For Sarah and the Poomer

The most beautiful thing we can experience is the mysterious. It is the source of all true and all sciences. He to whom this emotion is strange, who can no longer pause to wonder and stand rapt in awe, is as good as dead; his eyes are closed.

Albert Einstein

The Stranger: Take it easy, Dude.
The Dude: Yeah, well. The Dude abides.
The Stranger: The Dude abides. I don't know about you but I take comfort in that. It's good knowin' he's out there. The Dude. Takin' her easy for all us sinners.

"The Big Lebowski", Ethan Coen and Joel Coen

Acknowledgements

It is a cliché that scientific progress requires us to stand on the shoulders of giants, those who have come before us; however, reflecting on the path that lead me to Caltech, there are a number of people, from elementary school through college, who are responsible for my development in science and as a person. There are too many to acknowledge here; I hope it that it is enough to acknowledge that the completion of my doctorate is the culmination of the guidance given and friendships made in the communities from Concord, NH, Northfield, MN and Pasadena, CA.

I would like to thank all those who helped me through my time at Caltech. In particular my advisor Linda Hsieh-Wilson and my committee, Dennis Dougherty, Jackie Barton and Carl Parker, and the Hsieh-Wilson lab, all of whom helped me develop as a scientist. My parents, Lynda and David, my brother Jesse, my wife Sarah and our new son Cormac have taught me that true happiness can not be found in the lab, but with family and the smiles and joy that we bring to one another. Especially Sarah, who I rely on for companionship and as my intellectual equal who I can count on to challenge and encourage me as our scientific careers mature.

Abstract

The survival and development of organisms requires the ability of cells to communicate with the environment and with surrounding cells. This demand has led to the evolution of a number of methods used for communication. Chief among these is the ability to modify protein function with post-translational modifications (PTMs). PTMs allow cells to use a single protein for a variety of tasks and link protein activity with a specific environmental or cellular cue. Modification of transcription factors has arisen as a key model for the study of PTMs and their effects on cell processes. PTMs modulate transcriptional activity required for key processes such as development, differentiation and cell survival.

The eukaryotic transcription factor cAMP-response element binding protein (CREB) is a transcription factor that confers dynamic control of a number of cellular processes including neuronal and pancreatic cell survival, gluconeogenesis and neuronal long-term potentiation. CREB is activated by phosphorylation of single serine residue. The observation that a number of kinase signaling cascades converge on CREB has led to the question of how cells deal with the apparent loss of signal identity that occurs as a result of this convergence. In this thesis I describe the identification, characterization and functional analysis of a novel PTM of CREB, *O*-GlcNAc glycosylation, that provides an additional level of control of CREB activity. CREB glycosylation moderates phosphorylation-dependent CREB activity and reduces CREB-dependent gene expression in pancreatic β-cells, and as a result promotes β-cell death, as observed in type II diabetes. CREB glycosylation offers us an example of how cells use multiple PTMs to control protein function and how dysfunction in the regulation of these modifications may contribute to disease states.

Table of Contents

Acknowledgements		iv
Abstract		v
Table of Contents		vi-vii
List of Figures		viii-xiii
List of Schemes		xiv
List of Tables		xv
Chapter 1	Introduction: CREB-Mediated Regulation of Eukaryotic Gene Expression	1
Chapter 2	Introduction: *O*-GlcNAc Glycosylation	20
Chapter 3	Identification and Characterization of CREB *O*-GlcNAc Glycosylation	36
Chapter 4	CREB Glycosylation Represses Transcriptional Activity *In Vitro*	70
Chapter 5	CREB Glycosylation Moderates Phosphorylation-Dependent CREB Activity in Cultured Pancreatic Cell Lines	79
Chapter 6	CREB Glycosylation Potentiates Apoptosis in Pancreatic Cell Lines by Suppressing Gene Expression of Targets of CREB Regulation	98
Chapter 7	Work Towards Identifying Stimuli that Induce CREB Glycosylation	115
Appendix I Methods for Chapter 3	Methods for Identification of *O*-GlcNAc Glycosylated CREB from the Rat Brain.	139
Appendix II Methods for Chapter 3	Methods for Characterization of CREB *O*-GlcNAc Glycosylation by Mass Spectrometry	149
Appendix III Methods for Chapter 4	Methods for *In Vitro* Characterization of CREB *O*-GlcNAc Glycosylation.	175

Appendix IV Methods for Chapter 5	Methods for Characterization of CREB *O*-GlcNAc Glycosylation in Pancreatic Islets and Analysis of CREB Activity in Cultured β-cell Lines.	181
Appendix V Methods for Chapter 6	Methods for the Investigation of the Effect of Glycosylation on CREB-Dependent Gene Expression and β-cell Apoptosis.	192
Appendix VI Methods for Chapter 7	Methods for the Treatment of Male Sprague-Dawley Rats with Streptozotocin and Alloxan and Isolated Islet Drug Treatment.	204

List of Figures

Chapter 1

Figure 1-1.	Modes of Transactivation	2
Figure 1-2.	cAMP-Response Element Binding Protein (CREB) Domains and Functionality	3
Figure 1-3.	The CBP:phosphoSer133-CREB Paradigm of CREB Transactivation	9
Figure 1-4.	Cell-Specific Regulation of CREB Transactivation	11

