Nck1-dependent regulation of PERK activity impacts pancreatic β cell function and resilience

By

Lama Yamani

Division of Experimental Medicine
Department of Medicine
University of McGill
Montreal, Quebec, Canada
September 2015

A thesis submitted to McGill University in partial fulfillment of the requirements for the degree of Doctor in Philosophy.

ABSTRACT

PKR-like endoplasmic reticulum (ER) kinase (PERK), an ER transmembrane Ser/Thr protein kinase, is part of the unfolded protein response (UPR) and a key regulator of pancreatic β cell function and homeostasis. PERK phosphorylates the eukaryotic initiation factor 2 (eIF2) on its α subunit at Ser^{51} leading to attenuation of translation in stress conditions. Previously, we showed that the adaptor protein Nck1 modulates translation by directly interacting with the β subunit of eIF2 and assembling a phosphatase complex that dephosphorylates eIF2αSer^{51}. In this thesis, we unveil the role of Nck1 in modulating PERK in pancreatic β cells. Using pancreatic mouse insulinoma MIN6 β cells stably expressing an Nck1-specific shRNA, we show that silencing Nck1 enhances PERK basal activity and signaling. We also show that Nck1, through its SH2 domain, directly binds PERK phosphorylated on Tyr^{561} (pY^{561}) and thereby, limits PERK activity. In agreement with this concept, the activity of PERK Y561F, which escapes phosphorylation and Nck1 binding, was increased compared to wild-type PERK. Interestingly, Nck1 deficiency in MIN6 cells enhances insulin content in a PERK-dependent manner, by increasing insulin biosynthesis. In addition, Nck1-silenced MIN6 cells survive better to stress relevant to diabetes. Therefore, we hypothesized that PERK basal activity plays a role in determining β cell sensitivity to stress. Mechanistically, we provide evidence that silencing Nck1 in MIN6 cells enhances autophagy associated with PERK-induced sestrin2 and pAMPK signaling. Furthermore, we demonstrated that the PERK-Nrf2-dependent antioxidant response and the prosurvival Akt were constitutively activated in these cells. In conclusion, silencing Nck1 in pancreatic β cells enhances PERK basal activity, which in turn initiates signaling processes promoting a cellular adaptive response improving β cell function and resilience. Overall, my thesis provides insights on signaling

pathways that enhance the robustness of pancreatic β cells, making them more efficient in producing insulin and more resistant to stresses related to diabetes.

RÉSUMÉ

PKR-like ER kinase (PERK), une protéine kinase de type sér/thr exprimée à la membrane du réticulum endoplasmique (RE), est une composante importante de la réponse au stress causé par l'accumulation de protéines mal conformées dans le RE, connue sous le nom de UPR (*unfolded protein response*). Plusieurs études ont démontré que PERK joue un rôle régulateur important dans la fonction et l'homéostasie des cellules β du pancréas. En condition de stress, PERK phosphoryle la sous-unité α du facteur d'initiation eIF2 sur la Ser^{51}, ce qui réprime la traduction. Précédemment, nous avons montré que la protéine adaptatrice Nck1 module la traduction en interagissant directement avec la sous-unité β de eIF2 et en favorisant la formation d'un complexe incluant la phosphatase PP1 qui déphosphoryle eIF2αSer^{51}. Dans cette thèse, nous dévoilons le rôle de Nck1 dans la modulation de l'activité de PERK dans les cellules β pancréatiques. En utilisant des cellules MIN6 dérivées d'un insulinome de souris, nous avons montré que l'expression stable d'un shRNA contre Nck1, qui diminue significativement l'expression de Nck1, augmente spontanément l'activité basale et la signalisation de PERK. Nous avons également découvert que Nck1 atténue l'activité de PERK en se liant directement par son domaine SH2 à la tyrosine Y^{561} phosphorylée de PERK. En accord avec ces résultats, nous avons montré qu'une mutation de PERK qui empêche sa phosphorylation sur Y^{561} (Y561F) et son interaction avec Nck1 augmente son activité. Par ailleurs, il est intéressant de noter que la diminution de l'expression de Nck1 dans les cellules MIN6 améliore leur contenu en insuline suivant l'augmentation de la biosynthèse d'insuline de manière PERK-dépendante. De plus, la déplétion de Nck1 permet aux cellules MIN6 de mieux survivre en condition de stress reliés au diabète. Par conséquent, nous avons émis l'hypothèse que l'activité basale de PERK joue un rôle important dans la survie des cellules β face à un stress. En

accord avec cette hypothèse, nous avons mis en évidence que la diminution des niveaux de Nck1 dans les cellules MIN6 augmente l'autophagie à travers l'induction de la protéine sestrin2 et l'activation de l'AMPK. De même, nous avons démontré que l'expression de gènes cytoprotecteurs contre le stress oxydatif par le facteur de transcription Nrf2 (dépendante de PERK) et la protéine kinase Akt sont activées de façon constitutive dans ces cellules. En conclusion, la diminution de Nck1 dans les cellules β pancréatiques augmente l'activité basale de PERK, qui à son tour déclenche des processus de signalisation aboutissant à une réponse adaptative cellulaire qui améliore la fonction et la survie des cellules β pancréatiques. Dans l'ensemble, ma thèse fournit de nouvelles informations sur les voies de signalisation qui contribuent à la robustesse des cellules β pancréatiques en améliorant la production d'insuline et la résistance aux stress associés au diabète.

PREFACE

This thesis was written in accordance with the guidelines for a manuscript-based thesis issued by the Faculty of Graduate and Post-Graduate (doctoral) studies of McGill University.

This thesis consists of a general introduction, one published article, a manuscript under revision, a general discussion, and a section describing contribution to original research.

Appendix 1 describes additional original research I produced, related to my project and potentially useful for future research.

CONTRIBUTIONS OF AUTHORS

Chapters 1 and 4 were entirely written by myself, and Dr. Louise Larose added editorial comments.

Chapter 2: The design of experiments, lab work performed, and data analysis was contributed by myself and Dr. Mathieu Latreille (as a graduate student) as follows: I produced data presented in figures 2.1, 2.5, 2.6, 2.7, and 2.8A-C, as well as supplementary figures S2.2A-B, S2.3 and S2.4, while Dr. Mathieu Latreille produced data in figures 2.2, 2.3 and 2.4, and 2.8D, as well as supplementary figures S2.1 and S2.2C. I wrote a draft for this manuscript and with Dr. Louise Larose produced a final version.

Chapter 3: The design of experiments, lab work performed and data analysis was all done by myself except for the data presented in the following figures: 3.4A, 3.5B and 3.7C, which were performed by Dr. Bing Li. The first draft was entirely written by myself and the final version was achieved in collaboration with Dr. Louise Larose's editorial comments.

Appendix 1: The experiments and data analysis were performed by myself.

OTHER PUBLICATIONS:

Latreille M, Laberge MK, Bourret G, **Yamani L**, Larose L. Deletion of Nck1 attenuates hepatic ER stress signaling, improves glucose tolerance and insulin signaling in liver of obese mice. 2010. *Am J Physiol Endocrinol Metab.* 2011 Mar; 300 (3): E423-34. Epub 2010 Jun 29.

Contribution: Performed experiments and analysis for figures 2 and 3B-C, as well as addressing all reviewer's comments and writing the final draft with Dr. Louise Larose.

TABLE OF CONTENTS

ABSTRACT ii
RÉSUMÉ iv
PREFACE vi
CONTRIBUTIONS OF AUTHORS vii
TABLE OF CONTENTS viii
ACKNOWLEDGMENTS xiii
LIST OF FIGURES xiv
LIST OF TABLES xv
LIST OF ABBREVIATIONS xvi
CHAPTER 1. General introduction and literature review 1
1.1 Regulation of glucose homeostasis: overview 2
1.2 The pancreas 3
1.3 Pancreatic β cell physiology 4
1.3.1 Insulin biosynthesis, maturation and trafficking 4
1.3.1.1 Insulin synthesis and folding 5
1.3.1.2 Insulin granule packaging and maturation 6
1.3.1.3 Insulin granule trafficking and secretion 7
1.3.2 Glucose-stimulated insulin secretion (GSIS) 11
1.3.3 Fatty acid-stimulated insulin secretion 12
1.3.4 Insulin signaling in insulin-responsive tissues 13
1.3.4.1 PI3K-Akt pathway 16
1.3.4.2 Ras-MAPK pathway 18
1.3.5 Insulin signaling in pancreatic β cells 19
1.4 Pancreatic β cell-associated diseases 20
1.4.1 Type 1 diabetes (T1D) 20
1.4.2 Type 2 diabetes (T2D) 22
1.4.3 Maturity-onset diabetes of the young (MODY) 24
1.4.4 Diabetes as an ER stress disease 25
1.5 The unfolded protein response (UPR) and its role in pancreatic β cells 27
1.5.1 ER stress and the UPR: Overview 27

1.5.2 IRE1 28
1.5.3 ATF6 32
1.5.4 PERK 33
1.5.4.1 Structural basis of PERK activation 34
1.5.4.2 PERK signaling: phosphorylation of eIF2α 35
1.5.4.2.1 ATF4 translational upregulation and signaling 37
1.5.4.3 PERK signaling: phosphorylation of Nrf2 39
1.5.4.4 PERK links between ER and oxidative stress 40
1.5.4.5 Role of PERK in pancreatic β cells 41
1.5.4.6 Role of eIF2α in pancreatic β cells 44
1.5.4.7 Role of Nrf2 in pancreatic β cells 45
1.5.5 PERK signaling in regulating cell fate 45
1.6 The adaptor proteins Nck 48
1.6.1 Nck domains and binding partners 50
1.6.2 Nck regulation 51
1.6.3 Nck physiological functions 53
1.6.3.1 Nck and embryonic development 54
1.6.3.2 Nck and actin cytoskeletal remodeling 54
1.6.3.2.1 Cell migration 55
1.6.3.2.2 Axonal guidance 56
1.6.3.2.3 Pathogen infection 58
1.6.3.2.4 Nephrin signal transduction in podocytes 59
1.6.3.2.5 T cell receptor and FasL signaling 61
1.6.3.3 Nck and cellular proliferation 62
1.6.3.4 Nck and translation 63
1.6.3.5 Nck and the UPR 65
1.6.3.6 Nck and insulin signaling 66
1.7 Figures for Chapter 1 68
1.8 Rationale and Objectives of the study 88
CHAPTER 2. Interaction of Nck1 and PERK phosphorylated at Y^{561} negatively modulates PERK activity and PERK regulation of pancreatic β-cell proinsulin content **89**
2.1 Preface 90

2.2 Abstract 91
2.3 Introduction 92
2.4 Materials and methods 94
2.4.1 Cells 94
2.4.2 Cell treatments 95
2.4.3 Antibodies 95
2.4.4 Constructs 96
2.4.5 In vitro binding assays 96
2.4.6 In vitro kinase assays 97
2.4.7 Phosphoamino acid analysis 97
2.4.8 shNck1 MIN6 cells 97
2.4.9 Isolated pancreatic islets 98
2.4.10 Insulin biosynthesis 98
2.4.11 Statistics 99
2.5 Results 99
2.5.1 Nck regulates PERK 99
2.5.2 Nck and PERK interact *in vitro* 100
2.5.3 Identification of pY^{561} in the PERK juxtamembrane domain as the Nck-binding site 101
2.5.4 Phosphorylation of PERK at Y^{561} modulates PERK activity 102
2.5.5 PERK is phosphorylated at Y^{561} in MIN6 cells 103
2.5.6 Nck interacts with pY^{561} PERK in MIN6 cells 105
2.5.7 Nck1 modulates PERK and proinsulin levels in pancreatic β cells 105
2.6 Discussion 107
2.6.1 Nck directly interacts with and negatively regulates PERK 108
2.6.2 Nck1 modulates proinsulin biosynthesis and content in pancreatic β cells by regulating PERK 112
2.7 Acknowledgements 112
2.8 Figures for Chapter 2 113
CHAPTER 3. Nck1 deficiency improves pancreatic β cell survival to diabetes-relevant stresses by modulating PERK activation and signaling 131
3.1 Preface 132
3.2 Abstract 133

3.3 Introduction 134
3.4 Material and Methods 137
3.4.1 Cell Culture 137
3.4.2 Transmission Electron Microscopy 137
3.4.3 Cell Treatments 138
3.4.4 MTT viability assay 139
3.4.5 Western blot analysis and antibodies 139
3.4.6 mRNA expression analysis by qRT-PCR 140
3.4.7 Confocal microscopy 140
3.4.8 Statistical analysis 141
3.5 Results 141
3.5.1 Increased number of insulin secretory granules in Nck1-silenced MIN6 cells 141
3.5.2 Nck1-silenced MIN6 cells are protected against diabetes-relevant stresses 142
3.5.3 Silencing Nck1 in MIN6 cells attenuates PA-induced PERK activation and signaling 144
3.5.4 Silencing Nck1 in MIN6 cells promotes ATF4 expression without significantly inducing CHOP expression 145
3.5.5 Depletion of Nck1 in pancreatic β cells enhances autophagy 146
3.5.6 Silencing Nck1 in MIN6 cells increases sestrin2-pAMPK-mTORC1 signaling and Akt activation 147
3.5.7 Silencing Nck1 in MIN6 cells increases Nrf2 nuclear localization and expression of antioxidant genes 148
3.6 Discussion 149
3.7 Acknowledgements 154
3.8 Figures for Chapter 3 155
CHAPTER 4. General discussion and future perspectives 172
4.1 Nck binds to PERK and regulates PERK activity 173
4.2 Other PERK binding proteins 175
4.3 Role of protein tyrosine phosphatases 177
4.4 Nck1 silencing and pancreatic β cell insulin levels 179
4.5 Nck1 silencing and pancreatic β cell survival 180
4.6 Possible role for Nck1 in other tissues 185
4.7 Future perspectives 185

CHAPTER 5. Contribution to original research 189
Summary and Conclusion 192
REFERENCES 194
Appendix 1 250

ACKNOWLEDGMENTS

First, I would like to thank my supervisor and role model, Dr. Louise Larose. You were a constant motivation and inspiration to me. Thank you for all your encouragement and for your efforts to teach and guide me throughout my Ph.D. studies. Thank you for the time you spend teaching me how to perform good research and for all the discussions and advice you were always ready to offer. Thank you for making my Ph.D. experience a great journey.

I would like to thank the members of my advisory committee Dr. Stephane Laporte, Dr. Jun-li Liu and Dr. Elizabeth Fixman for all their advice and guidance during my Ph.D. studies.

I would like to thank all members of the Larose laboratory starting with the great companions during my whole Ph.D. journey, postdoctoral fellow Dr. Julie Dusseault, and Ph.D. student Hui Li. I would like to thank research associate Dr. Bing Li, and graduate students Nida Haider and George Kefalas. I couldn't have asked for better colleagues and friends, with whom I could discuss science, experiments, failures, successes, weekend plans, thesis writing and much more.

I would also like to thank all members of the polypeptide lab for being the best group of students, technicians, research assistants, associates and principal investigators to work with. Special thanks to Victor Dumas for great discussions we've had together, and Mary Lapenna for all the work she's doing for the whole polypeptide group.

Finally,

To my father Aouni and my mother Salwa: Thank you for your unconditional love, support, and encouragement throughout my Ph.D. studies (with its ups and downs). You are the greatest inspiration for my accomplishments. Your work and sacrifice throughout my life made me who I am today. I hope that I will remain a source of pride to you. And to the great sister/best friend trio Hiba, Hala and Leen: You guys are awesome!

LIST OF FIGURES

Chapter 1

Figure 1. 1 Regulation of glucose homeostasis by insulin and glucagon. 68
Figure 1. 2 Organization of the pancreas. 69
Figure 1. 3 Insulin structure and processing. 70
Figure 1. 4 Insulin secretion pattern. 71
Figure 1. 5 Insulin granule trafficking. 72
Figure 1. 6 Insulin granule exocytosis. 73
Figure 1. 7 Glucose-stimulated insulin secretion. 74
Figure 1. 8 Insulin signaling pathways. 75
Figure 1. 9 Insulin signaling in peripheral tissue. 77
Figure 1. 10 Unfolded protein response (UPR) signaling. 78
Figure 1. 11 PERK structure. 79
Figure 1. 12 eIF2α regulation. 80
Figure 1. 13 Translational regulation of ATF4. 81
Figure 1. 14 ATF4 direct and indirect activation of autophagy. 82
Figure 1. 15 Nrf2 regulation. 83
Figure 1. 16 Nck linear structure and identified binding partners. 84
Figure 1. 17 Nck mediates membrane receptor signaling to effector proteins regulating actin cytoskeletal rearrangement. 85
Figure 1. 18 Nck modulation of eIF2α downstream signaling and protein translation. 87

Chapter 2

Figure 2. 1 Lack of Nck enhances PERK activity. 113
Figure 2. 2 Nck and PERK interaction. 114
Figure 2. 3 PERK juxtamembrane pY^{561} is the Nck-binding site. 116
Figure 2. 4 PERK Y561F mutation enhances PERK catalytic activity. 118
Figure 2. 5 PERK phosphorylation at Y^{561} delays PERK activation and signaling in MIN6 cells. 119
Figure 2. 6 Nck interacts with PERK phosphorylated at Y^{561} in MIN6 cells. 121
Figure 2. 7 Nck1 modulates PERK activation and phosphorylation at Y^{561} in MIN6 cells. 122
Figure 2. 8 Nck1 modulates proinsulin levels and insulin biosynthesis in MIN6 cells and insulin content in isolated pancreatic islets. 123

Supplementary figure S2. 1 PERK Y561F mutation enhances ER stress-induced PERK-mediated peIF2αS^{51}. 125
Supplementary figure S2. 2 PERK phosphorylation at Y^{561} delays PERK activation and signaling in MIN6 cells. 126

Supplementary figure S2. 3 Nck interacts with PERK and modulates PERK activity. 128
Supplementary figure S2. 4 Overexpression of PERK modulates proinsulin levels in MIN6 cells. 130

Chapter 3

Figure 3. 1 Electron microscopy of control and Nck1-silenced MIN6 cells. 155
Figure 3. 2 Silencing Nck1 protects MIN6 cells against diabetes-related stresses. 157
Figure 3. 3 Stress-induced PERK activation in control and Nck1-silenced MIN6 cells. 159
Figure 3. 4 ATF4 and CHOP expression in control and Nck1-silenced MIN6 cells. 160
Figure 3. 5 Silencing Nck1 enhances autophagy in pancreatic β cells. 162
Figure 3. 6 Silencing Nck1 in MIN6 cells enhances Sesn2-pAMPK-mTORC1 signaling and Akt activation. 164
Figure 3. 7 Silencing Nck1 in MIN6 cells promotes Nrf2 nuclear localization and expression of Nrf2-target antioxidant genes. 166
Figure 3. 8 PERK regulation of adaptive or apoptotic response. 167

Supplementary Figure S3. 1 Proliferation in control and shNck1 MIN6 cells. 168
Supplementary Figure S3. 2 Time course of PERK activation in palmitate (PA)- and thapsigargin (Tg)-treated control and shNck1 MIN6 cells. 169
Supplementary Figure S3. 3 Enhanced autophagosomes formation in shNck1 MIN6 cells. 170

Chapter 4

Figure 4. 1 Nck1 modulation of the UPR through PERK signaling increases the robustness of pancreatic β cells in response to stresses. 188

LIST OF TABLES

Supplementary Table S3. 1 Primer sequences used for qRT-PCR analysis. 171

LIST OF ABBREVIATIONS

4EBP1	eIF4E-binding protein 1
aa	amino acid
ACC	acetyl CoA carboxylase
ADP	adenosine diphosphate
Ala	alanine
AMPK	5' adenosine monophosphate-activated protein kinase
Arg	arginine
Arp2/3	actin related proteins 2/3
AS160	Akt Substrate of 160 kDa
ASK1	apoptosis signal-regulating kinase 1
ATF	activating transcription factor
ATG7	autophagy-related 7
ATP	adenosine triphosphate
BafA1	bafilomycin A1
BCAP	B-cell adaptor for PI3K
Bcl-2	B-cell lymphoma/leukemia 2
Bcl-XL	B-cell lymphoma extra large
BCR	B cell receptor
Bid	BH3-interacting domain
Bim	BH3-only protein
Bip	binding immunoglobulin protein
bpVPhen	bis-peroxovanadium 1,10-phenathroline
bZIP	basic leucine zipper
Ca^{2+}	calcium
CALR	calreticulin
cAMP	cyclic adenosine monophosphate
cbl-b	casitas B-lineage lymphoma-b
Cdc42	cell division control protein 42
cDNA	complementary deoxyribonucleic acid
CE	capping enzyme
CEBP/β	CCAATenhancer-binding protein β
CHOP	CCAAT/enhancer binding protein (C/EBP) homologous protein
CNC	cap ‘n’ collar
CNX	calnexin
CPE	carboxypeptidase E
CPT1	carnitine palmitoyltransferase 1
CreP	constitutive repressor of eIF2α phosphorylation
CRIB	Cdc42- and Rac-interacting binding
Cul3	Cullin 3
Dab-1	Disabled-1
DAG	diacylglycerol
DHAP	dihydroxyacetone phosphate
DMEM	Dulbecco’s Modified Eagle’s Medium

