changes that are (1) important for learning and memory and (2) last for hours after CREB's initial activation.[94] In addition to the cascade transcriptional changes promulgated by IEG expression, CREB changes the expression of synaptic growth-related genes underlying intrinsic neuronal

excitability including brain-derived neurotrophic factor (*Bdnf*), glutamate ionotropic receptor AMPA type subunit 1 (*Gria1*), and neuronal nitric oxide synthase (*Nos1*).[79] The CREB-mediated expression of these neuronal growth, activity, and excitability genes strengthens both short term memory (STM) and long term memory (LTM) formation.[95]

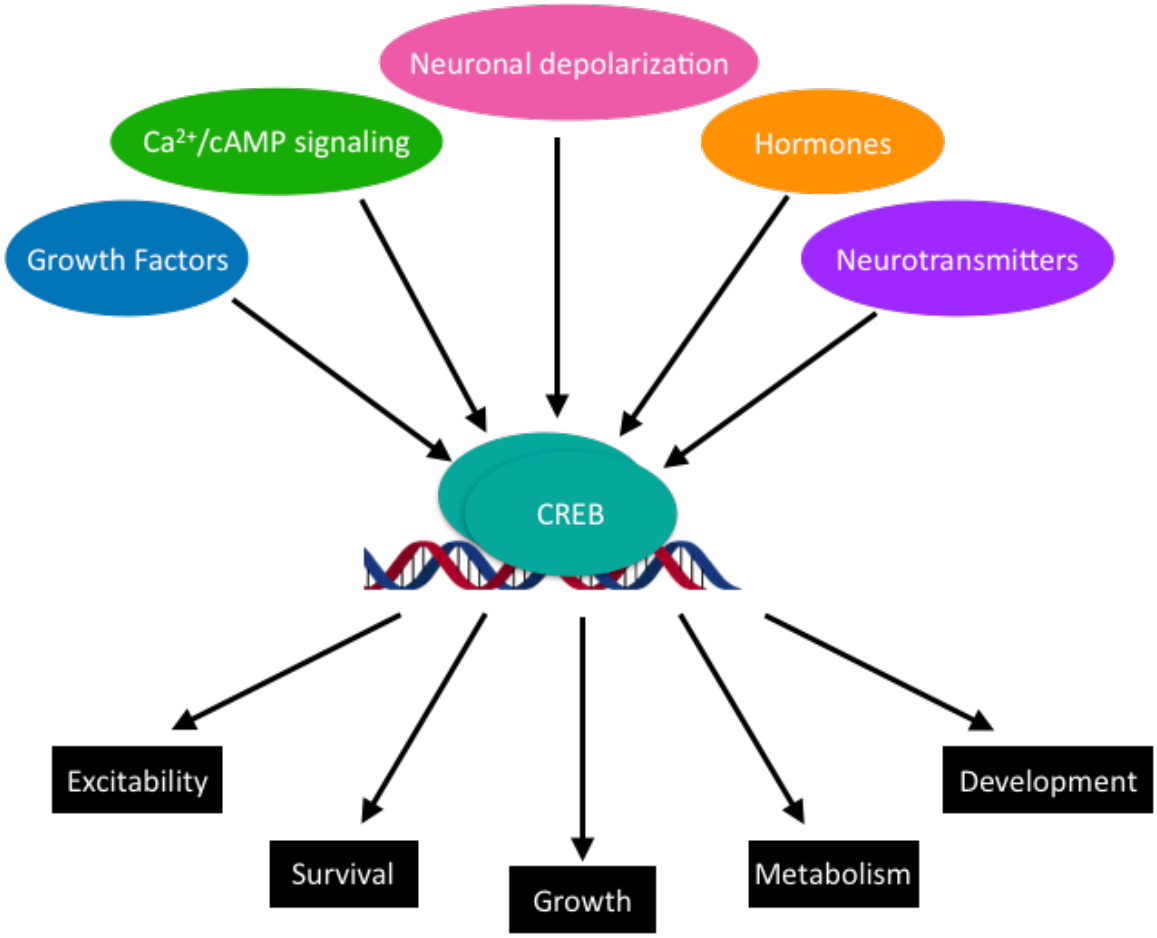

Figure 4.3 Overview of the stimuli that activate CREB and gene networks regulated by CREB activity. CREB is activated through phosphorylation at serine 133 and other mechanisms in response to growth factors, calcium, depolarization, cAMP, and neurotransmitters. CREB leads to the transcription of genes critical to neuronal excitability, survival, growth, metabolism, and development.