Chapter 2

Figure 2-1.	*O*-GlcNAc Glycosylation	20
Figure 2-2.	*O*-GlcNAc Transferase Domains and Functionality	21
Figure 2-3.	Regulation of OGT and *O*-GlcNAcase	23
Figure 2-4.	*O*-GlcNAcase Domains and Functionality Possible	25

Chapter 3

Figure 3-1.	CREB From the Rat Forebrain Can Be Separated into Distinct Isoforms by Two-Dimensional Electrophoresis	37
Figure 3-2.	CREB Is *O*-GlcNAc Glycosylated in the Rat Forebrain.	39
Figure 3-3.	OGT Glycosylates CREB *In Vitro*	40
Figure 3-4.	Insect Cell Expression Systems Will *O*-GlcNAc Glycosylate Recombinant Proteins, and Co-Expression of OGT with Target Protein Increases the Stoichiometry of Glycosylation.	41

Figure 3-5.	*In Silico* Digests of CREB	45
Figure 3-6.	Tryptic Digest and MALDI-ToF Analysis, 1000-4000 *m/z* Peptides	46
Figure 3-7.	Tryptic Digest and MALDI-ToF Analysis, 8000+ *m/z* Peptides	47
Figure 3-8.	Chymotryptic Digest and MALDI-ToF Analysis	51
Figure 3-9.	Glu-C Digest and MALDI-ToF Analysis	53
Figure 3-10.	Cyanogen Bromide Digest and MALDI-ToF Analysis	54
Figure 3-11.	Summary of CREB Identified Sites of *O*-GlcNAc Glycosylation	55
Figure 3-12.	In-Line Reverse Phase Liquid Chromatography and Tandem-Mass Spectrometry Analysis of Tryptic CREB Digest	57
Figure 3-13.	In-Line Reverse Phase Liquid Chromatography and Tandem-Mass Spectrometry Analysis of Chymotryptic CREB Digest	58
Figure 3-14.	Elucidation of Q2 Domain Glycosylation Via β-Elimination and Modification of Ser/Thr Modified Peptides	62
Figure 3-15.	Alanine Mutation of Sites of CREB Glycosylation Significantly Reduces CREB Glycosylation *In Vitro*	64
Figure 3-16.	In-Line Reverse Phase Liquid Chromatography and Tandem-Mass Spectrometry Analysis of CREB Enriched From the Rat Forebrain	66

Chapter 4

Figure 4-1.	Use of Recombinant CREB From Insect Cell Expression Systems to Produce Varied Levels of CREB Glycosylation	71
Figure 4-2.	*O*-GlcNAc Glycosylation Does Not Affect CREB:DNA Binding *In Vitro*.	73

Figure 4-3. *O*-GlcNAc Glycosylation Reduces the CREB:hTAF$_{II}$130 Interaction *In Vitro*. 74

Figure 4-4. *O*-GlcNAc Glycosylation Reduces CREB-Dependent Transcription *In Vitro*. 76

Figure 4-5. *In Vitro* CREB Glycosylation Suppresses Transcription by Disrupting CREB Recruitment of the RNA Polymerase II Initiation Complex. 76

Chapter 5

Figure 5-1. Identification of CREB *O*-GlcNAc Glycosylation in Pancreatic Islets 81

Figure 5-2. Stoichiometry of CREB *O*-GlcNAc Glycosylation in HIT-T15 Cells 82

Figure 5-3. Elevation of Cellular *O*-GlcNAc Glycosylation and CREB Glycosylation by Glucosamine Treatment 83

Figure 5-4. Glucosamine Treatment Increases Cellular O-GlcNAc Glycosylation, but Has No Effect on the Levels of CREB Phosphorylation. 85

Figure 5-5. CREB Glycosylation and Phosphorylation Are Not Mutually Exclusive. 86

Figure 5-6. PUGNAc Treatment Blocks *O*-GlcNAcase Activity and Increases CREB *O*-GlcNAc Glycosylation. 87

Figure 5-7. Glucosamine Treatment Reduces Forskolin-Induced CREB Activity. 88

Figure 5-8. Glucosamine Treatment Reduces CREB Activity and Not the Activity of the General RNA Polymerase II Complex. 89

Figure 5-9. PUGNAc Treatment Reduces CREB Activity. 90

Figure 5-10. Triple Alanine Gal4-CREB Chimeras Display a Gain-of-Function. 92

Figure 5-11. Transient Expression of AAA-Mutant CREB Reduces Glucosamine-Induced Inhibition of CREB Transactivation. 93

Figure 5-12. *O*-GlcNAc Glycosylation Allows an Additional Level of Control Over CREB Transactivation. 95

Chapter 6

Figure 6-1. Survival and Apoptotic Signaling in Pancreatic β-Cells: The Role for Insulin Receptor Substrates and CREB 99

Figure 6-2. IRS2 Expression Is Reduced by Glucosamine Treatment. 100

Figure 6-3. CREB-Dependent Gene Expression is Suppressed by Glucosamine in HIT-T15 Cells. 101