DMSO	dimethyl sulfoxide
Dock	dreadlock
DR5	death receptor 5
Dscam	Down syndrome cell adhesion molecule
DTT	dithiothreitol
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
eIF	eukaryotic initiation factor
EIF2AK3	eukaryotic initiation factor 2α kinase 3
EPEC	Enteropathogenic *Esherichia coli*
ER	endoplasmic reticulum
ERAD	ER-associated degradation
Erk	extracellular signal-regulated kinase
ERO1α	ER oxidoreductin 1α
ERO1β	ER oxidoreductin 1β
ESRE	ER stress response element
F-actin	filamentous actin
FasL	Fas ligand
FFA	free fatty acid
FOXO1	forkhead box O1
FP	foot process
GADD34	growth arrest and DNA damage-inducible protein 34
GAP	GTPase activating protein
GCN2	general control non-derepressible-2
GDP	guanosine 5'-diphosphate
GFP	green fluorescent protein
Git	GPCR-kinase-interacting protein
GLUT	glucose transporter
Gly3P	glycerol-3-phosphate
GMB	glomerular basement membrane
GPCR	G-protein-coupled receptor
Grb2	growth factor receptor-bound protein 2
GRP78	glucose-regulated protein of 78 kDa
GRP94	glucose-regulated protein of 94 kDa
GSIS	glucose-stimulated insulin secretion
GSK3	glycogen synthase kinase 3
GST	glutathione S-transferase
GTP	guanosine 5'-triphosphate
HepG2	hepatocellular carcinoma cell line
HFD	high fat diet
HNF4A	hepatic nuclear factor 4A
HO-1	Heme-oxygenase 1
HRI	heme-regulated inhibitor
HSP	heat shock protein
IAPP	islet amyloid polypeptide

ICA	islet cell autoantibodies
InR	Drosophila insulin receptor
IP3	inositol 1, 4, 5-triphosphate
IR	insulin receptor
IRE1	inositol requiring enzyme 1
IRS	insulin receptor substrate
ISR	integrated stress response
ITAM	immunoreceptor tyrosine-based activation motif
JNK	Jun N-terminal kinase
K_{ATP}	ATP-sensitive potassium channel
kDa	kilodalton
Keap1	kelch-like ECH-associated protein 1
LC3B	microtubule-associated protein 1 light chain 3 beta
LC-CoA	long-chain acyl-coenzyme A
MAPK	mitogen-activated protein kinase
MEFs	mouse embryonic fibroblasts
MEK	MAP kinase/Erk kinase
MHC	major histocompatibility complex
MIN6	mouse insulinoma pancreatic β cells
MODY	maturity-onset diabetes of the young
MTT	(3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide)
mRNA	messenger ribonucleic acid
mTORC	mammalian target of rapamycin complex
MUC18	melanoma-associated protein
Munc13	mammalian uncoordinated 13
Munc18	mammalian uncoordinated 18
Na_3VO_4	sodium orthovanadate
NaF	sodium fluoride
NCD	normal chow diet
Nck	non-catalytic region of tyrosine kinase
NOD	non-obese diabetic
NQO1	NADPH dehydrogenase quinoneoxireductase 1
Nrf2	nuclear factor erythroid 2-related factor 2
P58IPK	protein kinase inhibitor of 58 kDa
P90RSK	p90 ribosomal S6 kinase
PA	palmitate
Pak1	p-21 activated serine/threonine kinase 1
PARP	poly ADP-ribose polymerase
PERK	PKR-like endoplasmic reticulum kinase
PC	prohormone convertase
PDGF	platelet-derived growth factor
PDGFR	platelet-derived growth factor receptor
PDI	protein disulfide isomerase
PDIA6	protein disulfide isomerase A6
PDK1	3-phosphoinositide-dependent protein kinase 1

PDX1	pancreatic and duodenal homeobox 1
PH	pleckstrin homology
PI3k	phosphatidylinositol 3-kinase
PIP2	phosphatidylinositol 4, 5-diphosphate
PIP3	phosphatidylinositol 3, 4, 5-triphosphate
Pix	Pak-interacting exchange factor
PKB	protein kinase B
PKR	double-stranded RNA-dependent protein kinase
PLC	phospholipase C
PP	pancreatic polypeptide
PP1c	protein phosphatase 1
PTB	phospho-tyrosine binding
PTP1B	phospho-tyrosine phosphatase 1B
RIDD	regulated IRE1-dependent decay
RRP	readily releasable pool
ROS	reactive oxygen species
RTK	receptor tyrosine kinase
S1p	site-1 protease
S2p	site-2 protease
S6K	S6 protein kinase
SD	slit diaphragm
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
Ser	serine
Sesn2	sestrin 2
SFK	src family kinase
SH	src homology
Shc	src-homology 2 domain-containing protein
shRNA	short-hairpin ribonucleic acid
SM	Sec1/Munc18-like proteins
SNAP25	synaptosome associated protein of 25
SNARE	Soluble *N*-ethylmaleimide-sensitive factor attachment protein Receptors
Sos	son of sevenless
SRP	signal recognition particles
T1D	type 1 diabetes
T2D	type 2 diabetes
TBL2	Transducing β-like 2
TCPTP	T cell protein tyrosine phosphatase
TCR	T cell receptor
Tg	Thapsigargin
TG	triglycerides
TGN	trans golgi network
Thr	threonine
Tir	translocated intimin receptor
Tm	tunicamycin
TNF	tumour necrosis factor

TRAF2	TNF receptor-associated factor 2
tRNA	transfer ribonucleic acid
TSC	tuberous sclerosis complex
t-SNARE	target SNARE
Tyr	tyrosine
uORF	upstream open reading frame
UPR	unfolded protein response
VAMP2	vesicle associated membrane protein 2
v-SNARE	vesicle SNARE
WASP	Wiskott-Aldrich syndrome Protein
WAVE	WASp-family veprolin homologous protein
WIP	Wiskott-Aldrich syndrome Protein-interacting Protein
WRS	Wolcott-Rallison Syndrome
XBP1	x-box binding protein 1
Zn^{2+}	zinc

CHAPTER 1. General introduction and literature review

1.1 Regulation of glucose homeostasis: overview

In order to sustain a healthy body function, vertebrates including mammals must be able to maintain blood glucose levels within a limited range (4-7 mM) regardless of food intake and energy expenditure (Saltiel & Kahn, 2001). The pancreas is a central organism in the regulation of blood glucose (Figure 1.1). To do so, the pancreas synthesizes and secretes two hormones: insulin and glucagon. Insulin decreases blood glucose levels by stimulating responsive tissues to uptake glucose, while glucagon increases glucose release into the blood from storage tissues. When blood glucose levels increase upon food intake, the pancreas releases insulin, which activates insulin signaling in a number of insulin-responsive tissues, namely liver, skeletal muscle and adipose tissue to control blood glucose levels (also collectively called peripheral insulin-sensitive tissue). The primary role of insulin is to stimulate glucose uptake by the skeletal muscle, adipose tissue and liver (Pessin et al, 1999). In skeletal muscle and liver, glucose undergoes glycogenesis to be stored in the form of glycogen, while in adipose tissue, glucose is stored as triglycerides (TG). In addition, insulin prevents hepatic glucose production and release by inhibiting glycogenolysis, the breakdown of glycogen into glucose, and gluconeogenesis, a process involving glucose production from non-carbohydrate sources such as lactic acid and certain amino acids (Pilkis & Granner, 1992). Finally, insulin inhibits secretion of glucagon from glucagon-producing cells (Bonner-Weir & Orci, 1982). On the other hand, when the body faces conditions leading to low blood glucose levels, such as fasting, the pancreas releases glucagon, which stimulates the breakdown of glycogen to glucose (glycogenolysis) in liver, as well as activates hepatic gluconeogenesis to promote release of glucose (Chiasson et al, 1975; Magnusson et al, 1995). In addition, adipose tissue responds to glucagon by breaking down triglycerides into free fatty acids (lipolysis)

(Lefèbvre & Unger, 1972; Lefebvre, 1983). Overall, all these processes contribute to maintain blood glucose levels under control, allowing for a healthy body state. It is well known that perturbations arising in one or more of these processes lead to important diseases such as diabetes.

1.2 The pancreas

The pancreas displays exocrine and endocrine functions involved in regulating digestion and metabolism in mammals. In fact, the pancreas consists of two types of tissues: the exocrine and the endocrine tissues (Figure 1.2). The exocrine tissue composed mainly of acinar and centroacinar cells accounting for about 90% of the pancreas, is responsible for the production of digestive enzymes. The endocrine tissue, accounting for less than 10% of the pancreas, resides in clusters called islets of Langerhans that are responsible for maintaining glucose homeostasis by producing and secreting hormones. Islets of Langerhans are made up of different cell types: the α, β, δ, ε and the pancreatic polypeptide (PP) cells (Weir & Bonner-Weir, 1990). Pancreatic β cells synthesize and secrete insulin in the blood in response to various stimuli, primarily glucose (McGarry & Dobbins, 1999), while pancreatic α cells synthesize and secrete glucagon. The remaining cell types make up a minority of cells in the islets. δ cells secrete somatostatin, which inhibits the secretion of insulin and glucagon as well as slows down absorption of nutrients from the gastrointestinal tract (Fujimoto et al, 1974). The pancreatic polypeptide (PP) cells secrete pancreatic polypeptide that inhibits the release of somatostatin and digestive enzymes, and the ε cells secrete ghrelin, which regulates appetite by acting as a neuropeptide in the central nervous system (Wierup et al, 2002; Prado et al, 2004; Dickson et al, 2011). β cells make up 50-80% of the islets, and display different distribution patterns depending on the species. In murine islets, β cells are concentrated in the middle of the islet and surrounded by α cells, while in humans, α and β

cells are heterogeneously distributed in the islets (Brissova et al, 2005; Cabrera et al, 2006) . The following section is dedicated to describing pancreatic β cell physiology, including mechanisms involved in insulin biogenesis and secretion, and their role in regulating glucose metabolism and the development of pathological conditions.

1.3 Pancreatic β cell physiology

As already mentioned, the main function of pancreatic β cells is to secrete insulin in response to glucose (Campbell et al, 1982). The process of insulin production and secretion involves highly efficient mechanisms responsible for ensuring biosynthesis, packaging, transportation, and finally release of insulin, and this area has been the subject of extensive research since the discovery of insulin.

1.3.1 Insulin biosynthesis, maturation and trafficking

Insulin was first isolated in 1921 by the collaborative work of Frederick Banting, Charles Best and James Collip in the laboratory of James Macleod at the University of Toronto in Canada. Later, insulin's tertiary monomeric structure was crystallized in 1926 by x-ray crystallography (Abel, 1926). Insulin is a small protein (6 kDa) composed of two chains (A and B), which are connected together by two disulfide bonds. A third disulfide bond within chain A is also present to help establish the tertiary structure of insulin (Figure 1.3C). In most species, chain A is 21 aa long while chain B is 30 aa long. The amino acid sequence, as well as the position of the 3 disulfide bonds are highly conserved among species. These similarities in insulin among species allowed the use of insulin from animals in humans (De Meyts, 2004). While insulin is encoded by one gene

is most species, rats and mice have 2 insulin genes that are expressed equally in islets (Clark and Steiner, 1969).

1.3.1.1 Insulin synthesis and folding

Being made up of two chains, it was initially thought that insulin is synthesized as two separate peptides. However, early studies showed that insulin is synthesized as a single polypeptide precursor named preproinsulin (Steiner & Oyer, 1967). Preproinsulin is composed of a signal peptide, the insulin B-chain, a connecting 23-31 aa long peptide called a C-peptide, and the insulin A-chain (Figure 1.3A). The amino terminal signal peptide of preproinsulin interacts with cytosolic nucleoprotein signal recognition particles (SRP) and drives co-translational translocation, a process whereby preproinsulin is moved across the endoplasmic reticulum (ER) membrane into the ER lumen as it is translated (Egea et al, 2005). During this process, the signal peptide of preproinsulin is enzymatically cleaved by a signal peptidase to generate proinsulin (Patzelt et al, 1978) (Figure 1.3B). Such enzymatic cleavage is essential to prevent misfolding of proinsulin (Stoy et al, 2007). Proinsulin, now composed of an amino terminal B-chain, a carboxyl terminal A-chain and the C-peptide, aligns itself to take its tertiary structure. The C-peptide is believed to promote parallel alignment of chains A and B in order for the disulfide bonds to be created (Markussen & Schiff, 1973). The three intramolecular disulfide bonds establishing proinsulin's native tertiary structure are formed by the action of a number of ER-resident chaperones including the thiol-disulfide oxidoreductase protein disulfide isomerase (PDI) (Tang et al, 1988) and the ER oxidoreductins 1α (ERO1α) and 1β (ERO1β) (Zito et al, 2010; Wright et al, 2013).

1.3.1.2 Insulin granule packaging and maturation

Once its tertiary structure is established, proinsulin is delivered to the trans-Golgi network (TGN) where it gets concentrated with other granular components to be sorted in clathrin-coated immature granules. The sorting process in pancreatic β cells is highly efficient, with more than 99% of proinsulin molecules getting sorted in granules (Rhodes & Halban, 1987). As opposed to other related secretory proteins which are released continuously through a constitutive secretory pathway (Nagamatsu & Steiner, 1992), proinsulin undergoes sorting and secretion through a regulated secretory pathway (Rhodes & Halban, 1987). How proinsulin, as opposed to other secretory proteins, is uniquely recognized by the regulated secretory machinery to be sorted in immature granules is still under debate. Different models have been proposed for this, but all agree that proinsulin must possess unique features allowing its recognition as a target for sorting and packaging in the TGN (Molinete et al, 2000). Whether this recognition involves membrane receptors or not, remains to be elucidated. In one model, the sorting of proinsulin into immature granules involves recognition by specific receptors on the TGN membrane. A slightly different version of this model reports the involvement of other accessory proteins in the recognition and association between proinsulin and a TGN membrane receptor. In a third model, the aggregation of proinsulin and other granular constituents in slightly acidic Ca^{2+}- and Zn^{2+}-rich areas around the TGN membrane by itself triggers membrane reorganization and recruitment of cholesterol in preparation for vesicle formation. In all these models, the association of proinsulin at the membrane of the TGN induces the formation of immature granules budding from the TGN (Molinete et al, 2000).

The maturation process of insulin granules includes acidification of the granule internal environment, conversion of proinsulin to insulin, and loss of the clathrin coating (Orci et al, 1986; Orci et al, 1987). Mild acidification of the immature granules is already achieved by the presence of Ca^{2+} and Zn^{2+}. Further acidification requires the action of an ATP-dependent proton pump present on the vesicles, which contributes to the decrease of intravesicular pH levels (Rhodes et al, 1987; Davidson et al, 1988). This leads to optimal activation of endoproteolytic enzymes prohormone convertases (PC1/3 and PC2) that cleave proinsulin at both ends of the C-peptide to generate intermediate products. The final step to complete the processing of insulin requires carboxypeptidase E (CPE), which trims out basic residues at the carboxyl termini, leading to production of equimolar amounts of mature insulin and C-peptide (Figure 1.3C), with very small percentage of residual proinsulin (Kemmler et al, 1972; Steiner & Philipson, 2000). Potential biological activity for the C-peptide has been reported in several studies. Indeed, C-peptide administration was found to be beneficial for vascular permeability, ATPase activity and microcirculation in diabetic patients (Wahren et al, 1994), and prevented neural and vascular dysfunctions in diabetic rats (Ido et al, 1997). Nonetheless, a physiological role for the C-peptide still remains controversial. Time-wise, the maturation process of granules takes between 30 and 150 minutes after synthesis (Liu et al, 2014a). Based on pancreatic islet studies, the number of insulin secretory granules ranges from 5-10,000 per cell, with each granule carrying about 300,000 insulin molecules (Pouli et al, 1998; Fava et al, 2012) .

1.3.1.3 Insulin granule trafficking and secretion

The secretion pattern of insulin from pancreatic β cells has long been established to be biphasic (Curry et al, 1968). The first phase of insulin secretion occurs rapidly, within the first 5

minutes upon glucose stimulation, while the second phase of insulin secretion, although lower in intensity than the first phase, is prolonged, reaching a plateau at 25-30 minutes (Curry et al, 1968). Further investigation indicated that the two phases of insulin secretion pattern correspond to two distinct pools of mature insulin granules: the readily releasable pool (RRP), pre-docked at the plasma membrane, corresponding to the first phase of insulin secreted, and the reserve pool found internally within the cytoplasm, corresponding to the second phase of insulin secretion (Figure 1.4) (Wang & Thurmond, 2009). Pre-docked RRP insulin granules readily fuse with the plasma membrane and release insulin upon an influx of Ca^{2+} into cells, thereby establishing the first phase of secretion (Daniel et al, 1999). Pre-docked insulin granules, as opposed to the reserve pool, reside within 50 nm from the plasma membrane, localized with Ca^{2+} channels to increase efficiency in Ca^{2+} detection and subsequent fusion (Wiser et al, 1999; Gaisano, 2014). The second phase of insulin secretion implies more complex events since insulin has to be newly synthesized, processed, and secreted for the duration of the increase of blood glucose and to replenish the stores of insulin initially depleted in the first phase secretion, therefore explaining the time needed for this second phase to be established. These steps involve a number of mechanisms including trafficking to the plasma membrane, docking, priming and finally fusing with the plasma membrane to release insulin. In addition, the second phase insulin secretion needs recruitment of granules through actin cytoskeleton for fusion (Wang & Thurmond, 2009).

Early transport of insulin granules in the cell requires microtubule formation and kinesin machinery driven by ATP. In agreement, glucose stimulation has been shown to induce formation of microtubules (Howell & Tyhurst, 1986). This subsequently helps deliver granules to the cortical actin filaments near the periphery of the cell. Numerous studies reported that nutrient stimulation

also leads to actin remodeling in pancreatic β cells (Figure 1.5A), which then facilitates mobilization of insulin granules to the plasma membrane (Wang & Thurmond, 2009). On their surface, insulin granules express proteins belonging to a highly conserved family of proteins called SNAREs (Soluble *N*-ethylmaleimide-sensitive factor Attachment Protein REceptors), which mediate mobilization of vesicles, and ensure recognition and attachment between vesicles and target membranes (Sollner et al, 1993). Insulin granules express vesicle associated membrane protein 2 (VAMP2), a vesicle-SNARE (v-SNARE), while the plasma membrane expresses syntaxin1 and synaptosome associated protein of 25 kDa (SNAP25) target-SNAREs (t-SNAREs). Under basal condition, filamentous actin (F-actin) acts as a transportation track for insulin granules, but prevents interaction between v-SNAREs on insulin granules and t-SNAREs on the plasma membrane. Upon glucose stimulation, transient disruption of the interaction between F-actin and t-SNAREs allows access of the insulin granules to the plasma membrane (Thurmond et al, 2003). Remodeling of F-actin upon nutrient stimulation has been shown to mechanistically involve activation of the small GTPases Cdc42 (cell division control protein 42) and Rac1, whose activation leads to recruitment, binding and activation of p21-activated kinase 1 (Pak1) (Nevins & Thurmond, 2003; Li et al, 2004). Paks are known regulators of actin cytoskeletal reorganization in yeast and mammals (Harden et al, 1996; Sells et al, 1997; Leeuw et al, 1998) and Cdc42 was shown to play a major role in 2nd phase insulin secretion. Indeed, ablation of either Cdc42 or Pak1 led to disruption of glucose-induced 2nd phase insulin secretion (Wang et al, 2007; Wang et al, 2011). Using specific inhibitors of several Ser/Thr kinases, it was demonstrated that the signaling cascade downstream of Pak1, which includes activation of Ser/Thr kinases Raf1, MEK1/2, and Erk1/2 is responsible for F-actin remodeling and granule mobilization to the plasma membrane in pancreatic β cells (Figure 1.5B) (Kalwat et al, 2013).

Insulin exocytosis involves a machinery of various proteins that regulate tethering, priming and fusion (Figure 1.6). Tethering is the process by which physical interaction between the granule and plasma membrane occurs, prior to the interaction between SNARE complexes (Whyte & Munro, 2002). It requires the formation of a multi-subunit complex of different exocyst proteins such as Sec5 (Kee et al, 1997; Xie et al, 2013) in a small GTPase activity-dependent manner (Moskalenko et al, 2002), thereby establishing tethering of granules on plasma membrane (Figure 1.6A). Although exocyst formation deficiency does not inhibit tethering, it does however lead to a loss of subsequent SNARE interaction (Grote et al, 2000). Priming of granules requires the presence of SNAREs on both the granules and the plasma membrane, as well as Sec1/Munc18-like proteins (SM), which are involved in the formation of the SNARE complex (Sudhof & Rothman, 2009). V-SNAREs and t-SNAREs assemble together in a complex to allow priming of insulin granules on the plasma membrane (Sollner et al, 1993). First, the SM protein Mammalian uncoordinated 18 (Munc18), binds to syntaxin1 in its closed conformation. With the aid of priming factor Mammalian uncoordinated 13 (Munc13), Munc18 induces a conformational change of syntaxin1 allowing it to take an open conformation (Rhee et al, 2002). Subsequently, Munc18 acts as a clamp to hold together VAMP in between SNAP25 and syntaxin1 (Figure 1.6B), allowing the granules to reach the priming state on the plasma membrane (Gaisano, 2014). The final stages leading to fusion require accessory protein complexin (a clamping fusion protein) to hold the SNARE assembly close enough to the plasma membrane to allow fusion of the lipid bilayers as soon as the protein synaptotagmin (a phospho-lipid binding protein, Ca^{2+}- binding protein) detects Ca^{2+} influx, and induces a conformational change to the SNARE assembly (Figure 1.6C-D) (Krishnakumar et al, 2011; Li et al, 2011).