CREB's role in memory formation has been demonstrated repeatedly across species leading to its designation as "the memory gene".[94] Although CREB has been shown to enhance STM, CREB has mostly been shown to be important for LTM formation, a process that requires protein synthesis. Increasing expression of CREB can

lead to enhanced STM and LTM through its ability to increase neuronal excitability.[96] In neurons, either elevated CREB levels or higher intrinsic excitability preceding training leads to the preferential recruitment of these neurons into the memory trace and strengthening of the memory recall.[97-100] In accordance with these studies, the expression of the constitutively active CREB (caCREB) mutants, Y134F-CREB, DIEDML-CREB, and VP16-CREB, enhances LTM formation.[98,100-107] Reducing CREB activity through CREB knockout or the expression of a dominant-negative CREB mutant that lacks the DNA-binding domain (dnCREB, A-CREB, or K-CREB) results in severe LTM and synaptic plasticity impairments.[108-110] Integrating the results from experiments with higher and lower CREB activity lead to the proposal that there was a CREB dosage-dependent memory effect where higher CREB activity resulted in facilitated CREB activity.[111]

In addition to its role in learning and memory, CREB has been shown to have a key role in neuronal development, growth, and neurodegeneration. Günther Schütz's lab developed the first $CREB^{-/-}$ total knockout mouse, which could not be assessed for memory deficits due to its perinatal lethality resulting from a lack of sufficient pulmonary surfactant.[112] The $CREB^{-/-}$ mice were born at a reduced frequency (only 15% instead of the expected Mendelian 25% from breeding heterozygotes) and displayed reduced birth weight (70% of wildtype littermates) suggesting a developmental disadvantage.[112] Overexpressing S133A-CREB caused impaired synaptogenesis and neurogenesis demonstrating that CREB phosphorylation at serine 133 was important for neuronal and synaptic growth and development.[113] S133A-CREB mutant mice showed reduced allelic

frequencies (only 11% instead of the expected 25%) as in the CREB$^{-/-}$ mice, supporting a pivotal role of phosphorylation at serine 133 in early mouse development.[114]

Ginty and colleagues further explored the role of CREB in neuronal development by showing that CREB$^{-/-}$ mice displayed defects in axonal growth and increased neuronal apoptosis due to impairments in nerve growth factor (NGF) signaling.[115] Downstream of NGF and other growth factors, CREB regulates the expression of genes critical for neuronal growth and survival such as the anti-apoptotic protein, B-cell lymphoma 2 (*Bcl-2*), the Bcl-2 family member, *Mcl-1,* and neuronal growth factors such as insulin-like growth-factor 1 (IGF-1, *Igf1*), leptin (*Lep*), pituitary adenylate cyclase-activating peptide (PACAP, *Pacap*), *Bdnf*.[116-122] CREB also mediates the expression of the neuronal growth factor receptors including tyrosine receptor kinase B (TrkB, *Ntrk2*), the receptor for BDNF/NT-3 family of growth factors.[123] Through studies knocking out CREB, expressing dnCREB, or expressing an unphosphorylatable CREB mutant, S133A-CREB, researchers have observed widespread neuronal apoptosis through the inability to respond to and express pro-survival and growth neurotrophins including nerve growth factor (NGF), BDNF, IGF-1, and Bcl-2.[124,125] Increasing levels of wild-type (WT) CREB increases pro-survival genes (a.k.a. activity-regulated inhibitor of death (AID) genes) including *Atg3*, *Btg2, Gadd45β*, and *Gadd4γ* in mouse hippocampal neurons.[126] Through the mediation of the expression of these AID and pro-growth genes, CREB is able to direct neuronal development and protect neurons from apoptosis. Finally, CREB regulates a suite of metabolic genes in neurons including the gluconeogenic genes pyruvate carboxylase (*Pc*), phosphoenolpyruvate carboxykinase 1 (*Pepck1*), nuclear receptor subfamily 4 group A1 (*Nr4a1*), and glucose-6-phosphatase (*G6pc*).[79,127] CREB

is found within the mitochondria and mediates the major Complex I components NADH:ubiquinone oxidoreducatase subunits 2, 4, and 5 (*Nd2, Nd4, Nd5*).[128] Loss of CREB results in perturbations in the genes responsible for maintaining cholesterol homeostasis in response to neuronal activity.[129] While CREB has been shown to be important for neuronal development, survival, activity, and metabolism and mediating specific genes involved in these processes, the vast majority of CREB-mediated genes that are required for synaptic plasticity and memory formation are unknown.