Figure 6-4. OGT Is Associated Strongly with CRE-Dependent Promoters and RNA Polymerase II-Dependent Genes. 104

Figure 6-5. *O*-GlcNAc Glycosylation of CREB Attenuates β-Cell Survival. 105

Figure 6-6. Silencing of OGT Expression Prevents Glucosamine-Induced Apoptosis in MIN6 Cells. 106

Figure 6-7. Blocking CREB Glycosylation Rescues β-Cells from GlcN-Induced Cell Death. 107

Figure 6-8. CREB Glycosylation and the Regulation of β-Cell Survival 109

Figure 6-9. Role of CREB Glycosylation in Cell Survival and Possible Alternative Functions 110

Chapter 7

Figure 7-1. Diabetes and β-Cell Apoptosis 117

Figure 7-2. Diabetes, β-Cell Dysfunction and *O*-GlcNAc Glycosylation 119

Figure 7-3. High Glucose Treatment Reduces CREB Activity in a Time-Dependent Manner. 120

Figure 7-4. High Glucose Treatment Increases Cellular *O*-GlcNAc Glycosylation in HIT-T15, MIN6 and Pancreatic Islets in Culture. 122

Figure 7-5.	No Change in CREB Glycosylation Was Observed in Response to High Glucose Treatment.	123
Figure 7-6.	Animal Models of Type II Diabetes: Alloxan Treatment, Streptozotocin Treatment, Zucker Fatty Rats and Goto-Kakizaki Rats	124
Figure 7-7.	Alloxan Treatment of Rats Causes Ablation of Endocrine Cells in the Pancreas.	125
Figure 7-8.	Streptozotocin Treatments Leads to Increase in *O*-GlcNAc Glycosylation Concomitant With Pancreatic Islet Loss, But No Observed Change in CREB Glycosylation.	127
Figure 7-9.	O-GlcNAc Glycosylation Is Enhanced in Hyperglycemic Zucker Fatty Rats Without an Observable Increase in CREB Glycosylation.	128
Figure 7-10.	Effect of Cytokine and Growth Factors on IRS2 Expression	130

Appendix I

Appendix Figure 1.	Ni-NTA, Hisx6-tag Purification of CREB and OGT	145

Appendix II

Appendix Figure 2.	MALDI Mass Spectrometry Spectra of Tryptic CREB Digest	153
Appendix Figure 3.	In-Line Reverse Phase Liquid Chromatography and Tandem-Mass Spectrometry Analysis	156
Appendix Figure 4.	In-Line Reverse Phase Liquid Chromatography and Tandem-Mass Spectrometry Analysis of Chymotryptic CREB Digest	158-159
Appendix Figure 5.	In-Line Reverse Phase Liquid Chromatography and Tandem-Mass Spectrometry and Single Reaction Monitoring Analysis of OGT Labeling of CREB	161

Appendix Figure 6.	In-Line Reverse Phase Liquid Chromatography and Tandem-Mass Spectrometry Analysis of Glu-C CREB Digest	163-165
Appendix Figure 7.	In-Line Reverse Phase Liquid Chromatography and Tandem-Mass Spectrometry Analysis of Trypsin and 2-AET Treatment of Recombinant CREB	168-171
Appendix Figure 8.	MALDI-ToF MS Analysis of Sequential Trypsin, 2-AET, and Trypsin Treatment of Recombinant CREB	172
Appendix Figure 9.	In-Line Reverse Phase Liquid Chromatography and Tandem-Mass Spectrometry Analysis of Native CREB Purified from Rat Brain	173

Appendix III

Appendix Figure 10.	CREB:hTAF$_{II}$130 Binding Assay	178

List of Schemes

Chapter 2

Scheme 2-1. The Hexosamine Biosynthesis Pathway 24

Chapter 3

Scheme 3-1. Enzymatic Labeling of *O*-GlcNAc Modified Proteins Using β-1,4-Galactosyltransferase (GalT) and Peptido-*N*-Glycosidase F (PNGase F) Treatment 38

Scheme 3-2. Proteolytic Mapping of O-GlcNAc Glycosylation 43

Scheme 3-3. β-Elimination and Modification of O-GlcNAc Glycosylated Proteins 60

Chapter 5

Scheme 5. Method of Ketone Labeling for the Measurement of CREB O-GlcNAc Stoichiometry and Relative Changes in CREB Glycosylation 80

List of Tables

Chapter 1

Table 1-1.	CREB phosphoSer133 Kinases	7
Table 1-2.	CREB-Interacting Proteins	10
Table 1-3.	Post-Translational Modifications of CREB	12

Chapter 2

Table 2-1.	*O*-GlcNAc Glycosylated Proteins	29

Chapter 3

Table 3-1.	Tryptic Digest Map of CREB	49
Table 3-2.	Chymotryptic Digest Map of CREB	52

Appendix II

Appendix Table 1.	Summary of Chymotryptic Peptide (QIRTAPTSTIAPGVVM) In-Line Reverse Phase Liquid Chromatography and Tandem-Mass Spectrometry Analysis	160