1.3.2 Glucose-stimulated insulin secretion (GSIS)

The most potent stimulus of insulin secretion by β cells is glucose (Figure 1.7). When blood glucose levels increase as a result of food intake, glucose is transported into β cells through glucose transporter 2 (GLUT2) (Thorens, 1992). Once in the cell, glucose is converted to glucose-6-phosphate by glucokinase, as part of the glycolysis process. Taking place in the cytosol, glycolysis produces 2 molecules of ATP, NADH and pyruvate. Pyruvate then enter the mitochondria and get oxidized as part of the Krebs cycle, producing Acetyl-CoA and other products including NADH, ATP and CO_2. Finally, products from the Krebs cycle undergo oxidative phosphorylation at the cisternae membrane of the mitochondria. Briefly, NADH are utilized by the electron transport chain complexes to pump protons from the matrix into the intermembrane space, and eventually the ATP synthase utilizes the potential energy created by the electrochemical gradient of protons to generate ATP molecules (Newgard & McGarry, 1995; Henquin, 2000; Straub & Sharp, 2002). The increase of ATP to ADP ratio in cells will result in closing of ATP-sensitive potassium channels (K_{ATP}), leading to accumulation of potassium ions in the cell and membrane depolarization (Ashcroft et al, 1984; Cook & Hales, 1984; Aguilar-Bryan & Bryan, 1999). Subsequently, voltage-gated Ca^{2+}-channels are activated leading to an influx of Ca^{2+} into the cytosol, allowing fusion of insulin vesicles pre-docked at the plasma membrane and release of insulin into the blood (Kelly et al, 1991; Rolland et al, 2001). In addition to these mechanisms, glucose-6-phosphate can be metabolized into dihydroxyacetone phosphate (DHAP) and subsequently to glycerol-3-phosphate (Gly3P), which can generate lipid metabolic coupling factors that can also enhance insulin secretion (Eto K, 1999; Bender K, 2006).

1.3.3 Fatty acid-stimulated insulin secretion

Although glucose is the main stimulus for pancreatic β cell insulin secretion, studies have shown that free fatty acids (FFAs) amplify insulin secretion under both normal and elevated blood glucose levels (Pelkonen et al, 1968; Crespin et al, 1969; Stein et al, 1996; Dobbins et al, 1998). While acute exposure of β cells to FFAs potentiates GSIS (Stein et al, 1996), chronic exposure eventually leads to β cell dysfunction (Prentki et al, 2002; El-Assaad et al, 2003; Poitout et al, 2006). FFA regulation of insulin secretion may occur through different mechanisms. On one hand, FFAs can undergo facilitated diffusion into β cells at normal or low glucose levels (Hamilton & Kamp, 1999). Intracellular FFAs then gets converted to the lipid signaling molecule long-chain Acyl-coenzyme A (LC-CoA), which is transported to the mitochondria by the carnitine palmitoyltransferase 1 (CPT1). The process of Acyl-CoA oxidation in the mitochondria increases the production of NADH and FADH2, which generates ATP (Nolan et al, 2006). Under high glucose conditions, increase in ATP/ADP ratio leads to inhibition of the AMP-activated kinase (AMPK), and this activates the Acetyl-CoA carboxylase (ACC), which converts glucose-derived Acetyl-CoA to Malonyl-CoA, which in turn inhibits CPT1 activity. Therefore, Acyl-CoA produced from lipid metabolism cannot undergo lipid oxidation in the mitochondria given CPT1 is inhibited (Nolan et al, 2006). Increased cytosolic Acyl-CoA promotes accumulation of intracellular calcium, facilitating fusion of insulin granules at the plasma membrane, thereby promoting insulin exocytosis (Prentki et al, 2002). As already mentioned, glucose can also participate in triglyceride (TG) and FFA cycling, where esterification of LC-CoA to TG and diacylglycerol (DAG) is promoted by the glucose product Gly3p, concomitant with glucose activating lipolysis to reactivate LC-CoA, resulting in insulin secretion (Prentki & Corkey, 1996;

Prentki et al, 2002). On the other hand, FFAs can directly stimulate insulin secretion through direct binding and activation of the FFA receptor 1 G-protein coupled receptor (GPR40/FFA1) by a mechanism independent from the intracellular metabolism of FFAs (Briscoe et al, 2003; Itoh et al, 2003; Alquier et al, 2009). Different types of FFA have been shown to activate GPR40 including medium and long-chain saturated and unsaturated FFAs (Briscoe et al, 2003). Although not fully understood, it is believed that the signaling mechanism of FFA-stimulated insulin secretion through GPR40 involves the activation of the G protein α subunit $G_{q/11}$ which in turn activates phospholipase C (PLC) that hydrolyzes the phospholipid phosphatidylinositol 4, 5-diphosphate (PIP2) to 1, 2 diacylglycerol (DAG) and inositol 1, 4, 5-triphosphate (IP3). DAG then activates protein kinase D1 (PKD1), which triggers F-actin depolymerization leading to insulin granule exocytosis. In addition, IP3 production leads to opening of Ca^{2+} channels on the ER membrane that allow calcium entry into the cytoplasm, but this was shown to have a minor contribution towards exocytosis (Poitout, 2003; Ferdaoussi et al, 2012; Mancini & Poitout, 2013). Through GPR40 activation, FFAs contribute towards half of the acute insulin secretion (Mancini & Poitout, 2013). Although both phases of insulin secretion are enhanced by FFAs, the impact on second phase is stronger (Ferdaoussi et al, 2012).

1.3.4 Insulin signaling in insulin-responsive tissues

Once secreted into blood, insulin is accessible to insulin-sensitive tissues such as liver, adipose tissue and skeletal muscle. Insulin signaling initiated in these tissues results in uptake of glucose, which contributes to the regulation of glucose homeostasis. Insulin triggers signaling by binding to the insulin receptor (IR), a receptor tyrosine kinase (RTK) expressed on insulin-responsive tissues (De Meyts, 2004; Lawrence et al, 2007). Functional IR is a tetrameric protein

complex consisting of two extracellular α, and two transmembrane β subunits linked by disulfide bridges. Structure-wise, the insulin receptor is kept inactive by the inhibitory regulation of α subunits on the β subunits, which harbor the tyrosine kinase activity. When insulin binds to the α subunits, conformational changes allow activation of the β subunits by autophosphorylation of the kinase domains leading to additional conformational changes and increased kinase activity (Cohen, 2006; Taniguchi et al, 2006; De Meyts, 2008). Specific phosphorylated tyrosine residues on the β chains contribute to optimal IR tyrosine kinase activation while others create docking sites for phospho-tyrosine-binding proteins, such as insulin receptor substrates (IRS).

Six different members of the IRS family have been identified: IRS1-6 (Sun et al, 1991; Sun et al, 1995; Lavan et al, 1997; Fantin et al, 1998; Cai et al, 2003). While IRS1 and IRS2 are widely and highly expressed in different tissues, IRS3 is mainly expressed in adipocytes and brain, and IRS4 is limited to embryonic tissue. IRS5 is expressed ubiquitously but mostly in liver and kidneys, while IRS6 is expressed in skeletal muscle. Of all members identified, IRS1 and IRS2 are the most implicated in mediating insulin biological actions and glucose homeostasis. Indeed, liver or skeletal muscle-specific disruption of both *IRS1/2* highly phenocopied IR gene (*Insr)* deficiency in liver or skeletal muscle of mice, respectively (Michael et al, 2000; Biddinger et al, 2008; Dong et al, 2008; Long et al, 2011). Whole body $IRS1^{-/-}$ mice are smaller in size, glucose intolerant, and insulin resistant compared to wild-type mice (Araki et al, 1994; Tamemoto et al, 1994). However, the strongest phenotype was observed in $IRS2^{-/-}$ mice that developed diabetes, due to the dual effects of IRS2 deficiency in both insulin-responsive tissue which caused insulin resistance, and pancreatic β cells, which led to diminished insulin secretion (Withers et al, 1998). $IRS3^{-/-}$ mice displayed no defects in growth or glucose homeostasis (Liu et al, 1999), while *IRS4* deficiency in

mice was found to have very mild effects on glucose homeostasis (Fantin et al, 2000). Interestingly, overexpression of both IRS3/4 in cells impaired IRS1/2-dependent signaling (Tsuruzoe et al, 2001). Although IRS5 and IRS6 were activated upon IR activation, the signaling pathways triggered downstream were different from the classical pathway activated during insulin signaling involved in glucose homeostasis (Cai et al, 2003).

IRS proteins are composed of pleckstrin-homology (PH) and phospho-tyrosine-binding (PTB) domains near the N-terminus and both account for IRS interaction with IR. IRS center and C-terminus regions contain tyrosine residues that can be potentially phosphorylated by IR upon binding, and this generates binding sites on IRS for SH2 domain-containing proteins, leading to activation of various signaling pathways. IRS proteins can also undergo serine/threonine phosphorylation, which has been mostly associated with negative regulation of IRS activity and insulin signaling. Indeed, Ser/Thr phosphorylation of IRS1 was shown to decrease its tyrosine phosphorylation (Mothe & Van Obberghen, 1996), triggering dissociation from IR (Paz et al, 1997), and finally leading to its degradation (Pederson et al, 2001). In addition, Ser^{307} on IRS1 has been associated with negative regulation of insulin signaling (Aguirre et al, 2002). However, more recently, studies have introduced the view of Ser/Thr IRS phosphorylation as a positive regulator of insulin signaling. As opposed to the above-mentioned study, Ser^{307} phosphorylation on IRS was found to promote insulin sensitivity in mice (Copps et al, 2010).

The most-studied SH2-domain-containing binding proteins that associate with tyrosine phosphorylated IRS allowing for activation of insulin signaling transduction are phosphatidylinositol 3-kinase (PI3K) (Myers et al, 1994), adaptor protein growth factor receptor-bound protein 2 (Grb2) (Skolnik et al, 1993) as well as the protein tyrosine phosphatase SHP2

(Kuhne et al, 1993). Other binding proteins exist but they are less well-studied. PI3K is responsible for triggering the classical insulin signaling pathway involving Akt activation, while the Grb2/Sos activates the mitogen-activated protein kinases (MAPK) pathway (Figure 1.8). SHP2 also regulates insulin signaling at the MAPK pathway level.

1.3.4.1 PI3K-Akt pathway

PI3K is the most important SH2-domain-containing protein that binds IRS and mediates most of the metabolic effects of insulin. PI3K is composed of a regulatory (p85) and a catalytic (p110) subunits. Activation of the catalytic subunit occurs upon interaction of the SH2 domains of the regulatory subunit p85 with specific phospho-tyrosine residues on IRS proteins. This interaction relieves the inhibition imposed on the catalytic subunit by the regulatory subunit (Yu et al, 1998). Activated PI3K phosphorylates 3'-hydroxyl group of PIP2 to generate phosphatidylinositol 3, 4, 5-triphosphate (PIP3). PIP3 is a plasma membrane phospholipid that recruits proteins containing a PH domain such as the 3-phosphoinositide-dependent protein kinase 1 (PDK1) and the Ser/Thr protein kinase Akt/PKB. One of the most critical events in insulin signaling is the activation of Akt/PKB following its phosphorylation on Thr^{308} by PDK1 once recruited at the plasma membrane, because of the wide range of diverse pathways activated downstream of Akt (Mora et al, 2004). Further activation of Akt involves additional Akt phosphorylation on Ser^{473} by the mammalian target of rapamycin complex 2 (mTORC2) (Sarbassov et al, 2005). Three Akt isoforms, which share a high degree of aa identity exist in mammals (Akt1-3). The distribution of each isoform, its cellular localization and downstream signaling targets define the importance in glucose homeostasis (Taniguchi et al, 2006). While both Akt1 and Akt2 are widely expressed, with Akt2 being enriched in insulin-responsive fat and liver

tissues, Akt3 expression is limited to the nervous system (Chan et al, 1999). Indeed, Akt3 had no role in glucose homeostasis (Tschopp et al, 2005), and while Akt1 deficiency in mice leads to growth retardation, no metabolic abnormalities are observed (Chen et al, 2001; Cho et al, 2001b). On the other hand, Akt2 is the isoform most implicated in insulin signaling and glucose homeostasis. Akt2 deletion in mice triggers diabetes development due to dysfunctionality in glucose utilization and hepatic glucose output (Cho et al, 2001a). In agreement, human Akt2 mutations in the kinase domain trigger insulin resistance and diabetes development (George et al, 2004). Finally, Akt2 deficiency impairs insulin-stimulated glucose uptake in adipocytes (Bae et al, 2003; Jiang et al, 2003).

Activated Akt regulates the activity of various targets regulating proliferation, motility, growth, survival, glucose homeostasis, and cell survival (Figure 1.9). Among these, Akt activation induces plasma membrane translocation of the glucose transporter 4 (GLUT4) in skeletal muscle and adipose tissue, and activation of glycogen synthase in liver, thereby promoting glucose uptake and glycogen synthesis, respectively. It has been reported that Akt translocation of GLUT4 to the plasma membrane is mediated by the GTPase activating protein (GAP) Akt Substrate of 160 kDa AS160 (Kane et al, 2002; Sano et al, 2003). Indeed, Akt phosphorylates and inhibit AS160 GAP activity, thus allowing activation of Rab small GTPases involved in cytoskeletal reorganization enabling GLUT4 translocation (Sano et al, 2003). In addition, Akt phosphorylates and inhibits glycogen synthase kinase 3 (GSK3), which negatively regulates glycogen synthase and ATP citrate lyase, controlling glycogen and fatty acid synthesis respectively (Frame & Cohen, 2001). Moreover, Akt induces activation of the mammalian target of rapamycin complex 1 (mTORC1), which through activation of the Ser/Thr protein kinase S6 (S6K) and phosphorylation of the eIF4

binding proteins (4EBP1) increases protein synthesis, proliferation and cell growth (Wang & Proud, 2006). Briefly, Akt activates mTORC1 by phosphorylating and inhibiting the tuberous sclerosis complex 2 (TSC2) (Inoki et al, 2002), which along with TSC1 heterodimerizes and acts as a GAP for the small GTPase Rheb. Once TSC1/2 activity is inhibited by Akt activation, active Rheb participates in activating mTORC1 complex. Furthermore, Akt activation leads to phosphorylation of the transcription factor forkhead box protein O1 (FOXO1), leading to its nuclear exclusion, ubiquitination, and degradation (Brunet et al, 1999). In summary, Akt controls numerous pathways that in addition to promoting cell proliferation, survival and migration in response to extracellular signals, are also crucial regulators of whole body glucose metabolism and homeostasis.

1.3.4.2 Ras-MAPK pathway

Insulin signaling also leads to activation of the Ras-MAPK pathway by IR recruitment and tyrosine phosphorylation of IRS1 and Shc (Figure 1.8). Shc proteins are phospho-tyrosine binding adaptor proteins with an N-terminal PTB domain and a C-terminal phospho-tyrosine-binding SH2 domain (Rozakis-Adcock et al, 1992; Luschnig et al, 2000). Tyrosine phosphorylation of IRS1 and Shc at the IR allows recruitment and binding of Grb2 and guanyl nucleotide exchange factor son of sevenless (Sos) to IRS1 and Shc (Skolnik et al, 1993; White, 1998). IRS1-Grb2-Sos and Shc-Grb2-Sos complexes trigger activation of the small GTPase Ras, which then binds and activates Raf. Raf then initiates a cascade of phosphorylation and activation of dual-specificity kinases Mek1 and Mek2, which phosphorylate MAPK/Erk1 and Erk2 on Ser/Thr/Tyr residues resulting in their activation. Activated Erks have various targets including p90 ribosomal S6 kinase (p90RSK) and others to promote gene expression mediating cell growth, differentiation and

proliferation (Pouyssegur et al, 2002). Ras comes in different isoforms: H-Ras, N-Ras and K-Ras which are ubiquitously expressed (Hancock & Parton, 2005). The Raf proteins also come in different isoforms: A-Raf, B-Raf and C-Raf which are differentially regulated, and activate the MAPK pathway differentially in duration and intensity (Wellbrock et al, 2004).

1.3.5 Insulin signaling in pancreatic β cells

Studies on the role of insulin in pancreatic β cells used to generate controversial data. Initially, it was thought that since pancreatic β cells continuously produce and secrete insulin, insulin would have a minimal effect on β cell function due to the fact that continuous exposure to insulin may desensitize β cells. In agreement with this view, early studies showed that daily insulin injections reduced pancreatic insulin content and β cell mass (Best & Haist, 1941). However, increasing reports now support a positive role for insulin in regulating β cell proliferation, mass and survival. Indeed, β cell-specific *IR* knockout (*βIRKO*) mice display an age-dependent decrease in β cell mass due to β cell apoptosis, which leads to impaired glucose tolerance and GSIS (Kulkarni & Zisman, 2003; Otani et al, 2004). In addition, knockout of *IRS2* leads to development of a type 2 diabetes (T2D)-like phenotype with reduced β cell mass, impaired glucose tolerance and insulin secretion (Kubota et al, 2000). Also, diabetic and pre-diabetic patients carrying the IRS1 Arg^{972} polymorphism that impairs IRS1 function, display pancreatic β cell dysfunction linked to defects in insulin secretion (Federici et al, 2001). Moreover, pancreatic β cell-specific deletion of *PDK1* induce progressive hyperglycemia leading to diabetes. This was due to decreased proliferation and enhanced sensitivity to apoptosis leading to decreased β cell number and size, which ultimately causes loss of β cell mass (Hashimoto et al, 2006). Interestingly, overexpression of active Akt in pancreatic β cells enhanced cell size and total islet mass, while improving glucose

tolerance and robustness against stresses (Tuttle et al, 2001). Inversely, pancreatic β cell-specific expression of a kinase dead Akt impaired glucose tolerance due to a deficiency of insulin exocytosis, and increased mice susceptibility to high fat diet (HFD)-induced diabetes development (Bernal-Mizrachi et al, 2004). Furthermore, specific deletion of Rictor, the subunit involved in mTORC2 complex formation, in pancreatic β cells reduced glucose tolerance, β cell mass proliferation, insulin content and GSIS, which further emphasizes the importance of Akt activity in pancreatic β cells (Gu et al, 2011). To elucidate the role of mTORC1 signaling, conditional deletion of TSC2 ($\beta TSC2^{-/-}$) in pancreatic β cells was implemented, and mice displayed lower serum glucose levels, improved glucose tolerance and increased β cell mass (Rachdi et al, 2008). Similarly, β cell-specific ablation of TSC1 ($\beta TSC1^{-/-}$) enhanced β cell function due to increased mTORC1 activity (Mori & Guan, 2012). Finally, upregulation of mTORC1 signaling through Rheb overexpression in pancreatic β cells increased β cell mass and maintained euglycemia which further confirms the essential role of insulin signaling in pancreatic β cell function (Hamada et al, 2009).