Several studies have begun to delve into the global CREB-mediated gene networks underlying these processes. Genome-wide ChIP studies have shown that CREB binds to thousands of binding sites across the genome.[130-132] However, CREB-mediated transcription typically requires the recruitment of coactivators, which often means that only a small subset of genes bound by CREB are transcribed.[87] Also, despite similar genomic CREB occupancy across cell types, CREB-mediated, stimuli-specific gene expression is cell-type specific and restricted often to very few genes. For example, exposure to cAMP induces the expression of genes involved in cellular growth and survival in beta islet cells, fasting glucose and lipid metabolism in hepatocytes, and neuronal growth and activity in neurons.[131,133] A few early studies attempted to explore the transcriptome-wide, CREB-mediated memory and plasticity-associated genes and have identified a few genes including 4 differentially-expressed (DE) genes from mouse hippocampal overexpression of a constitutively active CREB and 41 DE genes from rat hippocampi following LTP induction or fear conditioning training.[102,134] A 2011 study showed that overexpressing the caCREB, VP16-CREB, for 3 or 6DIV resulted in 10% of the entire transcriptome differentially expressed in WT mouse hippocampal neurons.[133]

Another study identified 757 CREB-dependent, LTM-associated DE genes by comparing CREB knockout, CREB rescue, and CREB overexpression from WT with and without LTM training in *C. elegans*.[135] In this study, Murphy and colleagues showed that CREB basally mediated the transcription of genes involved in cellular growth, development, and metabolism without altering either longevity or memory genes. Upon LTM training, CREB altered expression of genes involved in neuronal activity, synaptic plasticity, calcium signaling, ion channels, and neurotransmitter signaling. Importantly, the genes that were mediated by CREB transcription under basal conditions were distinct and generally mutually exclusive from those regulated by LTM (only 12 genes were shared between the 463 LTM CREB-dependent genes and 281 basal CREB-dependent genes).[135] While this paper has provided great insight into CREB-mediated transcriptional networks, it remains to be determined whether or not these networks will be conserved in other species. Given the stark contrast in phenotypes in the CREB knockout between the mouse and worm (CREB KO in *C. elegans* results in no lifespan impairments while CREB KO in mice is perinatal lethal), the role of CREB will likely diverge in many ways the mouse and worm. These studies begin to probe the CREB-regulon and demonstrated that CREB regulates the expression of many different gene subsets, but much remains to be determined about the activity-dependent global gene networks regulated by CREB.

4.5 The CREB family of transcription factors: CREB, ATF1 and CREM

CREB cooperates with a suite of coactivators in order to regulate its transcriptional targets. We mentioned in the previous section that CREB can form heterodimers and bind to CRE sites with other TFs. These partners include two highly

homologous TFs, ATF1 or activating transcription factor 1 (65% homology) and CREM or cAMP response element modulator.[136] ATF1 and CREM share high sequence homology within the bZIP domain (91% for ATF1, 75% or 95% for CREM depending on alternative splicing) and therefore, are able to heterodimerize stably.[137,138] The affinity of dimers for the CRE site is highest for CREB/CREB homodimers followed by CREB/ATF1 heterodimers and then ATF1/ATF1 homodimers.[139,140] ATF1 acts as an activator while CREM, depending on its alternative splicing, can act as an activator or repressor.[141] In particular, CREM α, β, γ, and ε isoforms act as repressors since they lack the Q2 domain required for recruitment of $TAF_{II}130$ while CREMτ acts as an activator.[87,141,142] CREM activity both as a repressor and as an activator has been shown to be critical for neurogenesis, learning, and memory.[143,144] Both ATF1 and CREM activity is modulated by cAMP as they both have a PKA consensus site, but their ability to activate or repress transcription varies by cell type and expression levels.[141,145-147] Importantly, both CREM and ATF1 in concert with CREB has been shown to be critical in neuronal development since $CREB^{-/-}/ATF1^{-/-}$ and brain-specific $CREB^{-/-}/CREM^{-/-}$ double mutants were embryonic lethal.[148,149]

Despite ATF1, CREB, and CREM sharing considerable homology with other bZIP family members (such as the activator protein 1 (AP-1) family including c-Jun and c-Fos), ATF1, CREB, and CREM cannot heterodimerize with them, leading to a distinct classification of ATF1, CREB, and CREM into the "CREB family" of TFs.[137] The AP-1 family can heterodimerize with other AP-1 members, bind to the AP-1 site on DNA, and be activated through phosphorylation by c-Jun N-terminal kinase (JNK).[150] Unlike the rest of the CREB family of TFs, the homologous ATF2, ATF3, and ATF4 TFs can

heterodimerize with the AP-1 family, are activated by phosphorylation by JNK, but remain understudied, so it is still unclear if they act as activators or repressors. While little is known about the roles of ATF2-4, they have been shown to role similar to CREM, as a dominant negative regulator of CREB in the emotional response to stress and amphetamines.[151,152] In addition, ATF2-4 has been shown to heterodimerize with c-Jun and change the affinity of the heterodimer from AP-1 sites to CRE sites.[153] Furthermore, a more recent study demonstrated that ATF4 and c-Jun are important mediators of long-term facilitation, a process akin to LTP in aplysia.[154] We discussed earlier how CREB activity leads to the expression of the AP-1 family members, c-Jun and c-Fos, suggesting that extensive crosstalk occurs between the CREB and AP-1 TF families. In summary, much remains unknown about the individual contributions of CREB and its family members to learning and memory as well as any potential crosstalk within and without the CREB family.

4.6 CREB coactivators: CBP/p300 and CRTCs

Aside from the recruitment of RNA polymerase II, $TAF_{II}130$, important for basal transcription discussed in section 4.4, CREB interacts with and requires the activity of several key transcriptional coactivators in order to fully activate its gene networks. Phosphorylation of CREB at S133 strengthens the binding of the coactivators CBP and p300.[155] In addition, studies have shown that transcriptional activation is dictated by the interaction between the CREB KID and the KIX domain of CREB-binding protein.[156] The mechanisms by which CBP and p300 enzymatic activity enhances CREB-mediated transcription are two-fold: CBP and p300 acetylate (1) histones leading to increased chromatin accessibility, and (2) CREB directly at K91, K94, and K136, which increases

CREB trans-activation.[157,158] It is important to note that CBP and p300 also bind to a variety of other transcription factors and the pair can mediate distinct genes and cellular fates.[159] Finally, a ChIP-Seq experiment revealed that the genomic occupancy of CBP in neurons highly dependent on neuronal stimulation.[160] Indeed, both CBP and p300 have been shown to be important for LTM, and CBP has been shown to also be necessary for STM as well.[161-164] While there is some debate over whether or not CREB can recruit RNA polymerase II independent of phosphorylation and CBP association, some studies have demonstrated that CBP reinforces the interaction with RNA polymerase II through direct association with RNA helicase A.[165-168] The debate still continues over whether CBP/p300 is required for basal CREB transcription or solely for activity-induced CREB transcription.

CRTCs (cAMP-regulated transcriptional coactivator, formerly known as TORCs) are a family of coactivators that have proven to be critical regulators of CREB functions. CRTCs bind to the bZIP domain of CREB where arginine 314 has been shown to be critical for mediating that interaction.[169] Prior to stimulation, CRTCs is phosphorylated at S171, S275, and S307 by AMPK, which leads to its association with 14-3-3 proteins and cytoplasmic sequestration.[170] Upon cAMP and calcium stimulation, calcineurin dephosphorylates CRTCs, leading to their translocation to the nucleus where they can interact with CREB and mediate transcription.[171] The three members of the CRTC family, CRTC1, CRTC2, and CRTC3, all share an N-terminal CREB-binding domain (CBD), a central regulatory domain, an alternative splicing domain, and a transactivation domain.[79] All three CRTCs have been shown to mediate the CREB regulome through binding of the CRTC CBD and CREB bZIP domain to the CRE site (with a 2:2:1 of

stoichiometry), which facilitates the complex's association with $TAF_{II}130$.[169,172] The CRTCs have been shown to be nutrient sensors and master regulators of metabolic processes including gluconeogenesis, lipogenesis, mitochondrial activity, and stress.[173,174] In particular, CRTC1 was shown to reduce the expression of lipogenic genes including fatty acid synthase (*Fasn*) and nuclear sterol regulatory element binding protein 1c (*Srebp1c*).[173] CRTC2 has been shown to maintain glucose homeostasis through the facilitation of gluconeogenesis in response to fasting in the liver.[175] In addition, CRTCs has been shown to be an important regulator of lifespan and circadian rhythm.[176,177] The CRTC mediation of lifespan has been shown to be regulated through mitochondrial metabolism and catecholamine signaling.[178,179] Aside from the role of CRTCs as metabolic and lifespan regulators, CRTC1 and CRTC2 have been shown to be calcium and cAMP coincidence detectors, which, in the case of CRTC1, leads to the facilitation of synaptic plasticity and LTM.[171,180,181] Following learning, CRTC1 is translocated from synapses to the nucleus and modulates the strength of memory formation through regulation of CREB-mediated transcription of fibroblast growth factor 1b (*Fgf1b*), an important mediator of hippocampal-dependent associative memory.[182] Importantly, several studies have demonstrated that CRTC1 translocation is required for associative and context-dependent LTM.[182-184]

Due to the demonstrated roles of both CBP/p300 and CRTCs in memory formation, it has been proposed that the full activation of CREB for memory formation involves two phosphorylation-related events: (1) phosphorylation of CREB at serine 133 leading to the recruitment CBP/p300 to CRE sites and (2) dephosphorylation of the CRTCs, leading to their translocation into the nucleus where they can mediate

transcription.[79] While most studies have focused on either CBP/p300 or CRTCs, a few studies have begun to dissect the interplay and differential contribution of these two sets of coactivators on CREB transcription. One study found that the presence of CRTC2 could rescue CREB-mediated transcription of specific genes in CBP/p300 null cells.[185] A study performed in drosophila by the Saitoe lab showed that spaced training memory formation required the activity of CBP while fasting-dependent learning required CRTC2 activity suggesting that CBP and CRTC2 regulate distinct memory paradigms.[181] In a follow-up to this study, the Saitoe lab later showed that LTM formation requires the shifting from CBP-dependent to CRTC-dependent CREB transcription.[186] Another study showed that associative learning was enhanced by the recruitment of both CBP and CRTC to CREB on CRE sites, but CRTC alone is recruited in a graded fashion proportional to the amount of training.[182] Altogether, these studies suggest that both CBP and CRTCs are important for memory formation, but further studies are necessary to tease apart the individual contributions of each, their potential interdependence, and the memory-related transcriptional networks mediated by both.[84]