1.4 Pancreatic β cell-associated diseases

1.4.1 Type 1 diabetes (T1D)

Type 1 diabetes, formerly known as insulin-dependent diabetes, was first described in the 1970s as a chronic autoimmune disease caused by T-lymphocyte cell-mediated autoimmune attack on pancreatic β cells leading to their destruction and absolute insulin deficiency (Bottazzo et al, 1974; Atkinson & Maclaren, 1994). T1D results in hyperglycemia and ketoacidosis, where the body switches to metabolizing fatty acids and producing ketone bodies, which if left untreated leads to death. It affects people with genetic susceptibility in combination with certain

environmental factors that together contribute to the development of autoimmune attack on β cells (Atkinson & Eisenbarth, 2001). By the time of diagnosis, 70-80% of pancreatic β cells are destroyed (Rowe, 1923). T1D accounts for about 5-10% of diabetic cases and mostly appears during childhood. In fact, it is the primary cause of diabetes in children under 10 years (Daneman, 2006). Standard treatment for type 1 diabetic patients consists of administration of exogenous insulin, that could be provided manually or through an automatic insulin pump. More experimental treatments include artificial pancreas, which combines glucose monitoring and insulin pump, and islets transplantation. However, the success rate of artificial pancreas is still quite low and islets transplantation requires permanent use of immunosuppressive drugs to avoid destruction of the transplanted islet cells, which makes these approaches still not widespread. Therefore, T1D still remains a major cause of morbidity in affected patients. Many studies have been dedicated to identifying and understanding risk factors that could serve as predictors of this disease to prevent its development. Combination screenings of genetic, immune and metabolic markers provided interesting candidates potentially predicting the risk to develop T1D. One of these markers is the islet cell autoantibodies (ICA), which helped in understanding T1D in terms of classification and diagnosis (Bottazzo et al, 1974). One of the most well studied animal model for T1D is the non-obese diabetic mouse (NOD) (Makino et al, 1980) which revealed that genetic predisposition comes from mutations in major histocompatibility complex (MHC), but other loci also influence the risk factor for the disease (Melanitou et al, 2003; Atkinson & Rhodes, 2005). Other susceptibility genes identified are HLA haplotypes DR3 and DR4. In addition, T1D can be caused by environmental factors such as viral infection (Fairweather & Rose, 2002), toxins or stress that lead to autoimmune destruction of pancreatic β cells. Although T1D is unpreventable, it is suggested that immunosuppressive drugs can prevent pancreatic β cell destruction during the latent

autoimmune stage (Bluestone et al, 2010). Immunosuppressive therapies were also found to slow T1D progression (Herold et al, 2013; Lebastchi et al, 2013).

1.4.2 Type 2 diabetes (T2D)

T2D, which accounts for 80% of overall diabetes incidence, is a growing disease estimated to affect 300 million people worldwide by 2025 (Zimmet et al, 2001). The fast growth of T2D is attributed to the modern changes in lifestyle, where nutrient intake increase came in parallel with a decrease in physical activity. The main hallmarks of T2D are insulin resistance and β cell dysfunction. It is well-accepted that prior to becoming diabetic, people develop insulin resistance in insulin-responsive tissues, leading to fasting hyperglycemia (Martin et al, 1992). As a result of insulin resistance, pancreatic β cells undergo expansion in order to compensate for the increased demand to produce more insulin (Dor et al, 2004; Rankin & Kushner, 2009). Resilient β cells are capable of handling increased insulin demand to keep the glucose levels under control. However, sustained demand imposed on β cells triggers damage and exhaustion leading to apoptosis, and consequently development of T2D. Therefore, the turning point in the development of T2D is the onset of β cell dysfunction (Porte, 2001; Prentki et al, 2002). T2D is strongly associated with insulin resistance and obesity. In particular, visceral obesity is a leading factor in developing insulin resistance, and an important risk factor to develop T2D. However, not all insulin resistant subjects develop T2D, and only 20% of obese subjects develop T2D (Meier, 2008). Studies on human islets from obese non-diabetic patients showed an increase in β cell volume, indicating an ability for compensatory expansion in response to obesity-induced insulin resistance (Butler et al, 2003). On the other hand, T2D patients, whether obese or non-obese, displayed a decrease in β cell mass compared to weight-matched non-diabetic subjects, indicating that loss of β cell mass is

a determinant factor in T2D development (Butler et al, 2003). In addition, it was determined that β cell apoptosis is the main mechanism by which β cell mass is lost (Butler et al, 2003). These studies confirm that not all obese subjects develop diabetes, depending on the ability of their pancreatic β cells to compensate, which can be influenced by genetic factors (Marchetti et al, 2008). It is reported that at the time of diagnosis, more than 50% of β cell mass is lost, and this loss starts years before diagnosis (Holman, 1998).

Pancreatic β cells synthesize and secrete insulin in response to glucose to ensure tight regulation of glucose/energy metabolism and homeostasis. Although physiological levels of glucose and fatty acids are required to establish a healthy pancreatic β cell function, chronic exposure to nutrients contributes to β cell dysfunction through various mechanisms. The terms used in literature to refer to these conditions are glucotoxicity, lipotoxicity and glucolipotoxicity (Unger & Grundy, 1985; Unger, 1995; Prentki et al, 2002). In studies on rat insulinoma pancreatic INS832/13 β cells and human islets, the effects of glucolipotoxicity on β cell dysfunction were found to be synergistic compared to the separate effects of glucotoxicity or lipotoxicity (El-Assaad et al, 2003). One of the main reasons that continuous exposure of β cells to increased levels of glucose and fatty acids leads to pancreatic β cell dysfunction is the fact that increased insulin demand triggers reactive oxygen species (ROS) production (Tanaka et al, 2002). Indeed, insulin biosynthesis by β cells involves extensive endoplasmic reticulum oxidative folding activity to support the formation of disulfide bonds that are required for assembly of proper folded insulin precursor. Experimental data demonstrated that insulin biosynthesis results in extensive production of side products such as reactive oxygen species (ROS) (Tu & Weissman, 2004; Sevier & Kaiser, 2008). Accumulation of such reactive side products or any defect in processes regulating

them can significantly contribute to β cell dysfunction on the long term (Prentki & Nolan, 2006). Pancreatic β cells are particularly vulnerable to oxidative stress (Robertson RP, 2003) due to low expression of anti-oxidant enzymes (Baynes, 1991) and eventually, insulin synthesis decreases in exhausted pancreatic β cells. In addition, glucolipotoxicity shifts the fatty acid metabolism mechanism to produce toxic material such as ceramides (Chang-Chen et al, 2008). Moreover, glucolipotoxicity leads to aberrant DNA methylation of genes involved in β cell function, which impairs insulin at the transcriptional and translational levels (Hu et al, 2014). Although the characteristics and features of pancreatic β cell failure have been the focus of many studies, the importance of each feature and the mechanisms underlying them are still subject of investigation. Research aiming at identifying markers of pancreatic β cell dysfunction and providing a better mechanistic understanding of pancreatic β cell physiology are crucial determinants for the development of novel therapies to prevent and/or cure T2D in humans.

1.4.3 Maturity-onset diabetes of the young (MODY)

Maturity-onset diabetes of the young (MODY) is one of the different subtypes of monogenic diabetes. MODY is a non-autoimmune, autosomal dominantly inherited single gene disorders resulting in primary defects of pancreatic β cell function. These underlying causes of β cell defects are mutations in single genes involved in developmental and/or functional aspects of pancreatic β cell physiology (Murphy et al, 2008). MODY, as the name implies is usually diagnosed at a very young age up to young adulthood in subjects presenting with hyperglycemia and typical diabetes symptoms, even though many do not represent symptoms and are identified for their strong familial diabetes inheritance. Although MODY patients have defects in β cell function, residual endogenous insulin secretion persists in patients. Several subtypes of MODY

have been identified, each caused by mutations in a specific gene involved in pancreatic β cell function. MODY1 is caused by mutations of the transcription factor hepatic nuclear factor 4α (*HNF4A*), which is essential for early pancreatic development as well as expression of essential β cell genes (Vaxillaire & Froguel, 2008). MODY2 is caused by mutations in the glucokinase enzyme (*Gck*) involved in glycolysis of glucose (Froguel et al, 1993). MODY3 is caused by mutations in the hepatocyte nuclear factor 1α (*HNF1A*) also involved in differentiation of pancreatic β cells. MODY4 results from mutations of the important β cell transcription factor pancreatic and duodenal homeobox 1 (PDX1) (Murphy et al, 2008). MODY1/2/3 are the most frequent subtypes of MODY, with MODY cases accounting for 1-2% of all T2D cases. It is estimated that prevalence of MODY based on the UK population is 70-110 per million (Shields et al, 2010). The importance of correctly diagnosing MODY patients lies in the fact that MODY patients, compared to T2D patients, may not require insulin for treatment and are more sensitive to sulfonylurea medication, a treatment that induces insulin secretion by binding and inhibiting K_{ATP} channels (Vaxillaire et al, 2012). Although MODY is classified as a monogenic form of diabetes, several studies have identified common polymorphisms in genes implicated in MODY to associate with increased risk for T2D (Winckler et al, 2007; Vaxillaire et al, 2008; Voight et al, 2010).

1.4.4 Diabetes as an ER stress disease

A substantial amount of research describes diabetes (T1D and T2D) as an ER stress disease. ER stress is a condition where the ER luminal homeostatic environment is perturbed, by accumulation of improperly folded proteins. Many studies provide evidence showing that diabetes is an ER-stress induced disease. In the case of T1D, pancreatic β cells undergo apoptosis as a result

of the T-cell and macrophage-mediated autoimmune attack, which acts by releasing cytokines into pancreatic β cells. Cytokines induce ER stress by depleting the ER Ca^{2+} reserve, leading to β cell death (Araki et al, 2003; Cardozo et al, 2005). Indeed, restoring the capacity of the β cells to withstand ER stress protected mice against T1D (Engin et al, 2013). In relation to T2D, one of the examples of ER stress-induced T2D is the Akita mouse model (Hong et al, 2007). Akita mice harbor a point mutation in the Insulin 2 gene ($Ins2^{C96Y}$), which impairs folding of proinsulin in the ER. This prevents proinsulin trafficking into Golgi apparatus, and misfolded proinsulin accumulates in the ER, leading to ER stress. Eventually, the unresolvable stress induces dysfunction and apoptosis in pancreatic β cells and leads to development of diabetes (Wang et al, 1999). In this model, the second murine insulin gene *Ins1*, generates a non-mutant proinsulin but is trapped in the ER along with the mutant proinsulin leading to ER stress-induced β cell apoptosis (Wang et al, 1999; Hodish et al, 2010). In humans, accumulation of islet amyloid polypeptide (hIAPP) in pancreatic β cells contributes to development of T2D. IAPP is a polypeptide expressed and secreted along with insulin in human β cells, but accumulation of proIAPP in β cells can generate toxic oligomers leading to ER stress (Haataja et al, 2008). Indeed, islet amyloid deposits were found in islets of 90% of T2D patients (Cnop et al, 2005). In addition, genetic studies showed that mutations in the insulin gene leading to misfolded insulin also lead to diabetes (Steiner et al, 2009). Furthermore, increased expression of ER stress mediators in transplanted islets during isolation may contribute to cell death of transplanted islets (Negi et al, 2012). In conclusion, the development of T2D can evolve as a result of ER stress mediating pancreatic β cell dysfunction. Therefore, cellular mechanisms involved in responding to ER stress must play an essential role in pancreatic β cell function.

1.5 The unfolded protein response (UPR) and its role in pancreatic β cells

1.5.1 ER stress and the UPR: Overview

The ER is the site of secretory and transmembrane protein synthesis, folding, post-translational modification, and vesicle-mediated export in cells. It also acts as a dynamic huge reservoir for Ca^{2+}, as well as a site for lipid synthesis and modification. In the ER, synthesized polypeptides undergo a number of modifications including glycosylation, lipidation, and oxidative disulfide bond formation in order to reach their 'native' correctly folded conformation. Different ER-resident chaperones and enzymes bind to synthesized polypeptides and participate together to generate properly folded proteins, which then undergo anterograde trafficking into the TGN and finally get packaged into vesicles. Thiol-disulfide oxidoredcutases, such as PDI, is one family of chaperones responsible for oxidation, isomerization and reduction of disulfide bonds (Kaufman, 1999; Kaufman, 2002; Ron, 2002). In addition, lectin chaperones such as calnexin (CNX) and calreticulin (CALR) are involved in binding and assisting in the folding of proteins carrying monoglycosylated *N*-linked glycans (Ellgaard & Frickel, 2003). Moreover, chaperone families HSP70, with its main member Bip/Grp78, and HSP90, and its member Grp94 bind to newly synthesized proteins to facilitate folding (Ellgaard & Helenius, 2003). In order to ensure best cellular output and quality control, misfolded or unfolded proteins are recognized by the chaperone proteins and retained in the ER through direct interaction. These improperly folded proteins are then cleared from the ER through activation of ER-associated degradation (ERAD), a process that targets unfolded/misfolded proteins and expels them from the ER to the cytoplasm where they undergo proteasomal degradation (Tsai et al, 2002). ER homeostasis can be perturbed by various conditions including nutrient deprivation, depletion in calcium levels, mutations in proteins

synthesized or ER chaperones, as well as pharmacological agents, all leading to accumulation of unfolded/misfolded proteins in the ER lumen giving rise to ER stress (Lee, 1992). In other words, disequilibrium between proteins entering the ER and the folding capacity of the ER leads to ER stress. ER stress induces activation of a network of signaling pathways called the unfolded protein response (UPR), which counteracts the stress imposed on the ER by increasing the folding capacity of the ER to overcome the stress. Three ER transmembrane proteins are responsible for sensing ER stress and executing the UPR signaling pathways: IRE1α (Inositol-requiring enzyme 1α), ATF6 (Activating transcription factor 6), and PERK (PKR-like endoplasmic reticulum kinase) (Figure 1.10). Once activated, these proteins trigger translational modulation of cellular proteins and transcriptional activation of UPR genes to serve three main purposes: attenuate general protein translation to reduce overload of newly synthesized proteins to process by the ER, increase expression of chaperones and other proteins that contribute to expand the folding capacity of the ER, and finally enhance the clearance of misfolded proteins in the ER. These steps are all aimed to re-establish ER homeostasis. However, when these stress conditions cannot be resolved, damage becomes irreversible and apoptosis is induced by the UPR machinery. Being mainly secretory cells, pancreatic β cells are under constant demand to synthesize and secrete insulin, making it essential for the ER to ensure tight control over production of insulin as well as ensure efficient clearance of any accumulation of misfolded proteins. In the next sections I will discuss the UPR in detail, as well as its role in pancreatic β cells.

1.5.2 IRE1

IRE1 is a type I ER transmembrane protein, which gets anchored to the lipid membrane by a stop signal sequence that directs its N-terminal domain to face the ER lumen side during its

synthesis. IRE1 was first discovered in yeast as a stress sensor (Cox et al, 1993; Mori et al, 1996). As opposed to higher eukaryotes, IRE1 is the only stress sensor expressed in yeast. In mammals, 2 isoforms of IRE1 exist: IRE1α, ubiquitously expressed, and IRE1β, expressed in epithelial cells of the intestine and the stomach (Tirasophon et al, 1998; Wang et al, 1998). IRE1α possesses an N-terminal ER luminal domain, an ER transmembrane domain and a C-terminal cytoplasmic segment harboring distinct Ser/Thr protein kinase and endoribonuclease activities (Cox et al, 1993; Sidrauski & Walter, 1997). IRE1α is kept inactive through its luminal domain interaction with BiP in normal conditions. Upon ER stress, BiP dissociates from IRE1α to bind misfolded proteins, allowing IRE1α dimerization and trans-autophosphorylation. Dimerization of IRE1α appears to be driven by its luminal domain (Zhou et al, 2006), but besides dimerizing, IRE1α also has the ability to oligomerize and form clusters in response to ER stress (Credle et al, 2005). Following dimerization and activation of the kinase activity, the endoribonuclease activity of IRE1α is enhanced mainly due to conformational change of the IRE1α cytoplasmic segment following activation of the kinase domain (Shamu & Walter, 1996; Welihinda & Kaufman, 1996). Endoribonuclease-active IRE1α induces splicing of the X-box binding protein 1 (XBP1) mRNA to remove a 26-base pair intron to produce the spliced XBP1 (XBP1s) upon religation by the tRNA ligase RTCB (Yoshida et al, 2001a; Calfon et al, 2002; Lee et al, 2002; Tanaka et al, 2011; Jurkin et al, 2014; Kosmaczewski et al, 2014). XBP1s is a stable form of the XBP1 mRNA that upon production translocates to the nucleus and transcriptionally activates UPR gene targets (Yoshida et al, 2001a; Calfon et al, 2002). XBP1s has been shown to regulate transcription of UPR-related genes involved in protein folding, protein entry into the ER and ERAD (Lee et al, 2003; Shaffer et al, 2004). In addition, active IRE1α mediates the regulated IRE1-dependent decay (RIDD), a process of regulated decay of specific mRNAs to alleviate protein folding overload imposed on

the ER during ER stress (Hollien & Weissman, 2006; Han et al, 2009; Hollien et al, 2009). In *Drosophila melanogaster*, RIDD was shown to target mRNAs encoding secretory pathway-related proteins (Hollien & Weissman, 2006). The kinase-active IRE1α also recruits Tumour necrosis factor receptor-associated factor 2 (TRAF2), which activates MAP kinase kinase kinase ASK1 leading to Jun N-terminal kinase (JNK) activation (Urano et al, 2000; Nishitoh et al, 2002). JNK activation is believed to promote apoptotic signaling from IRE1α through phosphorylation and inhibition of Bcl-2 and Bcl-XL anti-apoptotic proteins, as well as activation of pro-apoptotic proteins such as Bim and Bid (Weston & Davis, 2007).

IRE1α and its signaling are essential for body function. Systematic deletion of *IRE1α* in mice is embryonically lethal at E12.5 due to liver hypoplasia caused by placental loss of IRE1α (Iwawaki et al, 2009). Re-expression of IRE1α in the placenta rescues the lethal phenotype and the liver function but mice still develop hyperglycemia, hypoinsulinemia, as well as display decreased immunoglobulin (Ig) levels and abnormalities in exocrine pancreatic and salivary gland tissues (Iwawaki et al, 2009; Iwawaki et al, 2010). Liver-specific deletion of *IRE1α* renders liver susceptible to lipid accumulation when exposed to ER stress (Zhang et al, 2011). On the other hand, *IRE1β*$^{-/-}$ mice survive but have increased susceptibility to experimental induction of colitis (Bertolotti et al, 2001). In addition, whole body *XBP1* knockout in mice is embryonically lethal at E12-13.5 due to anemia, hepatic hypoplasia and reduced hematopoiesis (Reimold et al, 2000). Although re-expression of XBP1 in the liver rescues mice from lethality, they die shortly after birth due to deficiencies in secretory organs such as the exocrine pancreas and salivary glands (Lee et al, 2005). Indeed, targeted deletion of XBP1 in B lymphocytes (Reimold et al, 2001; Iwakoshi et al, 2003), macrophages (Martinon et al, 2010), acinar gastric cells (Huh et al, 2010), or

pancreatic acinar cells (Hess et al, 2011) causes dysfunctionalities of these cells. However, liver-specific *XBP1*$^{-/-}$ mice display hypocholesterolemia, hypotriglyceridemia, reduced hepatic lipogenesis (Lee et al, 2008), and are protected against hepatotoxicity (Hur et al, 2012). In conclusion, while XBP1 function was shown to be important in different cell types, XBP1 deficiency in the liver seemed to be beneficial for hepatic cells.

Both IRE1α and XBP1 activities are implicated in pancreatic β cell function. A physiological role for IRE1α in pancreatic β cells was specifically demonstrated and it has been proposed that depending on the intensity and duration of IRE1α activation, different cellular processes and functions are influenced (Lipson et al, 2006). Indeed, transient glucose stimulation triggers physiological activation of IRE1α, which mediates insulin biosynthesis, but chronic exposure to glucose induces IRE1α hyperactivation and XBP1 splicing associated with suppression of insulin biosynthesis (Lipson et al, 2006). Indeed, activation of IRE1α by chronic glucose exposure is believed to trigger decay of insulin mRNA through RIDD (Lipson et al, 2008). Interestingly, chronic activation of IRE1α is associated with insulin resistance and diabetes (Ozcan et al, 2004; Ozcan et al, 2006; Eizirik et al, 2008; Scheuner & Kaufman, 2008). In addition, recent studies suggest that ER stress, through production of XBP1s, and regulation of glucose metabolism and insulin secretion leads to T2D (Park et al, 2010; Winnay et al, 2010; Zhou et al, 2011). On the other hand, β cell-specific deletion of XBP1 leads to reduced GSIS, β cell loss and hyperglycemia, as well as hyperactivation of IRE1α which triggers RIDD-dependent degradation of proinsulin mRNA (Lee et al, 2011). Therefore, physiological activation of IRE1α and its downstream signaling pathway contributes to maintaining proper pancreatic β cell function, while chronic activation of the pathway can have detrimental effects on pancreatic β cell function.

1.5.3 ATF6

ATF6 is a type II ER transmembrane protein, which is targeted to the lipid membrane by a signal anchor sequence that targets its C-terminal domain towards the ER lumen side during synthesis at the ER membrane. ATF6 is also kept inactive through its association with BiP at the ER lumen (Yoshida et al, 1998; Haze et al, 1999). In mammalian cells, ATF6 exists in two isoforms, α and β, both ubiquitously expressed and localized at the ER (Yoshida et al, 1998; Haze et al, 2001). ATF6 consists of an ER luminal domain interacting with BiP, a transmembrane domain and a cytoplasmic segment containing a basic leucine zipper (bZIP) transcription factor moiety (Haze et al, 1999; Yoshida et al, 2000). Upon ER stress, BiP dissociates from the luminal portion of ATF6, allowing ATF6 to translocate to the Golgi (Chen et al, 2002; Shen et al, 2002). At the Golgi, ATF6 is cleaved within its luminal domain by Site-1 protease (S1p), and within its lipid bilayer spanning domain by Site-2 protease (S2p), releasing a N-terminal cytosolic fragment (ATF6f) that translocates to the nucleus to act as a transcription factor (Haze et al, 1999; Ye et al, 2000). Once in the nucleus, ATF6 binds to ER stress response element (ERSE) (Yoshida et al, 1998; Yoshida et al, 2000; Yoshida et al, 2001b) to activate UPR genes involved in protein folding and ERAD (Haze et al, 1999; Lee et al, 2002; Yamamoto et al, 2007).