4.7 The role of CREB and its coactivators in neurodegeneration

Consistent with CREB's critical role in neuronal activity and homeostasis, CREB and its coactivators have been shown to be deregulated in neurodegenerative diseases.[187] As mentioned in section 4.4.1, knocking out CREB (and CREM) or expressing a dominant negative form of CREB in the brain leads to apoptosis and neurodegeneration.[149,188] In AD mouse models and post-mortem human AD brains, lower *Creb1* and *Bdnf* expression and/or CREB and BDNF protein levels were observed.[189] Furthermore, treatment of rat hippocampal neurons with Aβ peptides decreased CREB

activity.[189] On the other hand, increasing CREB activity in CA1 region rescues spatial memory deficits due to AD and normal aging as well.[190,191] While expression of slightly more active CREB (Y134F/DIEDML-CREB) leads to enhanced LTM and neuronal growth, elevated CREB activity through the overexpression of the constitutively active VP16-CREB can lead to excitotoxicity-induced neurodegeneration.[102,106,192] In addition, higher CREB transcription was associated with Huntington's disease (HD) in a mouse model although CREB has also been shown to play a neuroprotective role in mHtt-expressing mice.[193,194] Contrary to this study, other studies have suggested that elevated CREB transcription is neuroprotective and diminished CREB activity is neurodegenerative in HD model mice.[195-197] Furthermore, CREB activity has been shown to be impaired in several different human diseases including Coffin-Lowry syndrome and Rubenstein-Taybi syndrome (RSTS).[137] Altogether, these studies show that fine tuning CREB activity is necessary for maintaining neuronal homeostasis and preventing neurodegeneration (Figure 4.4).

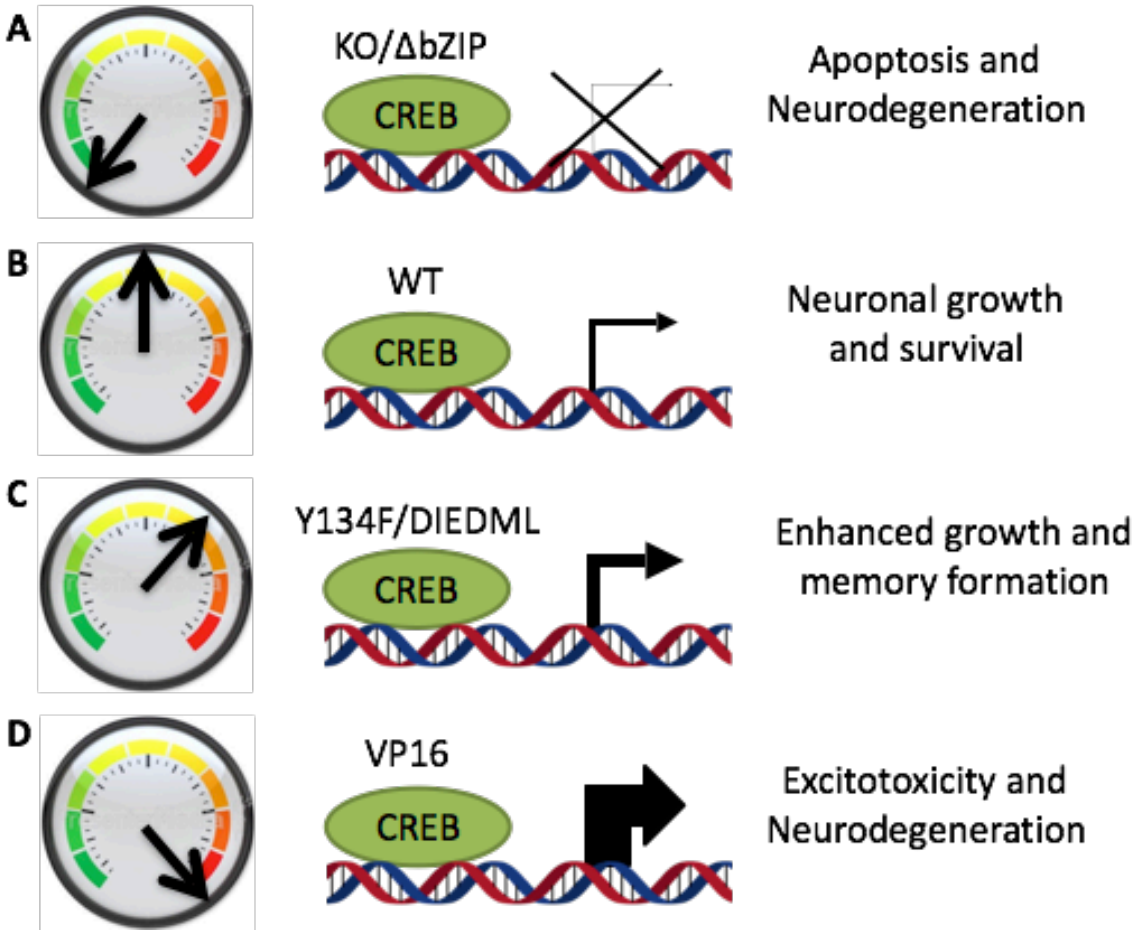

Figure 4.4 Fine tuning CREB activity is critical for neuronal growth and survival. Shown here is a summary of the different CREB mutants and their relative activity levels. (A) The lowest activity of CREB occurs in CREB knockout or the expression of the dominant negative mutants that lack the DNA-binding domain (dnCREB→ K-CREB or A-CREB) and leads to apoptosis-induced neurodegeneration. (B) WT CREB is able to regulate the transcription of neuronal growth and survival genes leading to thriving neurons. (C) The expression of the slightly more active Y134F/DIEDML mutants yields enhanced growth and memory formation. (D) Ramping up CREB activity through expression of the constitutively-active VP16-CREB mutant results in excitotoxicity-induced neurodegeneration.

In addition to CREB itself, CREB's coactivators CBP and CRTC have been linked to neurodegeneration. Loss of CBP or p300 has been shown to underlie Rubenstein-Taybi Syndrome (RSTS), a debilitating disease characterized by physical abnormalities and mental retardation due to histone acetylation and CREB transcriptional perturbations.[198-201] Supportive of a neuroprotective role for CBP, reduced CBP activity has been associated with HD while increased CBP function ameliorates learning and

memory deficits in a mouse model of AD.[202-204] CRTC1 has been shown to have a critical role in AD Aβ peptides disrupted calcium signaling leading to decreased dephosphorylation of CRTC1 leading to impaired CRTC- and CREB-mediated transcription in AD mouse models.[205] Saura and colleagues revealed that CRTC1-dependent transcription is impaired in AD mouse models during the early stages of AD mouse model cognitive decline, which is observed in the human hippocampus along with lower CRTC1 protein levels.[206] In two different AD mouse models (*PS* cDKO and $APP_{Swe,Ind}$), the authors showed a reversal in memory deficits upon overexpression of CRTC1 due to the recovery of CRTC1-mediated transcription.[183,206] In summary, CREB and its coactivators have proven to be critical mediators of neurodegenerative diseases underscoring the importance of precise regulation of CREB activity.

4.8 The CREB PTM code

The correct activation of CREB is critical for proper neuronal function: too little CREB activity either by expressing dominant negative CREB or knocking out CREB leads to apoptosis and neurodegeneration through a dearth of CREB-mediated pro-survival and growth genes; too much CREB activity from overexpression of the constitutively active VP16-CREB results in excitotoxicity and neurodegeneration through heightened expression of neuronal activity and excitation genes. Thus, it is critical to fine tune the activity of CREB in order to ensure proper neuronal activity and prevent neurodegeneration.

In order to modulate its activation, CREB is regulated by a variety of post-translational modifications (PTMs) including phosphorylation, acetylation, sumoylation, ubiquitylation, and glycosylation.[79] Many studies have focused on CREB's major

activity-induced phosphorylation site, S133, which resides in the kinase-inducible domain (KID). In neurons, this phosphorylation event occurs downstream of signaling in response to cAMP, calcium, hormones, growth factors, membrane depolarization, and stress.[94] Phosphorylation at S133 by protein kinase A (PKA), mitogen- and stress-activated kinase 1/2 (MSK1/2), ribosomal S6 kinase (RSK), protein kinase B (AKT), MAP kinase-activated protein kinase 2 (MAPKAP K2), and Ca^{2+}-calmodulin kinase IV (CaMKIV) recruits the coactivators, CBP/p300, which then acetylate CREB at K91, K94, and K136 and increase CREB trans-activation.[157] Several studies have shown that dephosphorylation by protein phosphatases 1 and 2A (PP1, PP2A) at S133 decreases CREB transcriptional activity.[207,208] Furthermore, overexpression of S133A-CREB results in neurodegeneration, some LTP deficits, and impaired LTM.[95,209-211] In stark contrast to these findings using S133A overexpression, S133A-CREB knock-in results in normal LTP in the hippocampus and amygdala and LTM, suggesting that phosphorylation at serine 133 may not be critical for learning and memory.[114,212] While S133A-CREB knock-in did not appear to affect learning and memory, Blendy and co-workers suggested a role of S133A-CREB in development.[114]