Since *ATF6α/β* knockout mice were embryonically lethal (Yamamoto et al, 2007), several studies focused on investigating the role of ATF6 α and β separately. Whole body knockout of *ATF6α* mice kept on HFD display hepatic and renal steatosis, accompanied by glucose intolerance, suggesting a role for ATF6 in regulating these processes (Usui et al, 2012). While the wild-type mice fed on HFD exhibited expansion of pancreatic β cell mass, *ATF6α*-null mice failed to do so and displayed pancreatic β cell ER stress (Usui et al, 2012). In addition, *ATF6α* deficiency in Akita

heterozygous mice exaggerated the Akita phenotype by displaying increased hyperglycemia and reduced insulin content compared to heterozygous Akita mice (Usui et al, 2012). Altogether, these studies suggest that ATF6 is important for pancreatic β cell homeostasis. Furthermore, depletion of ATF6α in rat insulinoma pancreatic β cells (INS-1 832/13) enhanced ER stress-dependent cell death, accompanied by reduced BiP mRNA and protein levels, and increased JNK activation (Teodoro et al, 2012). Although *ATF6β*$^{-/-}$ mice and mouse embryonic fibroblasts (MEFs) lacking *ATF6β* were indistinguishable from their wild-type counterparts (Yamamoto et al, 2007), silencing ATF6β in INS-1 832/13 cells increased susceptibility of β cells to apoptosis under chronic ER stress conditions, but did not impair induction of most UPR genes (Odisho et al, 2015).

1.5.4 PERK

PERK is a type I ER transmembrane protein that consists of an ER luminal domain, a transmembrane domain and a cytoplasmic domain which harbors a Ser/Thr protein kinase activity (Figure 1.11A) (Harding et al, 1999). The luminal domain of PERK regulates its activation by interacting with BiP. Under ER stress, dissociation of BiP allows PERK dimerization and activation following PERK autophosphorylation on several sites including Thr^{980} in the activation loop of the kinase domain (Harding et al, 1999; Bertolotti et al, 2000). PERK was isolated as a gene encoding an eIF2α kinase expressed at high levels in the pancreas (Shi et al, 1998; Sood et al, 2000). Indeed, activated PERK phosphorylates the eukaryotic initiation factor 2 α-subunit (eIF2α) on Ser^{51} (Harding et al, 1999; Marciniak et al, 2006). The uniqueness about PERK compared to the other ER stress sensors is that PERK by phosphorylating eIF2α, has the ability to attenuate general protein synthesis (Sood et al, 2000), thereby decreasing the protein load imposed on the ER during stress conditions. Paradoxically, PERK-induced attenuation of translation

increases translation of specific proteins such as the activating transcription factor 4 (ATF4), a transcription factor regulating the expression of a number of genes involved in cellular processing such as lipid and amino acid metabolism, protein folding, oxidative stress and apoptosis (Scheuner et al, 2001; Vattem & Wek, 2004). Overall, PERK regulates various mechanisms that aim to reduce ER load of new proteins to process, and produce proteins that contribute to re-establish ER homeostasis. In addition to regulating general protein synthesis through eIF2α phosphorylation in ER stress conditions, PERK also regulates the ability of cells to survive oxidative stress by phosphorylating and activating the nuclear factor erythroid 2-related factor 2 (Nrf2), a transcription factor involved in activating an antioxidant cell response (Cullinan et al, 2003).

Interestingly, apart from being a Ser/Thr protein kinase, recent studies confirmed that PERK also displays a tyrosine protein kinase activity. While earlier studies using mass spectrometry showed that PERK can be phosphorylated on tyrosine residues (Ma et al, 2001), autophosphorylation of a specific tyrosine residue (Y^{615}) in the PERK kinase domain was implicated in optimal activation of PERK in response to ER stress (Su et al, 2008). In Chapter 2, I will also report the identification of a novel phosphotyrosine residue (Y^{561}) in the PERK juxtamembrane domain, which plays a significant role in limiting PERK activation. Altogether, these findings reveal that in addition to being regulated by phosphorylation on threonine residues, tyrosine phosphorylation on specific sites differentially regulates PERK activation.

1.5.4.1 Structural basis of PERK activation

The crystal structure of the mouse PERK kinase domain reveals PERK as a homodimer and that PERK phosphorylated on Thr^{980} adopts a conformation ready to bind eIF2α (Cui et al,

2011). Based on structural analysis, a "line-up" model for activation of PERK was proposed, where a PERK dimer in a back-to-back conformation approaches another dimer to perform trans autophosphorylation to activate each other's kinase domain (Cui et al, 2011). It was also suggested that unfolded proteins could bind multiple PERK luminal domains, allowing oligomerization of PERK homodimers, but this model still needs additional validation. Also, this study shed light on the structural components of the kinase domain, which contains two distinct subdomains: the small N-terminal and the long C-terminal lobes. While the N-lobe provides an interface for PERK dimerization, the C-lobe forms a long activation loop where Thr^{980} is phosphorylated, providing further stabilization to the activation loop, which then acts as a platform for substrate phosphorylation (Cui et al, 2011). Based on the homology between the luminal domains of PERK and IRE1α, it was suggested that the mechanism by which unfolded proteins induce PERK and IRE1α activation is highly similar. PERK and IRE1α, both bind to BiP in an ATP-dependent manner, while ADP-bound BiP has a higher affinity for unfolded proteins than the sensors, leading to BiP dissociation when ATP levels are low during ER stress (Sou et al, 2012). Finally, a recent study reported a novel tetrameric organization of the luminal domain of PERK, which could correlate with higher state of PERK activation (Figure 1.11B) (Cararra et al 2015).

1.5.4.2 PERK signaling: phosphorylation of eIF2α

The eukaryotic initiation factor 2 (eIF2) is required for initiation of translation, and is regulated by guanine nucleotide exchange and phosphorylation. EIF2 is composed of three subunits: α, β, and γ. The α subunit is a regulatory subunit phosphorylated on a serine residue at position 51 (Ser^{51}) in various stress conditions (Harding et al, 1999; Sood et al, 2000). The β subunit is involved in interacting with mRNA and recognizing the initiation codon on mRNA. It

also interacts with other initiation factors such as eIF2B, and other proteins such as Nck1 (Kebache et al, 2002; Hinnebusch, 2005). The γ subunit is the main docking site for either GDP or GTP, and contains a tRNA binding cavity. When bound to GTP, active eIF2 forms the 43S pre-initiation complex with the methionine-loaded initiator methionyl tRNA (Met-tRNA) and the 40S ribosomal subunit to initiate translation. The 43S complex is required to associate with the target mRNA along with other eIFs to scan for the initiation codon AUG. Once the initiation codon is reached, GTP on eIF2 undergoes hydrolysis and eIF2 is released from the complex in an inactive form (Gebauer & Hentze, 2004). In order to reactivate eIF2, the guanine nucleotide exchange factor eIF2B interacts with eIF2 and promotes the exchange of GDP for GTP, then eIF2 is released in an active GTP-bound form (Pavitt, 2005) (Figure 1.12). When PERK gets activated, it phosphorylates eIF2α on Ser^{51}. eIF2B, which is expressed in limited amount compared to eIF2, has higher affinity for phosphorylated eIF2αSer^{51} than non-phosphorylated eIF2 (Sudhakar et al, 2000). This leads to sequestration of eIF2B and attenuation of global protein translation due to reduced GDP-GTP exchange on eIF2, and subsequently reduced initiation of translation (Rowlands et al, 1988). Early studies on the role of PERK in mouse embryonic stem cells showed that loss of PERK sensitizes cells to ER-stress induced cell death due to loss of eIF2α phosphorylation (Harding et al, 2000b). Decreasing the load of translation by cyclohexamide (inhibitor of protein biosynthesis) was able to ameliorate the cell phenotype (Harding et al, 2000b), showing that regulation of general protein synthesis through PERK-eIF2α is important for the cellular adaptive response to stress.

Interestingly, eIF2α is also a substrate for other eIF2α kinases which are activated under different cellular stress conditions. In addition to PERK activated upon ER stress, those kinases include: the heme-regulated inhibitor (HRI) activated upon iron or heme deficiency (Han et al,

2001), the general control non-derepressible-2 (GCN2) activated upon amino acid deprivation by uncharged tRNA (Natarajan et al, 2001; Zhang et al, 2002b) and the double-stranded RNA-dependent protein kinase (PKR) activated upon viral infection (de Haro et al, 1996). Although activated by different stresses, the signaling pathway common to eIF2α phosphorylated by all eIF2α kinases is defined as the integrated stress response (ISR).

1.5.4.2.1 ATF4 translational upregulation and signaling

In ER stress conditions, phosphorylation of eIF2αSer^{51} by PERK results in attenuation of protein translation, as a mechanism to reduce protein load on the ER machinery (Harding et al, 1999; Marciniak et al, 2006). But this paradoxically upregulates translation of ATF4 mRNA (Karpinski et al, 1992; Harding et al, 1999; Koumenis et al, 2002; Ameri et al, 2004; Lu et al, 2004). ATF4 belongs to the ATF/CREB (activating transcription factor/ cyclic AMP response element binding proteins) family of basic-region leucine zipper (bZIP) transcription factors (Hai & Curran, 1991). ATF4 mRNA contains two upstream open reading frames (uORFs), which act differentially on ATF4 translation (Vattem & Wek, 2004). UORF1 is a positive-acting element, which in conditions of low availability of active eIF2 ternary complex allows initiation of ATF4 translation. On the other hand, during abundance of eIF2 ternary complex, ribosomal scanning and re-initiation occurs also at the uORF2. UORF2 acts as an inhibitory element in ATF4 translation, because it overlaps the coding region of ATF4 and engages a stop codon (Figure 1.13). Under ER stress conditions, phosphorylation of eIF2α leads to a decrease in active ternary complex formation. This gives time for the ribosomes to scan through uORF2 of ATF4 without re-initiation. By the time the ternary complexes associate with the scanning ribosome, the coding region of ATF4 has been reached (Vattem & Wek, 2004). As an active transcription factor, increased levels

of ATF4 promote transcriptional activation of genes involved in amino acid biosynthesis transport, metabolism and resistance to oxidative stress (Harding et al, 2003). In addition, ATF4 transcriptionally activates genes encoding ER chaperones including BiP and Grp94, UPR-associated transcription factors such as XBP1, and components of the machinery for ATF6 processing. One of the most characterized targets of ATF4 is the CCAAT/enhancer binding protein (C/EBP) homologous protein (CHOP), a bZIP transcription factor and mediator of ER stress-induced apoptosis (Ron & Habener, 1992; Ma et al, 2002). CHOP activates a number of pro-apoptotic genes including death receptor 5 (DR5) and the pro-apoptotic member of Bcl-2 family, Bim (Puthalakath et al, 2007). CHOP also induces the expression of growth arrest and DNA damage-inducible 34 (GADD34), a regulatory subunit of the phosphatase complex PP1 responsible for dephosphorylating eIF2α (Connor et al, 2001). GADD34 is part of a negative feedback mechanism that controls protein translation re-initiation (Brush et al, 2003; Ma & Hendershot, 2003; Novoa et al, 2003). Indeed, induced expression of an active GADD34 that induces dephosphorylation of eIF2α leads to inhibition of ATF4 expression (Blais et al, 2004). Moreover, ATF4 enhances expression of autophagy genes including *MAP1LC3B*, *ATG5*, *ATG12*, and *BECN1* (Rouschop & Wouters, 2009; Rzymski et al, 2009; Cao & Kaufman, 2012). Autophagy is a conserved lysosomal-based degradation process of intracellular constituents. It has been proposed that autophagy plays a prosurvival role as part of the adaptive response of the UPR (Bernales et al, 2006; Yorimitsu et al, 2006). Stable ATF4 expression was shown to protect cells by inducing autophagy through upregulation of the microtubule-associated protein 1 light chain 3 beta (LC3B), an important component in the autophagy process (Milani et al, 2009). ATF4 also indirectly enhances autophagy through upregulation of sestrin2 (Sesn2) protein expression (Bruning et al, 2013). Sesn2 belongs to a family of stress-inducible proteins known to maintain

metabolic homeostasis against age- and obesity-related diseases (Budanov et al, 2002). Sesn2 expression leads to activation of AMPK, which in turn plays a role attenuating mTORC1 activity, thereby releasing the inhibition imposed by mTORC1 activity on the autophagy process (Budanov & Karin, 2008; Lee et al, 2010). Therefore, this pathway is proposed to play an important role in regulating cell survival upon ER stress (Figure 1.14).

1.5.4.3 PERK signaling: phosphorylation of Nrf2

The second identified direct PERK substrate is the transcription factor Nrf2 (erythroid-derived 2-like 2), involved in PERK-dependent cell survival (Cullinan et al, 2003). Nrf2, part of the Cap 'n' Collar (CNC) family of basic leucine zipper (bZIP) transcription factors (Andrews et al, 1993), is ubiquitously expressed and activated in response to oxidative stress (Hayes & McMahon, 2001). Nrf2 is regulated by the E3 ubiquitin-protein ligase Cullin3 (Cul3) and the substrate adaptor Keap1 (Kelch-like ECH-associated protein 1). In normal conditions, Keap1 represses Nrf2 expression by directly binding to the Neh2 (Nrf2-ECH homology) domain of Nrf2 (Itoh et al, 1999), allowing Cul3-dependent ubiquitination and proteasomal degradation of Nrf2 (Cullinan et al, 2004; Zhang et al, 2004; Furukawa & Xiong, 2005). Under oxidative stress, Keap1 dissociates from Nrf2, allowing Nrf2 translocation to the nucleus (Figure 1.15). Different mechanisms were proposed to explain Keap1 dissociation from Nrf2. In one model, oxidative and electrophilic stress leads to an attack on the cysteine residues of Keap1, which induces Keap1 conformational change and disrupts interaction with Nrf2 (Zipper & Mulcahy, 2002). It was also proposed that phosphorylation of Nrf2 introduces a modification that favors dissociation of Nrf2 from Keap1 (Huang et al, 2000). This is likely the mechanism by which PERK enables Nrf2 translocation to the nucleus. Dissociation of the Nrf2-Keap1-Cullin3 complex allows Nrf2

stabilization in the cytoplasm and subsequent translocation to the nucleus. Once in the nucleus, Nrf2 heterodimerizes with the small Maf transcription factors, and induces transcriptional activation of antioxidant response element (ARE)-containing genes such as HO-1 (Heme-oxygenase 1) and NQO1 (NADPH dehydrogenase quinoneoxireductase 1) (Itoh et al, 1997; Chan & Kan, 1999). Interestingly, Nrf2 is also reported to dimerize with ATF4 to promote expression of survival genes (He et al, 2001). In addition to its activation of antioxidant gene expression, Nrf2 contributes to expression of genes involved in proteasome-mediated ERAD in pancreatic β cells under ER stress (Lee et al, 2012b).

1.5.4.4 PERK links between ER and oxidative stress

Elucidating signaling pathways triggered by PERK helped in understanding its crucial role in coordinating various cellular stress responses. Indeed, Nrf2 being a master regulator of oxidative stress response and ATF4 being a key player in mediating the ER stress response are both under the regulation of PERK. In addition, both ATF4 and Nrf2 interact to promote expression of antioxidant response element-dependent genes (He et al, 2001). On the other hand, increasing the capacity of the ER for protein folding can enhance the production of ROS, which then initiates a strong antioxidant response to protect cells against damage. Therefore, PERK activation of both, ATF4- and Nrf2-pathways, contribute to promote cell survival against stress conditions (Cullinan & Diehl, 2006). It is known that ubiquitin-mediated proteolysis and ERAD machinery are also enhanced during ER stress (Jarosch et al, 2003). PERK-mediated Nrf2 activation leads to expression of genes also involved in proteasomal degradation (Kwak et al, 2003), further emphasizing the role of Nrf2 in enhancing the ability of the cell to survive during stresses.

1.5.4.5 Role of PERK in pancreatic β cells

The first indication of a role for PERK in pancreatic β cell physiology was the discovery of mutations in the gene encoding PERK, the eukaryotic initiation factor 2α kinase 3 (*EIF2AK3*), in Wolcott-Rallison Syndrome (WRS) patients (Delepine et al, 2000). WRS was first described by Drs. Wolcott and Rallison, who identified 3 affected siblings with an early-onset form of diabetes (Wolcott & Rallison, 1972). WRS is an insulin-requiring diabetes that manifests during the first 6 months of life, where patients are diagnosed with hyperglycemia. In addition, WRS patients manifest other characteristics including skeletal dysplasia, hepatic dysfunction, renal failure, exocrine pancreatic insufficiency, as well as mental retardation (Senee et al, 2004; Rubio-Cabezas et al, 2009; Ozbek et al, 2010). Although WRS is a rare autosomal recessive disease, it is the most frequent cause of permanent neonatal diabetes mellitus in consanguineous families (Rubio-Cabezas et al, 2009; Julier & Nicolino, 2010). Different mutations in *EIF2AK3*, all resulting in PERK loss of function have been reported (Senee et al, 2004; Rubio-Cabezas et al, 2009; Ozbek et al, 2010). These include nonsense or frameshift mutations leading to premature termination of PERK or missense mutations in the PERK kinase domain. All missense mutations are located within or in the vicinity of the PERK kinase domain, whereas frameshift and nonsense mutations are spread out over the length of the protein (Julier & Nicolino, 2010).

The mouse model of *PERK* deficiency remarkably reproduces the WRS characteristics. Briefly, *PERK* deficient mice display permanent neonatal diabetes, growth retardation, skeletal dysplasia, hepatic dysfunction and pancreatic exocrine deficiency. These mice display hyperglycemia and reduced insulin serum levels shortly after birth, associated with a progressive loss of pancreatic β cells, which was initially attributed to apoptosis (Harding et al, 2001; Zhang

et al, 2002a). Exocrine pancreatic insufficiency and luminal distension of the ER in both endocrine (islets) and exocrine (acinar) tissues were also observed (Harding et al, 2001). In addition, *PERK* knockout mice display skeletal dysplasia and bone anomalies making mice smaller in size compared to wild-type mice (Zhang et al, 2002a). Later on, extensive studies performed using different pancreatic and β cell-specific *PERK* deletion demonstrated that specific loss of PERK in pancreatic β cells during fetal development led to diabetes. Indeed, the loss of β cell mass due to *PERK* deficiency was a result of insufficient β cell differentiation and proliferation, as opposed to β cell death (Zhang et al, 2006). At birth, β cell mass was lower in *PERK* deficient mice, and the difference was exacerbated with time. The fact that β cell mass expands during late fetal and neonatal stage (Kaung, 1994) accounts for the progressive difference seen between wild-type and *PERK* knockout mice, where wild-type mice expand β cell mass while *PERK* knockout mice fail to do so, and with time hyperglycemia develops (Zhang et al, 2006). In addition, induced expression of PERK in pancreatic β cells in *PERK* knockout mice rescued β cell function and diabetic phenotype (Zhang et al, 2006). Further studies using adenoviral-mediated expression of a dominant negative PERK (AdDN-PERK) in rat insulinoma β cells (INS832/13) demonstrated that PERK activity is required for insulin transcription, content and secretion, as well as β cell proliferation (Feng et al, 2009). Although insulin content was decreased by expressing a dominant negative PERK in INS832/13 cells, retention of proinsulin in the ER was also observed, without evidence of increased protein synthesis. Confirmed in *PERK*-deficient pancreatic β cells (Gupta et al, 2010), these findings challenge the belief that dysfunction of *PERK*-deficient β cells is due to ER stress secondary to uncontrolled protein translation associated with overload of the ER. Interestingly, accumulation of proinsulin was attributed to defects in ER and Golgi anterograde trafficking as supported by the finding that downregulation of PERK using shRNA in INS832/13

cells impaired proinsulin trafficking (Gupta et al, 2010). Recently, attempts to uncouple the role of PERK in pancreatic embryogenesis from its role in mature pancreatic β cells was made possible using PERK small molecule inhibitors in pancreatic β cells (Harding et al, 2012). In fact, inhibition of PERK activity in β cells using the small inhibitor GSK2606414 led to increased proinsulin synthesis, as well as accumulation of immature proinsulin in the ER. These findings on the role of PERK on insulin trafficking contrast those previously reported by Gupta et al. (2010). However, the degree of inhibition of PERK activity can account for the difference. In fact, in Gupta et al. (2010) study, PERK was downregulated using shRNA, while in the other, Harding et al. (2012) used a pharmacological PERK inhibitor, which might lead to a more profound inhibition of PERK activity than shRNA. Nonetheless, a significant role of PERK in proper trafficking of proinsulin through the ER and Golgi has been clearly established.