Despite many studies showing the importance of serine 133 phosphorylation, its transcriptional consequences are still poorly understood.[94] Several genes have been shown to be mediated by CREB phosphorylation, but CREB phosphorylation itself and the genes it regulates are stimuli- and cell type-specific.[131,213] Robust CREB phosphorylation occurs in pancreatic β cells upon exposure to cAMP, but not upon depolarization.[213] Furthermore, elevation of cAMP leads to increased expression of (1) anti-apoptotic and pro-growth genes in islet cells, (2) glucose and lipid metabolism genes

in hepatocytes, and (3) regulation of cell death and transcription factor genes in HEK293T cells.[131] In hippocampal neurons, tetracycline-inducible knock-in of S133A-CREB leads to a decrease in the levels of glutamate receptors (specifically AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid) and NMDA (*N*-Methyl-D-aspartic acid) subunits), which shifts synaptic plasticity from LTP to LTD.[95,209] In other cell types, heme oxygenase-1 (*Hmox1*), amphiregulin (*Areg*), interleukin-6 (*Il6*), and nuclear receptor subfamily 4 group A members 1 and 3 (*Nr4a1, Nr4a3*) were downregulated when S133A-CREB was expressed while *Crem* and cyclin A2 (*Ccna2*) were upregulated.[214-216]

In brain microarray and RNA-Seq studies, the transcriptional effects of S133A-CREB have been further expanded. Silva and colleagues reported only a single gene, 14-3-3ζ (*Ywhah*), that is downregulated upon overexpression of S133A-CREB following fear conditioning.[211] In the nucleus accumbens, an area important for addiction, the overexpression of S133A-CREB for 8 weeks resulted in upregulation of 4 genes and downregulation of 20 genes when compared to control WT mice without CREB overexpression.[217] The genes downregulated upon S133A-CREB overexpression included genes important for neuronal growth and development including *Bdnf*, T-box brain protein 1 (*Tbox1*), and cholecystokinin (*Cck*) in addition to genes for synaptic proteins such as synaptophysin (*Syp*) and synaptotagmin XVII (*Syt17*).[217] Antithetically, a more recent RNA-Seq study found that no genes were differentially expressed in a S133A-CREB knock-in mouse.[114] By integrating the results from the behavioral and transcriptional studies, the effects of ablating CREB phosphorylation at S133 appear to differ depending on the S133A-CREB expression method *videlicet* S133A

overexpression shows behavioral and transcriptional alterations while S133A knock-in expressed at endogenous CREB levels appears to have little or no memory or gene expression consequences. These studies reveal two major findings: (1) the level of CREB dictates CREB-mediated transcription and (2) the exact transcriptional effects of CREB phosphorylation at serine 133 in the KID remain unclear.

Also within the CREB KID, ataxia telangiectasia-mutated (ATM) kinase phosphorylates S111 followed by casein kinases 2 (CK2) phosphorylation at S108 and then by casein kinases 2 (CK1) phosphorylation at S114 and S117 in response to genotoxic stress.[218,219] Only after the sequential phosphorylation of all of these sites can ATM phosphorylate S121, which only occurs upon significant DNA damage.[219] CREB phosphorylation at S108, S111, S114, S117, and S121 (called the "ATM/CK cluster") has been shown to regulate the transcription of genes involved in metabolic homeostasis in response to cAMP and genotoxic damage (Figure 4.5).[220] In addition, phosphorylation at serine 142 by CaMKIIα or CK2 in response to Ca^{2+} signaling plays a critical role in circadian entrainment and inflammatory nociception.[221-223] Phosphorylation of the ATM/CK cluster or S142 inhibit the interaction with CBP/p300 and decrease CREB-mediated transcription.[219,220,224] Interestingly, phosphorylation of S142 *and* S143 in addition to S133 leads to full activation of Ca^{2+}- and CREB-mediated transcription in neurons despite disrupting the interaction with CBP.[221] Likewise, phosphorylation of S108, S111, and S114 in addition to S133 is required for full activation of cAMP- and CREB-mediated transcription in MEFs by mediating CREB-DNA binding, which in turn mediates its association with the co-activator CRTC2.[220] Furthermore, phosphorylation of CREB at S270 and S271 by cyclin-dependent kinase 1 (CDK1) in response to genotoxic

stress minimizes the binding of CREB to DNA thereby disrupting with downstream CREB-mediated transcription.[224] Aside from CREB phosphorylation, exposure to TNFα leads to (1) ubiquitylation and subsequent proteosomal degradation of CREB and (2) K285 and K304 SUMOylation of CREB, which spares CREB from proteolysis.[225] Altogether, these PTMs, both individually and combinatorially, determine CREB activity in response to a variety of stimuli.