Even though studies using various mice models mainly limited the role of PERK to the early stages of neonatal development (Zhang et al, 2006), a recent study reported that PERK is also essential to maintain adult pancreatic β cell function (Gao et al, 2012). Through the generation of an acutely inducible *PERK* knockout model, it was shown that the loss of PERK in adult pancreas results in progressive development of hyperglycemia due to insufficient insulin production. This was associated with a reduction in islets size and integrity, preceded by accumulation of immature insulin and compromised β cell morphology associated with ER distension. Even though β cell proliferation markers were normally induced, proliferation was not sufficient to maintain β cell mass, as β cell death was observed secondary to the loss of *PERK*. Finally, β cell-specific *PERK* deletion in mice led to rapid development of diabetes, suggesting an autonomous effect of *PERK* deletion in β cells (Gao et al, 2012). The discrepancy between this

study and Zhang et al. study on PERK's role during embryogenesis (Zhang et al, 2006) might be explained by the approaches used to knockout *PERK*, as previously, embryonic *PERK* deletion was driven during late stages of embryogenesis, thereby probably led to a slow decline of PERK levels during embryogenesis (Hamanaka et al, 2005).

1.5.4.6 Role of eIF2α in pancreatic β cells

Further understanding of the role of PERK signaling in pancreatic β cell function came from studies investigating the role of eIF2α. Early studies showed that mice harboring homozygous knockin of eIF2αSer51Ala mutant ($eIF2\alpha^{A/A}$), a non-phosphorylatable form of eIF2α, die within hours after birth due to severe hypoglycemia and defective gluconeogenesis (Scheuner et al, 2001). Those mice also display decreased β cell mass leading to deficiency in insulin production, but no deficiencies in other cell types in the islets (Scheuner et al, 2001). To overcome the developmental defect and further understand the role of eIF2α phosphorylation in β cells, β cell-specific eIF2α transgenic mice were generated (Back et al, 2009). Briefly, ubiquitous expression of wild-type eIF2α was induced in $eIF2\alpha^{A/A}$ mice to restore the developmental defect, then using the estrogen receptor-Cre recombinase fusion protein under the control of the rat insulin II promoter, the expression of wild-type eIF2α was deleted. In this model, specific β cell loss of eIF2α phosphorylation led to a severe diabetic phenotype caused by unregulated proinsulin synthesis, defective intracellular ER trafficking, increased oxidative damage, and reduced stress response and gene expression (Back et al, 2009). This study provided a great demonstration for the crucial role of controlling protein synthesis in pancreatic β cells function, and how overloading β cells with proteins to synthesize and process leads to β cell dysfunction and death. Interestingly, these studies, along with the *PERK* deficiency studies also highlighted specificity in defects associated

with the loss of either PERK or eIF2α in pancreatic β cells. Indeed, defects associated with PERK depletion in INS832/13 cells was not related to increased protein translation (Feng et al, 2009), as opposed to pancreatic β cells expressing $eIF2\alpha^{A/A}$ displaying uncontrolled protein translation (Back et al, 2009).

1.5.4.7 Role of Nrf2 in pancreatic β cells

Pancreatic β cells are susceptible to oxidative stress due to low expression of anti-oxidant genes, and the intrinsic nature of β cells in producing ROS associated with insulin synthesis and processing (Li et al, 2008). Recent evidence highlighted a positive role for Nrf2 in protecting pancreatic β cells against various stress. Indeed, several studies reported that Nrf2-silenced β cells are more susceptible to ER stress- and oxidative stress-induced cell death (Lee et al, 2012b; Li et al, 2014b; Fu et al, 2015). In addition, enhanced Nrf2 activity suppressed the onset of diabetes and preserved pancreatic islets in *db/db* mice (Uruno et al, 2013). Finally, activation of Nrf2 secondary to *Keap1* deficiency in mice protects pancreatic β cells against nitrosative and oxidative stress in diabetic mouse models (Yagishita et al, 2014).

1.5.5 PERK signaling in regulating cell fate

Although UPR signaling initiates both prosurvival (Ron & Walter, 2007) and proapoptotic (Hetz et al, 2011) signals upon ER stress, the exact mechanism that switches the cell state from survival to apoptosis still remains an active topic of investigation. The type, intensity and duration of stress and the UPR arms temporal activation pattern are all probably involved in playing a role in determining cell fate. Related to the type of stress, pharmacological ER stress inducers such as Thapsigargin (Tg) can have fatal effects based on induction of irreversible damage, as opposed to

physiological stress conditions that induce the UPR in a milder fashion. Interestingly, secretory cells, such as pancreatic β cells are known to face constant challenges targeting ER homeostasis. For example, transient increase in blood glucose activates the UPR secondary to the acute need of insulin biosynthesis, but activation of the UPR in that case steers towards increasing the folding capacity of the ER, and then shuts off once glucose levels decrease. Therefore, physiological ER stress activates UPR signaling, but not UPR-mediated expression of apoptotic genes (Rutkowski et al, 2006). In addition, differential activation of the UPR arms at different stages during stress can tilt the balance towards cell death. For example, under persistent ER stress conditions, IRE1α activity is shut down in human cells (Lin et al, 2007), while PERK activity is maintained (Lin et al, 2009). In that case, the XBP1s prosurvival signaling is inhibited, while the proapoptotic CHOP expression is induced and leads to cell death (Hetz et al, 2011). Increasing evidence suggests that the structural organization of the UPR sensors upon ER stress is also very important in determining their activity level and type of signal that will be transduced. The structural pattern of organization of the UPR sensors may trigger different UPR activity as a reflection of the intensity of UPR signal in correlation with the ER stress inducer *per se*. For example, artificial dimerization of IRE1α leads to increased XBP1 splicing, but did not induce JNK activation and mRNA degradation (Han et al, 2009). Related to PERK signaling, it is believed that different states of PERK structural organization (dimers vs tetramers) is determinant of the intensity of PERK signaling in switching from prosurvival to proapoptotic (Carrara et al, 2015). Mechanistically, CHOP induction has been shown to be the main mediator of apoptosis in response to ER stress (Rutkowski et al, 2006). However, ER stress conditions that mildly activate the UPR fail to induce cell death even though CHOP levels are increased. This was attributed to the instability of CHOP and GADD34 mRNAs in these conditions. Indeed, it was shown that although low levels of Tg treatment initially

increased CHOP expression, it was lost 24 h later, allowing cells to survive. Therefore, ER stress-induced cell death occurs only when enhanced expression of proapoptotic proteins is maintained in response to prolonged ER stress and UPR activation (Rutkowski et al, 2006). To understand better how PERK signaling leads to cell death, the role of ATF4 and CHOP in this process was further investigated. In a recent study, CHOP function as a direct inducer of apoptotic signals was challenged by the findings that CHOP signal induced by ATF4 is actually enhancing expression of genes involved in protein synthesis, not apoptosis. Indeed, overexpression of CHOP alone did not induce cell death, while ATF4 overexpression did. Chromatin immunoprecipitation (CHIP) and RNA sequencing (RNAseq) analysis demonstrated that ATF4 and CHOP expression in MEFs transcriptionally activate target genes involved in protein synthesis, as opposed to genes involved in inducing cell death. In agreement, increased GADD34 expression following ATF4 and CHOP induction leads to increased protein translation. Thereby, induction of ATF4, CHOP and GADD34, by promoting protein synthesis, generates ROS, decreases ATP levels and leads to cell death if ER stress is not resolved (Han et al, 2013). On the other hand, ATF4 promotes autophagy at least through inducing the expression of sesn2. A remarkable study that distinguished between the effects of ATF4 on autophagy and CHOP induction in relation with cell survival established a strong correlation between ATF4-induced CHOP and cell apoptosis in response to ER stress while ATF4-induced autophagy correlated with enhanced cell survival (Matsumoto et al, 2013). Therefore, a model was proposed in which upon ER stress, ATF4 triggers autophagy, and according to the stress intensity and duration, later ATF4-dependent induction of CHOP leads to apoptosis (Matsumoto et al, 2013). Furthermore, treating cells with different ER stress conditions showed that GADD34 delays CHOP induction by negatively regulating eIF2αSer^{51} signaling, which induces ATF4 and CHOP expression leading to cell death. Indeed, depletion of GADD34

in ER stressed cells enhanced cell apoptosis in correlation with increased CHOP expression in response to tunicamycin-induced ER stress but not sodium fluoride (NaF). This indicates that in cells surviving to GADD34 depletion, an alternative mechanism favoring ATF4-autophagy signal might be activated as opposed to ATF4-CHOP signal (Iwasaki et al, 2015). Therefore, PERK activity and signaling is an essential determinant of cell fate in response to stress, and understanding the mechanisms regulated by PERK is important to understand how and when cells can undergo apoptosis.

1.6 The adaptor proteins Nck

Most cellular processes involve transduction of extracellular signals through RTKs and plasma membrane receptors devoid of catalytic activity to the intracellular environment, triggering a coordinated cellular response (Pawson, 1993). In these processes, adaptor proteins play an important role in assembling molecular complexes that enable signal transduction. Amongst different families of adaptor proteins, the Nck family has been well characterized in coupling signal transduction from activated plasma membrane receptor tyrosine kinases (RTKs) to specific intracellular effectors regulating crucial cellular responses such as actin cytoskeleton reorganization (Pawson & Scott, 1997).

Nck (non-catalytic region of tyrosine kinase) family includes Nck1 and Nck2, which are 47 kDa Src Homology (SH) domain-containing adaptor proteins consisting of three N-terminal SH3 and one C-terminal SH2 domains (Lehmann et al, 1990; Braverman & Quilliam, 1999). Nck are ubiquitously expressed, but at different levels in different tissues, and detected in various intracellular environments including cytosol, nucleus and cytoplasmic surface of the ER (Li et al,

1992; Park & Rhee, 1992; Lawe et al, 1997; Latreille & Larose, 2006). Lacking enzymatic activity, Nck adaptor proteins couple activated plasma membrane receptors with or without protein tyrosine kinase activity, as well as tyrosine phosphorylated cytosolic proteins to effector targets (Li et al, 1992; Park & Rhee, 1992; Nishimura et al, 1993; Holland et al, 1997; Schlaepfer et al, 1997; McCarty, 1998; Stein et al, 1998; Chen et al, 2000). Nck form molecular complexes with tyrosine phosphorylated proteins via the SH2 domain, which recognizes and binds specific phosphotyrosine residues, while the SH3 domains bind proline-rich sequences on effector proteins (Cohen et al, 1995; Pawson, 1995). *NCK1* cDNA was isolated in 1990 from a human melanoma expression library through its non-specific interaction with an antibody directed against the abundant melanoma associated protein MUC18 (Lehmann et al, 1990). Later, a mouse *Nck2* cDNA was isolated from the screening of an expression library using the autophosphorylated cytoplasmic region of the epidermal growth factor receptor (EGFR) (Margolis et al, 1992). Nck homologous genes have also been identified in various species including a unique *Nck* gene in *Drosophila melanogaster* (Dock, Dreadlock) and *Xenopus laevis* (Nck) while two genes encode Nck proteins in human (*NCK1* and *NCK2*), and mouse (*Nck1*, and *Nck2/Grb4*). In mouse, *Nck1* gene is located at q21 locus of chromosome 3 while *Nck2* is located at q21 locus of chromosome 2 (Huebner et al, 1994; Vorobieva et al, 1995). Although encoded by independent genes, Nck1 and Nck2 share 68% amino acid identity (Vorobieva et al, 1995; Chen et al, 1998; Braverman & Quilliam, 1999). The aa identity even increases to 82% in the SH2 domain, and to 78%, 85% and 75% in the 1st, 2nd and 3rd SH3 domains, respectively (Buday et al, 2002). This suggests the existence of functional redundancy between Nck1 and Nck2 to a certain extent, but increasing evidence support also specific roles for each Nck isoform. *In vivo* Nck functional redundancy was demonstrated by the absence of any obvious phenotype following the knockout of either *Nck* in mice (Bladt et al, 2003).

Nonetheless, early embryonic lethality of the double *Nck* knockout embryos revealed a crucial role for these proteins during development (Bladt et al, 2003). The exact cause of lethality is unknown, but fibroblasts derived from these embryos displayed defects in cell motility and lamellipodia formation that might be essential during embryogenesis.

1.6.1 Nck domains and binding partners

As mentioned above, both Nck1 and Nck2 consist of three SH3 domains and a single SH2 domain. Without differentiating between Nck1 and Nck2, the first identified Nck SH2 domain binding partners were activated epidermal growth factor receptor (EGFR) and platelet-derived growth factor receptor (PDGFR) (Chou et al, 1992; Li et al, 1992; Meisenhelder & Hunter, 1992; Park & Rhee, 1992). Via their SH3 domains Nck were found to bind to intracellular proteins such as IRS (Lee et al, 1993), Src (Chou et al, 1992), and Sos (Ras exchange factor) (Hu et al, 1995), thereby assembling molecular complexes involved in intracellular signal transduction. Overall, Nck binding proteins include a wide variety of proteins with different activities or associated with specific cellular functions and the list is growing as research on Nck binding partners keeps advancing (Figure 1.16).

Recent studies improved our understanding of Nck-dependent protein interactions. Indeed, the Nck SH2 domain mediates Nck interaction with phosphotyrosine residues in a specific amino acid sequence matching pYDXV(S/A/T/Y)X(D/E), where pY is the phosphotyrosine residue and X is any amino acid (Songyang et al, 1993), while the SH3 domains have been described to interact with classical PXXP proline-rich motif or non-classical core consensus motif RXXK (Mayer, 2001; Aitio et al, 2008). Although Nck1 and Nck2 share high identity in their SH2 domains,

specific binding partners for each SH2 domain have been reported (Frese et al, 2006). For example, PDGFR was reported to bind both Nck1 and Nck2, but this was involving two different pTyr residues. In fact, PDGFR pY^{751} appears to be a specific binding site for Nck1 (Nishimura et al, 1993) while pY^{1009} is Nck2-specific (Chen et al, 2000). Moreover, a phosphopeptide derived from Tir (translocated intimin receptor), a bacterial protein from enterohaemorrhagic *Eschericia coli*, that is translocated and phosphorylated into the host cell upon infection was identified as the strongest natural binding ligand of Nck1 and Nck2 (Frese et al, 2006). Using sequential mutations of the pTir peptide, optimal binding profiles were generated for both Nck1 and Nck2 SH2 domains. Based on this approach, the Arf GTPase-activating protein GIT1, involved in actin cytoskeletal reorganization, was identified as a binding partner of Nck1 and Nck2 through its pY^{383}. However, it was shown that PDGF pY^{1009} binds by both Nck1 and Nck2 while PDGFR pY^{751} failed to bind Nck, either Nck1 or Nck2 (Frese et al, 2006). Interestingly, few binding partners were found to be exclusive for Nck2. These include the RTKs Ephrin receptor B1 (Cowan & Henkemeyer, 2001; Bong et al, 2004) and Ephrin receptor B2 (Su et al, 2004), and Disabled-1 (Dab-1) (Pramatarova et al, 2003), all of which are proteins mediating neuronal signaling.

1.6.2 Nck regulation

Early studies revealed that Nck is phosphorylated upon activation of various membrane receptors in various cell types (Li et al, 1992; Meisenhelder & Hunter, 1992; Park & Rhee, 1992). Indeed, Park et al. demonstrated that following activation of a variety of membrane receptors such as EGFR and PDGFR, phosphorylation of Nck on Ser/Thr and tyr residues increased and they proposed that this might alter Nck's function (Park & Rhee, 1992). Similarly, epidermal growth factor (EGF) stimulation in A431 human skin carcinoma cells, platelet-derived growth factor

(PDGF) stimulation in murine fibroblast NIH 3T3 cells, and high-affinity receptor for IgE (FcεRI) stimulation in leukemia RBL-2H3 cells, all resulted in enhanced phosphorylation of Nck on tyr, ser and thr residues (Li et al, 1992). Furthermore, Nck was found to be constitutively phosphorylated on ser residues in resting NIH 3T3 cells. Finally, angiotensin II, which binds and activates the G-protein-coupled receptor (GPCR) angiotensin II receptor in rat aortic smooth vascular muscle cells was shown to induce phosphorylation of Nck on ser residues (Voisin et al, 1999). The biological relevance of Nck phosphorylation and Nck phosphorylated sites still remains to be identified, but it is believed that post-translational modifications such as phosphorylation may specifically and temporally regulate Nck interaction with its binding partners or its subcellular localization (Takeuchi et al, 2010).

Nck binding activity was proposed to be regulated by a self-inhibitory intramolecular interaction, where the basic amino acid sequence of the linker region between the SH3.1 and SH3.2 domains interacts with the acidic region of SH3.2 domain (Takeuchi et al, 2010). This intramolecular interaction was suggested to contribute to discriminate between low- and high-affinity binding partners for the SH3.2 domain, with only high-affinity binding partners being efficient in competing with the SH3.2 domain-mediated auto-inhibitory interaction. Interestingly, Nck phosphorylation in the linker region between the SH3.1 and SH3.2 domains can modulate the ability of Nck to interact with other proteins. Indeed, Nck phosphorylation can prevent Nck intramolecular interaction, thus keeping Nck in an open conformation favoring protein interactions (Takeuchi et al, 2010).

Only limited studies addressed regulation of Nck expression, but in the past it has been suggested that Nck expression is modulated by insulin sensitivity in insulin target tissues. In fact,

decreased levels of Nck proteins were observed in liver of streptozotocin-treated rats, a T1D insulin-dependent model (Bonini et al, 1995a), but were significantly increased in adipose tissue and liver of spontaneously obese insulin-resistant diabetic KKAy mice, a T2D non-insulin dependent model (Bonini et al, 1995b). In agreement with lower levels of Nck in insulin sensitive tissues, Nck mRNA levels were decreased in skeletal muscle of endurance-trained rats, which are more sensitive to insulin compared to sedentary rats (Kim et al, 1999). More recently, it was shown that upon T cell receptor (TCR) activation, Nck is ubiquitynated and subjected to proteasomal degradation. Specifically, interaction of the E3 ubiquitin-protein ligase Cbl-b with the SH3 domains of Nck upon TCR activation was reported to be required for Nck ubiquitination (Joseph et al, 2014). In addition, proteasomal-mediated degradation of Nck by fibroblast growth factor 2 (FGF2) prevents apoptosis in cancer cells (Li et al, 2013). Interestingly, Nck1 expression levels were significantly reduced in skeletal muscle from mice lacking the non-receptor tyrosine phosphatase PTP1B, but the underlying mechanism still remains to be elucidated (Panzhinskiy et al, 2013). Finally, upregulation of Nck2, but not Nck1, has been linked to melanoma progression (Labelle-Cote et al, 2011), but here again the mechanism regulating Nck2 expression needs further investigation.

1.6.3 Nck physiological functions

The first clues indicating a biological function for Nck came from studies showing that in *Drosophila melanogaster*, mutations in Dock, the Nck homolog, impair photoreceptor guidance and targeting (Garrity et al, 1996; Hing et al, 1999). From these studies, it was proposed that Nck links cell surface receptors to actin cytoskeletal reorganization (Figure 1.17). In the past decades,

many studies confirmed a role for Nck in regulating actin cytoskeletal dynamics, but through the years, Nck has been also shown to regulate other cellular processes that will be described below.

1.6.3.1 Nck and embryonic development

The earliest study revealing a role for Nck in embryogenesis was performed by Tanaka et al., in which microinjection of Nck mutants in *Xenopus laevis* embryos demonstrated that Nck contributes towards embryonic dorsoventral patterning. Specifically, dominant negative Nck mutants with non-functional SH domains led to embryos with altered mesoderm cell migration during gastrulation (Tanaka et al, 1997). Although this study provided evidence of a role for Nck in embryonic development in vertebrates, it was not until *Nck* knockout mice were generated that we further appreciated the function of Nck in embryogenesis. As reported above, the lack of apparent phenotype of the knockout of either *Nck1* or *Nck2* in mice suggests *in vivo* functional redundancy. However, early embryonic lethality of the double *Nck* knockout embryos at day E9.5 revealed an essential role for Nck during embryogenesis (Bladt et al, 2003). Even though the exact cause of lethality is unknown, *Nck* double knockout embryos failed to undergo chorion-allantoic fusion and axial rotation, as well as closure of cephalic neural folds, suggesting important defects in actin reorganization in these embryos.

1.6.3.2 Nck and actin cytoskeletal remodeling

A wide variety of Nck SH3 binding partners are involved in actin cytoskeletal dynamics (Figure 1.16), in agreement with Nck regulating this process. Indeed, Nck is well-known for its role in linking activated plasma membrane receptors with or without protein tyrosine kinase activity to effector proteins regulating actin polymerization associated with cytoskeletal

remodeling. In fact, through its SH3 domains Nck interacts with WASP (Wiskott-Aldrich syndrome protein) (Rivero-Lezcano et al, 1995), WIP (Wiskott-Aldrich syndrome protein-interacting protein) (Anton et al, 1998), Pak (Hing et al, 1999), and guanine nucleotide exchange factors (such as Trio and Vav) (Pauker & Barda-Saad, 2011) to control cytoskeletal rearrangement in response to a wide variety of external stimuli such as growth factors, stress conditions and extracellular matrix (Figure 1.17). Through these interactions, Nck is implicated in a wide variety of actin-based cellular events and some of them will be discussed below.

1.6.3.2.1 Cell migration

Reorganization of the cytoskeleton is the basis for morphological and functional changes inducing cell migration (Friedl & Wolf, 2010). Cell migration involves a number of steps which include establishing a front-rear axis polarity, cycling of membrane protrusions, followed by adhesion to surface, forward propulsion then disengagement of the rear end of the cell (Lauffenburger & Horwitz, 1996). Actin polymerization requires the activation of actin-related proteins 2 and 3 (Arp2/3) complex (Higgs & Pollard, 1999) under the control of the WASP family proteins (WASP, N-WASP, WAVE and WASH) (Welch & Mullins, 2002; Legg et al, 2007). Activation of WASP proteins is achieved by the small Rho GTPase Cdc42 in collaboration with the membrane phospholipid PIP2 (Egile et al, 1999; Higgs & Pollard, 1999; Prehoda et al, 2000; Rohatgi et al, 2000). The role of Nck in this process is to recruit WASP at the plasma membrane level in close proximity of Cdc42 and PIP2. Therefore, Nck cooperates to achieve WASP activation and stimulation of Arp2/3-dependent actin polymerization (Rohatgi et al, 2001; Tomasevic et al, 2007). Indeed artificial clustering of Nck SH3 domains at the plasma membrane led to localized actin polymerization (Rivera et al, 2004), through a mechanism involving

phosphoinositides and small Rho GTPases Cdc42 and Rac1 in mediating WASP- and N-WASP-activation of Arp2/3 complex (Tomasevic et al, 2007; Rivera et al, 2009). In agreement, Nck was shown to be essential for migration and establishing directionality of endothelial cells by maintaining the front-rear axis of polarity and formation of membrane protrusions and adhesion turnover by activating the small Rho GTPases Cdc42 and Rac1 (Chaki et al, 2013), further contributing to our understanding of the mechanisms by which Nck coordinates cytoskeletal dynamics.

1.6.3.2.2 Axonal guidance

Axonal guidance is a process during neural development by which neurons through their axon precisely reach the right target. Growing axons rely on sensorimotor structures at their leading edge called growth cones (Letourneau & Cypher, 1991; Letourneau et al, 1992). Growth cones direct axon projections based on signal transduction from extracellular cues to changes in actin cytoskeletal dynamics (Tanaka & Sabry, 1995). A well-known example of axonal guidance is the development of *Drosophila melanogaster* eye structure. Each unit of the Drosophila adult eye contains 8 photoreceptor neurons called R cells, each of which sends an axonal projection towards glial cells during the development of the eye structure at the larval/pupal stages. One of the most powerful genetic systems to identify essential components of axonal guidance was the study of mutations impairing R cell axonal projection in the Drosophila eye. Using this approach, Dock, the Nck orthologue, was found to be required for photoreceptor R cell axonal guidance based on the finding that mutations in *Dock* resulted in defects of R cell projection and targeting patterns (Garrity et al, 1996). In addition, Dock was detected at the growth cone of R cells (Garrity et al, 1996) and expression of wild-type human Nck in Dock mutants in Drosophila rescued the

defects in R cell projections (Rao & Zipursky, 1998), emphasizing the importance of Nck in linking growth cone receptor signaling to the actin cytoskeletal changes leading to directional guidance of R cell projections. Mutagenesis analysis also identified Nck domains required for proper axonal guidance in R cells. Through expression of different Nck proteins harboring specific non-functional SH domain mutations, it was found that only the 2nd SH3 domain (SH3.2) was required for R cell growth cone connectivity restoration. Other domains, including the SH2 were redundant and dispensable, although required for other processes such as fiber pattern formation in the inner optic ganglia (Rao & Zipursky, 1998). Furthermore, studies performed to identify signaling mechanisms implicated in axonal guidance involved Drosophila Pak in this process (Hing et al, 1999). Pak not only colocalized at the growth cone with Dock, but directly interacts with Dock SH3.2 domain through its N-terminal PxxP motif (Hing et al, 1999). In agreement with a functional relevance for the Dock-Pak interaction in Drosophila eye development, loss of Pak in R cells, like Dock mutations, resulted in normal axogenesis and extension of R cell axons into the brain, but growth cones failed to establish connectivity with their correct targets (Hing et al, 1999). Mechanistically, Pak comprises of a Ser/Thr kinase domain that gets activated by binding through its CRIB (Cdc42- and Rac-interacting binding) domain to Rho family GTPases Rac1 or/and Ccd42 (Manser et al, 1994; Manser et al, 1997; Sells & Chernoff, 1997). In addition, Pak constitutively binds to Pix (Pak-interacting exchange factor), a guanine nucleotide exchange factor for Rac/Cdc42 (Manser et al, 1998). Therefore, Dock binding to activated guidance receptor leads to the recruitment of the Pak/Pix complex at the growth cone plasma membrane, where by interacting with and activating Rac/Cdc42, results in activation of Pak and associated actin reorganization mediating axonal guidance (Hing et al, 1999).

Through its SH2 domain, Dock associates with at least three axonal guidance receptors, including the fly ortholog of human insulin receptor (InR) (Song et al, 2003), Down syndrome cell adhesion molecule (DSCAM) (Schmucker et al, 2000), and Repulsive guidance receptor Roundabout (Robo) (Fan X et al, 2003) and through it SH3 domains, it constitutively interacts with the netrin receptor DCC (Deleted in Colorectal Cancer) (Li et al, 2002). Dock not only propagates signal transduction from cell surface receptors to intracellular effector proteins, but it also controls signal intensity and duration. By recruiting the non-receptor protein tyrosine phosphatase PTP61F (homolog of human PTP1B), Dock modulates InR signaling in R cells (Wu et al, 2011). Indeed, compound eye overgrowth resulting from InR overexpression in Drosophila was abrogated by co-expressing Dock and PTP61F. Mechanistically, it was shown that Dock, by recruiting PTP61F to the InR, facilitates InR dephosphorylation, thus tightly regulates InR signaling (Wu et al, 2011). On the other hand, Dock was also detected in motor neurons axon growth cones in Drosophila embryo, and loss of Dock during axonogenesis delays synaptic formation by RP3 motor neurons, thus inducing central nervous system defects (Desai et al, 1999). The important role of Nck in motor neuron function has also been demonstrated in mice, where Nck-deficient mice displayed locomoter defects characterized by a hopping gait caused by multiple axon guidance defects (Fawcett et al, 2007).

1.6.3.2.3 Pathogen infection

Nck is implicated in pathogen infection through its role in regulating actin polymerization (Gruenheid et al, 2001; Rietdorf et al, 2001). Enteropathogenic *Esherichia coli* (EPEC), which is responsible for severe infant diarrhea (Chen & Frankel, 2005), following adhesion to intestinal enterocytes (Moon et al, 1983), introduces its bacterial protein Tir (translocated intimin receptor)

into the host cell membrane (Kenny et al, 1997). This allows binding of intimin (bacterial outer membrane protein) to Tir, inducing Tir phosphorylation on tyrosine residues by host cell protein tyrosine kinases (Luo et al, 2000). Gruenheid et al. demonstrated that Nck directly binds activated Tir phosphorylated on pY^{474} through its SH2 domain, mediating through its SH3 domains the recruitment and activation of N-WASP, consequently activating the Arp2/3 complex (Gruenheid et al, 2001). These events initiate actin polymerization and pedestal formation beneath adherent bacteria causing the disease. The role of Nck in this process was further confirmed by showing that *Nck* null host cells are resistant to Tir-induced actin polymerization (Gruenheid et al, 2001). Similarly, Nck is involved in *Vaccinia virus* infection (Frischknecht et al, 1999) . Indeed, following intracellular replication, *Vaccinia virus* triggers the expression of the viral envelope protein A36R, which once phosphorylated on Y^{112} recruits Nck through its SH2 domain. In host cells, this leads to actin polymerization and formation of motile plasma membrane projections beneath the virus, again through Nck-dependent N-WASP and Arp2/3 activation (Frischknecht et al, 1999).

1.6.3.2.4 Nephrin signal transduction in podocytes

Blood filtration by the kidney depends on the integrity of the glomerular filtration barrier, a specialized blood filtration interface consisting of three major components: the fenestrated endothelial cells, the glomerular basement membrane (GBM), and the podocytes (Kriz, 2007). Podocytes are highly differentiated and specialized epithelial cells, which comprise the outer layer of the kidney's blood filtration barrier. Their unique structure results from three structurally and functionally different regions: the main cell body, the major processes and the foot processes (FPs) (Mundel & Kriz, 1995). Major processes extend from the main body and split into FPs, which compose a network of interdigitating actin-rich extensions connected to other FPs from

neighboring podocytes via the slit diaphragm (SD), a specialized multiprotein intercellular junction (Mundel & Kriz, 1995). One key component of the slit diaphragm is a transmembrane adhesion protein called Nephrin (Ruotsalainen et al, 1999). Member of the immunoglobulin (Ig) superfamily, nephrin is critical for formation and maintenance of foot processes in podocytes (Ruotsalainen et al, 2000). Mutations in the nephrin gene, *NPHS1*, result in failure of FPs morphogenesis, and development of congenital nephrotic syndrome of the Finnish type (Kestila et al, 1998). Through its extracellular IgG domains, Nephrin creates *cis* and *trans*-hemophilic interactions with other nephrin molecules on adjacent podocytes, thereby forming the tight junctions of the slit diaphragm (Barletta et al, 2003; Khoshnoodi et al, 2003). Nephrin transduction of extracellular signals from SD to intracellular regulation of podocyte actin dynamics depends on its phosphorylation on tyrosine residues in its cytoplasmic domain by members of the Src family kinases (SFKs) (Verma et al, 2003). In two independent studies, a critical role for Nck in nephrin activation inducing actin remodeling and foot processes formation was uncovered (Jones et al, 2006; Verma et al, 2006). Indeed, podocyte-specific deletion of *Nck2* in *Nck1*$^{-/-}$ mice was shown to prevent formation of foot processes and led to development of nephrotic range proteinuria (Jones et al, 2006). It was shown that upon phosphorylation of nephrin on specific tyrosine residues, Nck binds nephrin through its SH2 domain and induces actin reorganization (Jones et al, 2006; Verma et al, 2006). Interestingly, three nephrin phosphorylated tyrosine residues, Y^{1176}, Y^{1193} and Y^{1217} are contained within the pYDXV sequence matching the perfect consensus binding motif for the SH2 domain of Nck (Jones et al, 2006; Li et al, 2006; Verma et al, 2006; Blasutig et al, 2008). Then, through its SH3 domains, Nck was found to bind N-WASP, leading to Arp2/3 activation and actin cytoskeletal rearrangement (Lu et al, 1997; Rohatgi et al, 2001). In addition, the nephrin-Nck-Pak signaling cascade was suggested to contribute to preserving the

morphological characteristics of podocytes (Zhu et al, 2010). Mechanistically, Nck binding to phosphotyrosine residues on nephrin allows recruitment of Pak to nephrin and Pak's subsequent phosphorylation (Zhu et al, 2010). Further confirming the role of Nck in maintaining the glomerular filtration barrier, loss of *Nck* in adult podocytes in mice led to foot processes abnormal morphology (Jones et al, 2009). Finally, Nck was recently reported to also play a role in nephrin tyrosine phosphorylation (New et al, 2013). Indeed, Nck1 and Nck2 curiously promote nephrin phosphorylation *in vitro* and this process depends on functional Nck SH3 domains. Supporting this, specific loss of *Nck* in mice podocytes, in which podocyte injury occurs, displays loss of nephrin tyrosine phosphorylation (Jones et al, 2009).

1.6.3.2.5 T cell receptor and FasL signaling

T cell receptor (TCR) signaling in T lymphocytes involves activation of signaling mechanisms targeting actin cytoskeletal reorganization. Nck has been reported to be recruited by the activated TCR complex, but its role in mediating TCR signaling and function is controversial (Lettau et al, 2009). T cell receptor signaling relies on activation of the protein tyrosine kinases Fyn, Lck and Zap-70, which induce the formation of molecular complexes implicating adaptor proteins such as Nck (Burkhardt et al, 2008). Nck is involved in TCR signaling regulating actin dynamics through its interaction with CD3ε, a component of the TCR complex, and regulators of actin cytoskeleton like the guanine exchange factor Vav and the adaptor protein SLP-76 (Wunderlich et al, 1999; Gil et al, 2002; Zeng et al, 2003; Takeuchi et al, 2008; Barda-Saad et al, 2010; Borroto et al, 2013). On the other hand, Nck regulates actin cytoskeletal organization in T cells by interacting with TSAd (T cell specific adaptor), which promotes Nck interaction with Lck and SLP-76 (Hem et al, 2015). Whether interaction of Nck with CD3ε, TSAd or both represents

the mechanism whereby TCR activation induces actin polymerization events needs further investigation. However, it is still believed that upon TCR stimulation, Nck modulates actin polymerization by recruiting WASP to molecular activator complexes (Zeng et al, 2003; Barda-Saad et al, 2010). Finally, Nck was recently implicated in survival and differentiation of T cells as well as maintaining the threshold for TCR responsiveness (Lu et al, 2015).

Nck also functionally interacts with the death factor Fas ligand (FasL), a type II transmembrane protein member of the tumor necrosis factors (TNF) family (Suda et al, 1993). In unstimulated T cells and natural killer cells, FasL is kept in special lysosomal vesicles to prevent uncontrolled activation. Upon recognition of a target cell expressing FasL receptor (CD95), FasL-containing lysosomal vesicles translocate to the cell-cell interaction site forming the immunological synapse, where FasL binds CD95 (Lettau et al, 2007; Lettau et al, 2008). Nck, by interacting with a proline-rich motif within the FasL cytoplasmic segment, recruits FasL-containing vesicles to cell-cell contact sites through actin-based modulation (Wenzel et al, 2001; Lettau et al, 2006).

1.6.3.3 Nck and cellular proliferation

The role of Nck in proliferation has been established downstream of EGFR and PDGFR (Chou et al, 1992; Li et al, 1992; Meisenhelder & Hunter, 1992; Park & Rhee, 1992). Nck has also been implicated in oncogenesis where overexpression of Nck in NIH3T3 cells altered cell morphology and induced cell transformation. Indeed, it was demonstrated that Nck overexpression by 5 fold of endogenous levels enhanced formation of foci in NIH3T3 cells (Chou et al, 1992; Li et al, 1992). It was also shown that Nck2 was overexpressed in human metastatic melanoma

compared to non-metastatic melanoma, further suggesting a role for Nck in human cancer (de Wit et al, 2005). Furthermore, human primary melanoma cells overexpressing Nck2 displayed enhanced cell proliferation, migration and invasion *in vitro*, as well increased melanoma-derived tumor growths *in vivo* (Labelle-Cote et al, 2011).

In addition, Nck has been implicated in B cell receptor (BCR)-mediated proliferation and B cell survival, but in contrast to Nck's effects in T cells, Nck appears to modulate B cell processes through actin-independent mechanisms (Castello et al, 2013). Indeed, Nck is recruited to the BCR complex by binding to the Src kinase Lyn, which associates with the immunoglobulin α subunits of the activated BCR complex through the non-ITAM (immunoreceptor tyrosine-based activation motif) phosphorylated pY^{204}. This results in recruitment of BCAP (B-cell adapter for PI3K) by Nck in the BCR complex and activation of PI3K signaling in B cells, thereby promoting B cell proliferation and survival (Castello et al, 2013).

1.6.3.4 Nck and translation

Our laboratory was the first to uncover a role for Nck in modulating protein translation through its direct interaction with the β subunit of eIF2 (Kebache et al, 2002; Kebache et al, 2004; Cardin et al, 2007). Indeed, we showed that Nck1 overexpression enhances cap-dependent and -independent protein translation and in parallel, Nck is detected in polysomes-enriched fractions. Mechanistically, we uncovered that Nck assembles a holophosphatase complex containing the Ser/Thr protein phosphatase PP1c and the adaptor protein CReP (constitutive repressor of eIF2α phosphorylation), which targets PP1c to eIF2 (Figure 1.18) (Latreille & Larose, 2006). Therefore, we proposed that Nck as a component of the CReP/PP1c holophosphatase complex contributes to

maintain eIF2α hypo-phosphorylated, thereby favoring translation. In agreement with a role for Nck in eIF2α phosphorylation, we reported that Nck deficiency in MEFs increases basal levels of eIF2αSer^{51} phosphorylation and signaling (Latreille & Larose, 2006). In addition, we discovered that overexpression of Nck1 selectively limits eIF2αSer^{51} phosphorylation by a subset of eIF2α-kinases, including PERK, HRI and PKR, but not GCN2 (Kebache et al, 2004; Cardin et al, 2007). Although the exact mechanism by which Nck1 modulates only a subset of eIF2α-kinases was not elucidated at that time, these findings suggest an additional mechanism besides mediating eIF2αSer^{51} dephosphorylation. Otherwise, the effects of Nck would have been non selective towards eIF2α kinases. In agreement, a few years later we demonstrated that Nck1 overexpression reduced PKR-mediated eIF2αSer^{51} phosphorylation by directly interacting with and limiting PKR activation (Cardin & Larose, 2008). However, whether this mechanism also accounts for Nck modulation of PERK- and HRI-induced eIF2αSer^{51} phosphorylation, and to what extent GCN2 differs to escape Nck1 regulation still remain to be addressed.

Nonetheless, further supporting a role for Nck in regulating translation, it was recently reported that Nck1 directly assembles a cytoplasmic capping complex including a capping enzyme (CE) and a 5'kinase involved in converting a 5'- end monophosphate mRNA to a 5' diphosphate for subsequent addition of guanylic acid (GMP) by the CE (Mukherjee et al, 2014). Therefore, the impact of Nck on protein translation can be through a combined modulation of eIF2αSer^{51} phosphorylation and cytoplasmic capping.

1.6.3.5 Nck and the UPR

As reported above, our laboratory uncovered that Nck1 modulates ER stress-induced PERK-dependent eIF2αSer^{51} phosphorylation while it also impacts ER stress-induced cell death, thus implicating Nck in the UPR (Kebache et al, 2004; Latreille & Larose, 2006). In addition, Nck was also implicated in IRE1α signaling, where Nck1 was shown to negatively regulate IRE1α-mediated activation of Erk-1 in an *in vitro* reconstitution system (Nguyen et al, 2004). Interestingly, in this study Nck was found to directly associate with IRE1α through two of its SH3 domains in unstimulated conditions, thereby potentially preventing IRE1α signaling leakage. However, in response to IRE1α activation, Nck was proposed to dissociate from IRE1α, probably through IRE1α conformational changes allowing IRE1α activation and signaling targeting Erk-1 activation (Nguyen et al, 2004).

Further evidence for a role of Nck1 in modulating the UPR was obtained from high fat diet fed *Nck1*$^{-/-}$ mice, which were protected against obesity-induced glucose intolerance and insulin resistance without a change in HFD-induced body weight gain (Latreille et al, 2011). In agreement with others (Ozcan et al, 2004), in HFD-induced obesity in wild-type mice, characterized by glucose intolerance and peripheral insulin resistance, we observed signs of ER stress in insulin target tissues such as liver, adipose tissue and to some extent skeletal muscle. ER stress was defined by hyperactivation of IRE1α and PERK, as well as their respective downstream signaling markers (pJNK, XBP1s, peIF2αSer^{51}, ATF4, CHOP) in these tissues. In these conditions, sustained activation of the IRE1α-JNK pathway, through direct JNK-mediated phosphorylation of IRS-1 at Ser^{307}, was linked to insulin resistance (Ozcan et al, 2004). Interestingly, *Nck1*$^{-/-}$ mice fed a HFD were less glucose intolerant and more insulin sensitive than obese wild-type mice. In addition

activation and signaling from IRE1α and PERK were attenuated in liver of HFD *Nck1*$^{-/-}$ mice, correlating with enhanced hepatic insulin signaling and improved glucose homeostasis. Accordingly, correlating with a role for Nck1 in activation of the UPR, we demonstrated that transient silencing of Nck1 in human hepatocellular carcinoma HepG2 cells reduced ER stress-induced IRE1α activation and signaling while promoting insulin signaling (Latreille et al, 2011). Furthermore, our group recently obtained strong evidence that the effects of silencing Nck1 on IRE1α in HepG2 cells might be mediated through downregulation of the non-receptor protein tyrosine phosphatase PTP1B, which is required for optimal IRE1α activation and signaling (Hui, L., unpublished data).