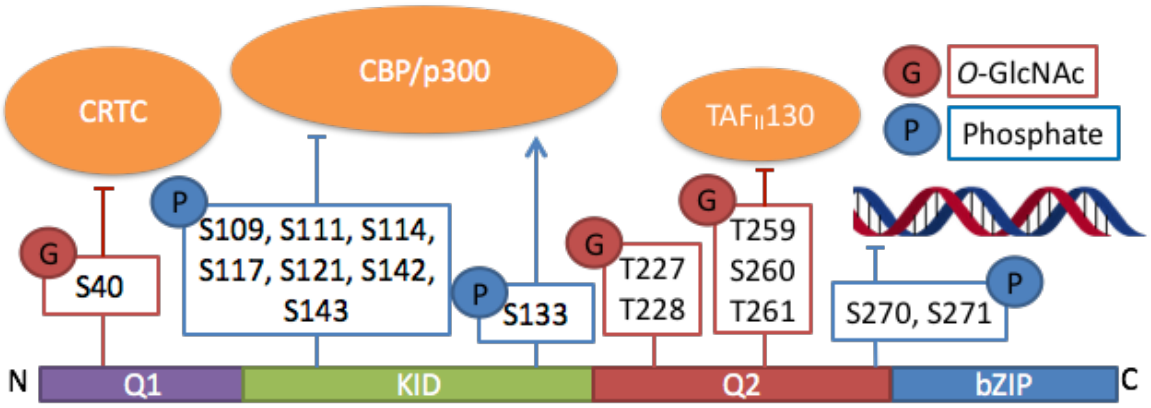

Figure 4.5 Overview of CREB phosphorylation and *O*-GlcNAc glycosylation sites. Shown here are all of the known CREB *O*-GlcNAc glycosylation and phosphorylation sites. In the *N*-terminal Q1 domain, CREB is glycosylated at serine 40, which disrupts the interaction with CRTC. Within the KID domain, phosphorylation at S133 enhances the binding of CBP/p300 to CREB while phosphorylation at S109, S111, S114, S117, S121, S142, and S142 interfere with this interaction. Glycosylation at T259, S260, T261 leads to impaired recruitment of the basal transcription factor, $TAF_{II}130$, to the Q2 domain of CREB. Finally, phosphorylation at S270 and S271 perturbs CREB binding to DNA.

Through mass spectrometric analysis, our lab identified several potential *O*-GlcNAc glycosylation sites where CREB including S40, T227/T228, and T259/S260/T261 (Figure 4.5).[226] Our lab went on to show the glycosylation of CREB at T259-T261 in the Q2 domain interferes with the association of CREB with $TAF_{II}130$.[227] The major glycosylation site on CREB, S40, is glycosylated in response to neuronal depolarization and occludes CREB's interaction with CRTC1 and CRTC2 thereby decreasing CREB-dependent transcription of certain key neuronal growth and activity genes including *Bdnf*, *c-fos, Wnt2,* and *Arc*.[226] Using an electrophoretic mobility shift

assay (EMSA), our lab showed that these transcriptional changes are not due to differences in the DNA binding ability of S40 glycosylated CREB. Interestingly, CREB glycosylation at S40 occurs preferentially on CREB phosphorylated at S133 suggesting interplay between CREB glycosylation and phosphorylation. Moreover, ablation of glycosylation in a S40A-CREB mutant leads to enhanced neuronal outgrowth and accelerated memory formation when compared to wild-type (WT) CREB in a fear conditioning paradigm.[226] In summary, glycosylation at S40 was shown to act as a brake to repress the expression of specific CREB-mediated genes involved in neuronal growth and activity. Therefore, S133 phosphorylation and S40 *O*-GlcNAcylation have been shown to have opposing effects on CREB transcription. However, the exact contribution of each of these PTMs to (1) overall CREB activity and (2) the CREB-mediated transcriptional networks globally have not yet been studied.

4.9 How are CREB phosphorylation and glycosylation integrated in order to confer biological outcomes?

Given the gaps in our understanding of the PTM code for CREB, several outstanding questions remain unanswered. While much is known about the modifications that adorn CREB, we still do not know how to decipher the complex PTM code that regulates CREB. Specifically, does phosphorylation at S133 globally increase and glycosylation at S40 globally decrease CREB transcription across all gene networks or are there specific gene networks affected by CREB PTMs? In addition, is there substantial interplay or overlap between the gene networks regulated by phosphorylation or glycosylation of CREB? Furthermore, are multiple PTMs on CREB integrated in order to determine cellular fate as seen in the multiple genotoxic-induced and Ca^{2+}-signaling

phosphorylation events discussed earlier? If indeed PTMs are integrated, then studying individual PTMs in isolation may provide an incomplete picture of the effects of TF PTMs. In our next chapter, we will leverage transcriptome-wide studies and targeted CREB mutants to begin to answer some of these questions toward the ultimate goal of determining the PTM code for CREB.

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