1.6.3.6 Nck and insulin signaling

Nck was implicated in insulin signaling by studies showing that through its SH2 domain, Nck associates with IRS1 phosphorylated on Y^{147} (pY^{147}) in insulin-stimulated cells (Lee et al, 1993). This binding site on IRS1 was found to be Nck-specific among the SH2 domain-containing proteins known to bind tyrosine phosphorylated IRS1 (Grb2, Shc, p85 of PI3K). In contrast, Nck2 was also reported to directly bind IRS1, but in a phosphotyrosine-independent manner involving two out of the three SH3 domains of Nck2 (Tu et al, 1998; Tu et al, 2001). Although these studies provide evidence that Nck could play a role in modulating insulin signaling by interacting with IRS proteins, the biological relevance of these Nck-dependent interactions with IRS proteins still await to be demonstrated. Nevertheless, we provided strong evidence that Nck1 is implicated in limiting insulin signaling (Li et al, 2014a). In fact, we demonstrated that silencing Nck1 in HepG2 cells and Nck1 deficiency in primary hepatocytes leads to enhanced basal and insulin-induced PI3K/Akt signaling, associated with FOXO1 nuclear exclusion and increased glycogen synthase

activity. Mechanistically, we proposed that loss of Nck1 in HepG2 cells and liver enhances insulin signaling by downregulating the non-receptor protein tyrosine phosphatase PTP1B, a known negative regulator of growth factor signaling including insulin (Li et al, 2014a).

1.7 Figures for Chapter 1

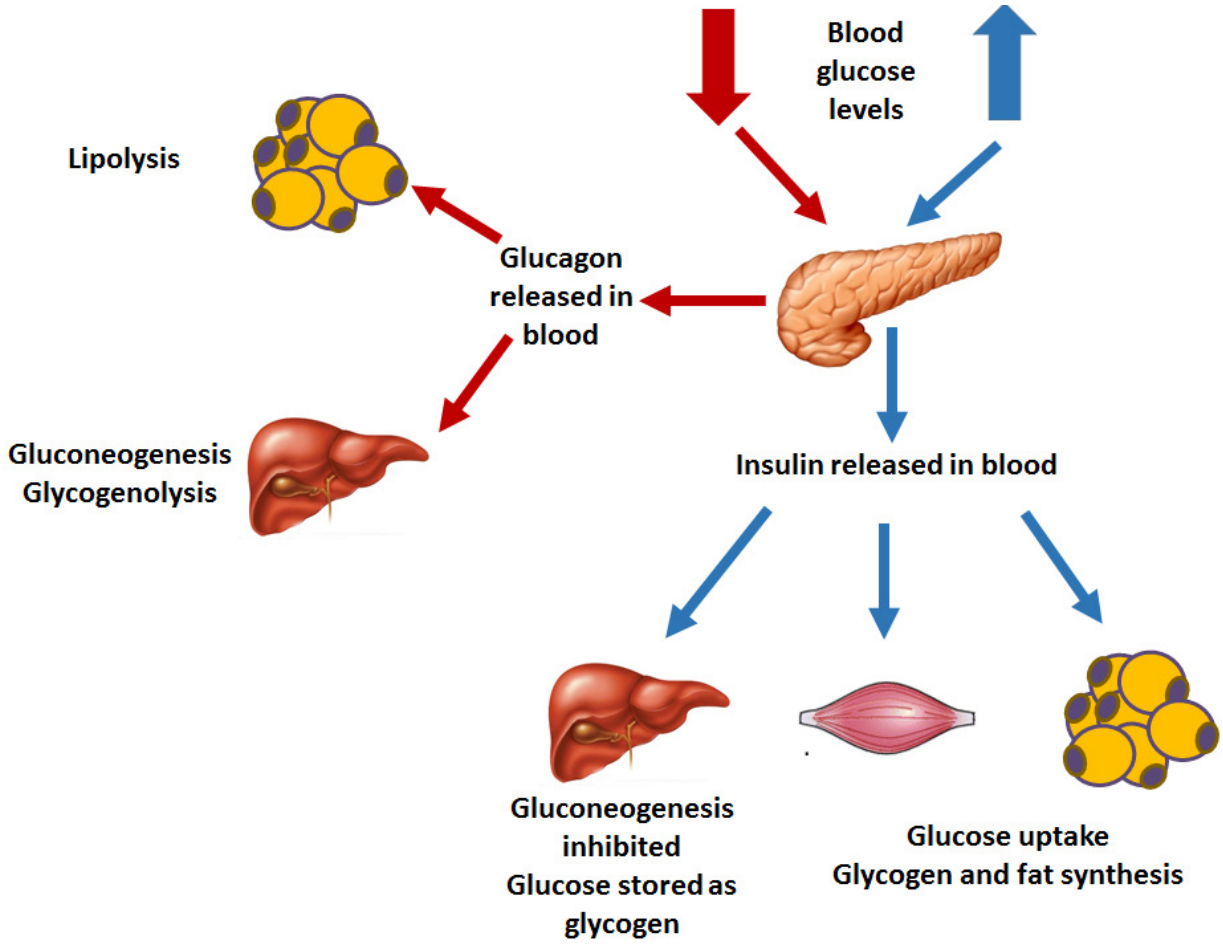


Figure 1. 1 Regulation of glucose homeostasis by insulin and glucagon.

When blood glucose levels increase, the pancreas releases insulin, which acts on peripheral tissues to induce glucose uptake and storage, as well as inhibit hepatic glucose production and release. On the other hand, a decrease in blood glucose levels induces release of glucagon from the pancreas, which triggers hepatic glucose production through breakdown of glycogen and gluconeogenesis, along with breakdown of fat in adipose tissue to free fatty acids, all contributing to an increase of glucose and free fatty acids in the blood.

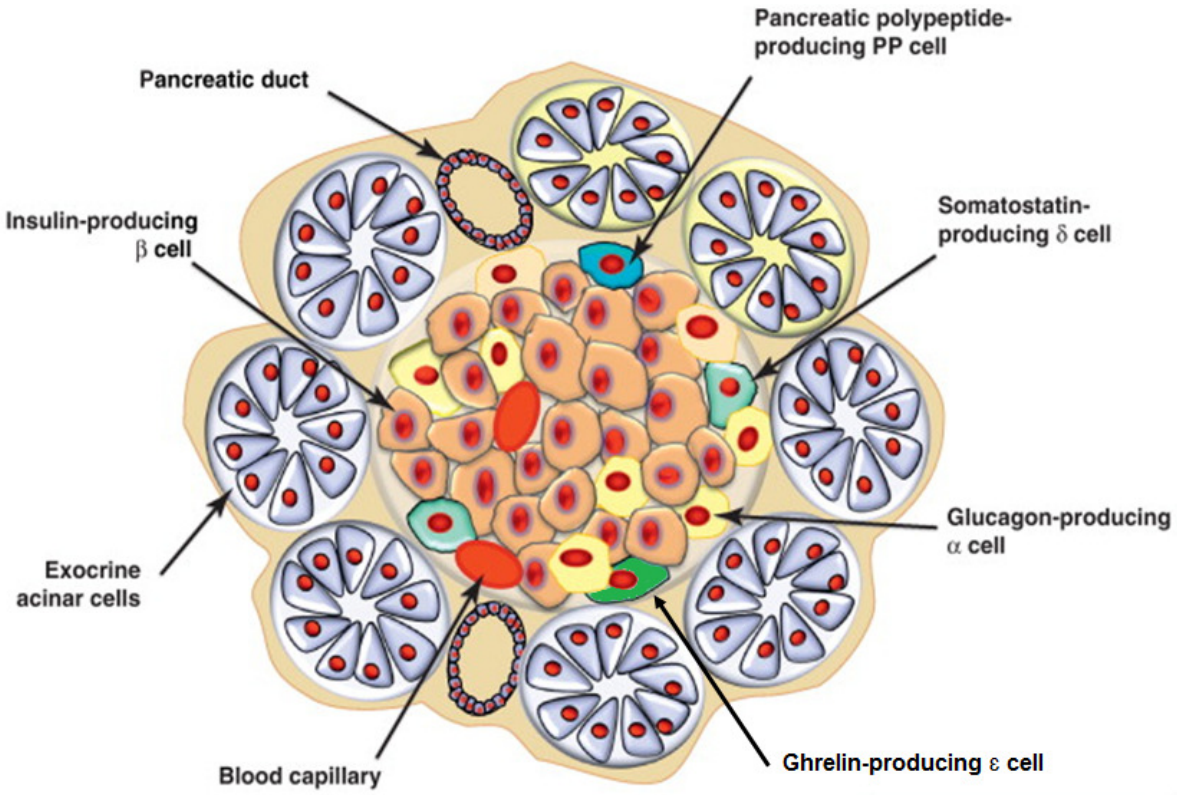


Figure 1. 2 Organization of the pancreas.

The pancreas is made up of exocrine tissue, which consists of acinar, centroacinar and ductal cells, and endocrine tissue composed of different types of cells (α, β, δ, ε, and pancreatic polypeptide (PP) cells) organized in structures called islets of Langerhans. Adapted from (Efrat & Russ, 2012).

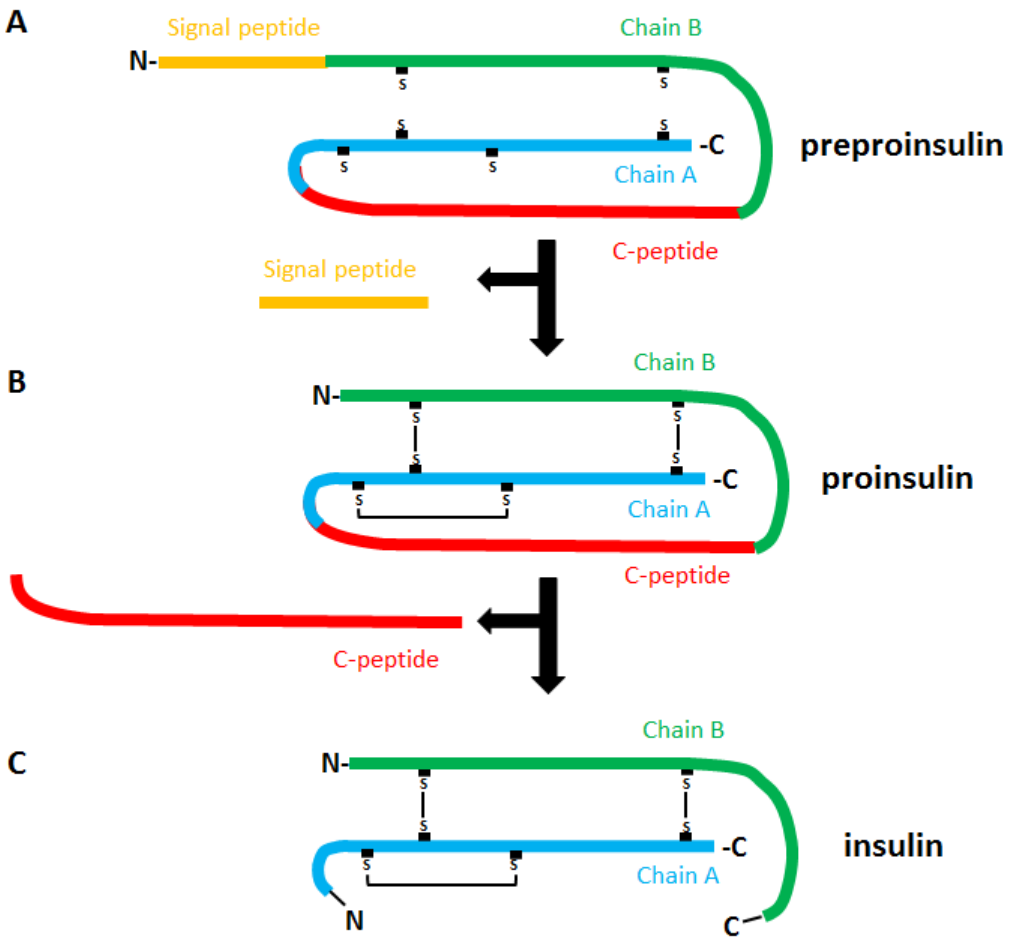


Figure 1. 3 Insulin structure and processing.

(A) Insulin is synthesized as a precursor polypeptide called preproinsulin composed of a signal peptide, insulin chains A and B and a C-peptide. (B) In the ER, the signal peptide is cleaved to produce proinsulin, and disulfide bonds are formed between chains A and B, and within chain A. (C) Proinsulin is transported to the TGN and gets packaged into vesicles, where it is finally cleaved to produce the C-peptide and mature insulin consisting of chains A and B.

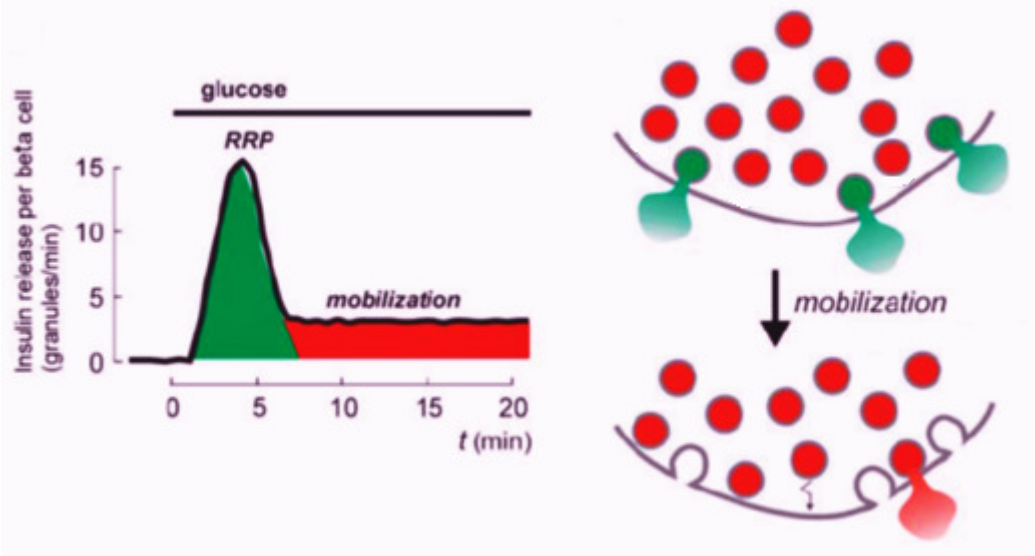


Figure 1. 4 Insulin secretion pattern.

Pancreatic β cells secrete insulin in a biphasic manner in response to nutrient stimulation. The first phase of secretion corresponds to an early and quick spike in release of a population of insulin granules known as readily releasable pool (depicted in green). The second phase of insulin secretion corresponds to a lower level but more continuous release of a population of insulin granules that requires synthesis of insulin and granule mobilization to the plasma membrane (depicted in red). Adapted from (Rorsman & Renstrom, 2003).

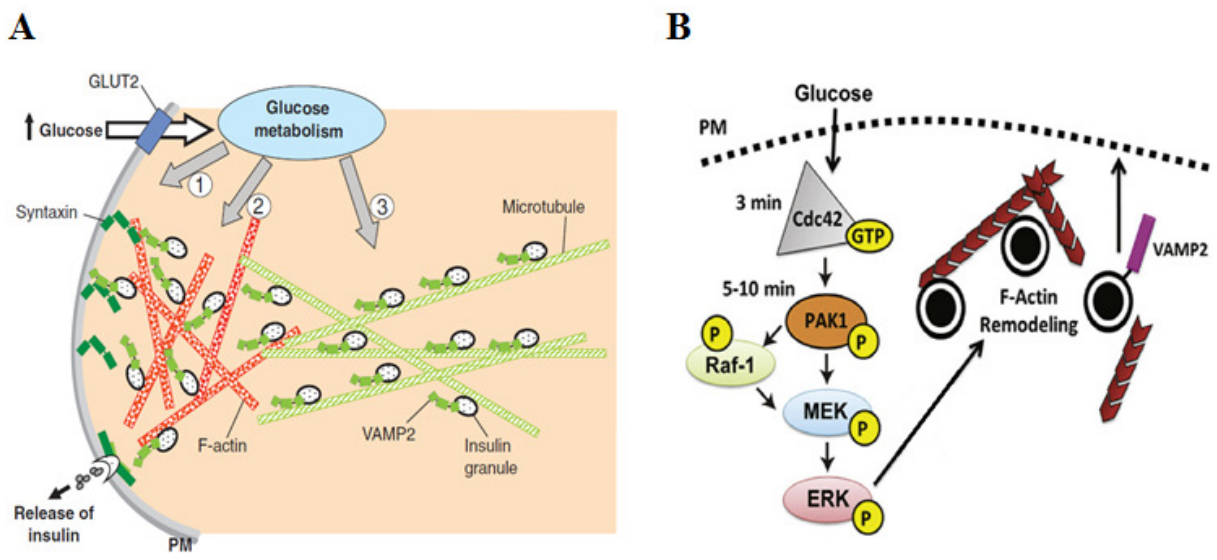


Figure 1. 5 Insulin granule trafficking.

(A) Processes involved in nutrient-driven insulin granule mobilization and exocytosis: Glucose increases intracellular Ca^{2+} levels in pancreatic β cells leading to fusion of pre-docked insulin granules with the plasma membrane (1). Glucose metabolism also stimulates actin filament remodeling to allow mobilization of the reserve pool to reach the plasma membrane, where VAMP2 expressed on insulin granules can interact with plasma membrane SNAREs Syntaxin and SNAP25 (2). In addition, glucose stimulates modulation of microtubule machinery in order to facilitate transport of insulin granules to the cortical actin filaments (3). (B) Mechanisms involved in actin filament remodeling: upon glucose stimulation, activated small Rho GTPase Cdc42 recruits Pak1 and activates it, which prompts activation of Raf/MEK/ERK signaling cascade. (A) adapted from (Wang & Thurmond, 2009) and B from (Kalwat et al, 2013).

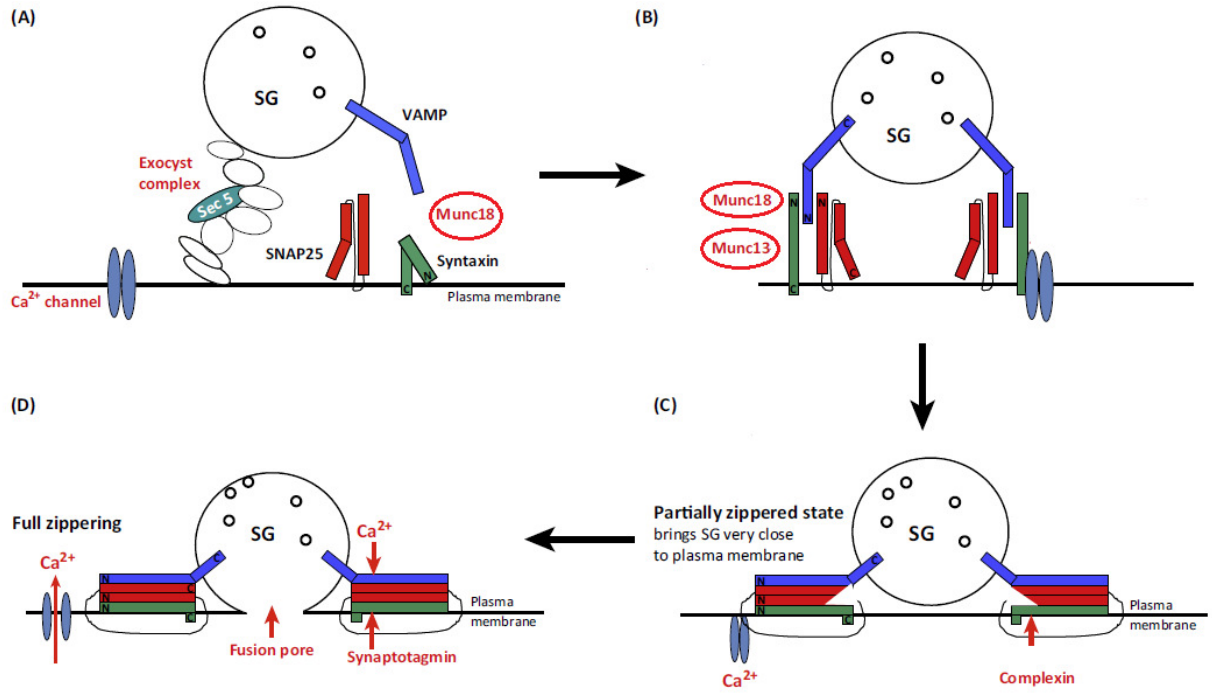


Figure 1. 6 Insulin granule exocytosis.

Secretory granule (SG) exocytosis includes tethering, priming and then fusing of the granules with the plasma membrane to release content. (A) Tethering of insulin granules involves formation of a multi-subunit exocyst complex (including Sec5) that allows tethering of the granules to the plasma membrane, and Munc18 binds to the closed form of Syntaxin. (B) Priming requires the assistance of Munc13 to change Syntaxin conformation to an open form, which allows the formation of the SNARE complex (Syntaxin-VAMP-SNAP25). The SNARE complex also binds Ca^{2+} channels to allow optimal response upon Ca^{2+} entry into cells. (C) Fully primed granules are now held close to the plasma membrane by the interaction of the SNARE complex. (D) Fusion is stimulated when Synaptotagmin detects Ca^{2+} influx, becomes activated and stimulates a conformational change of the SNARE complex. Figure adapted from (Gaisano, 2014).

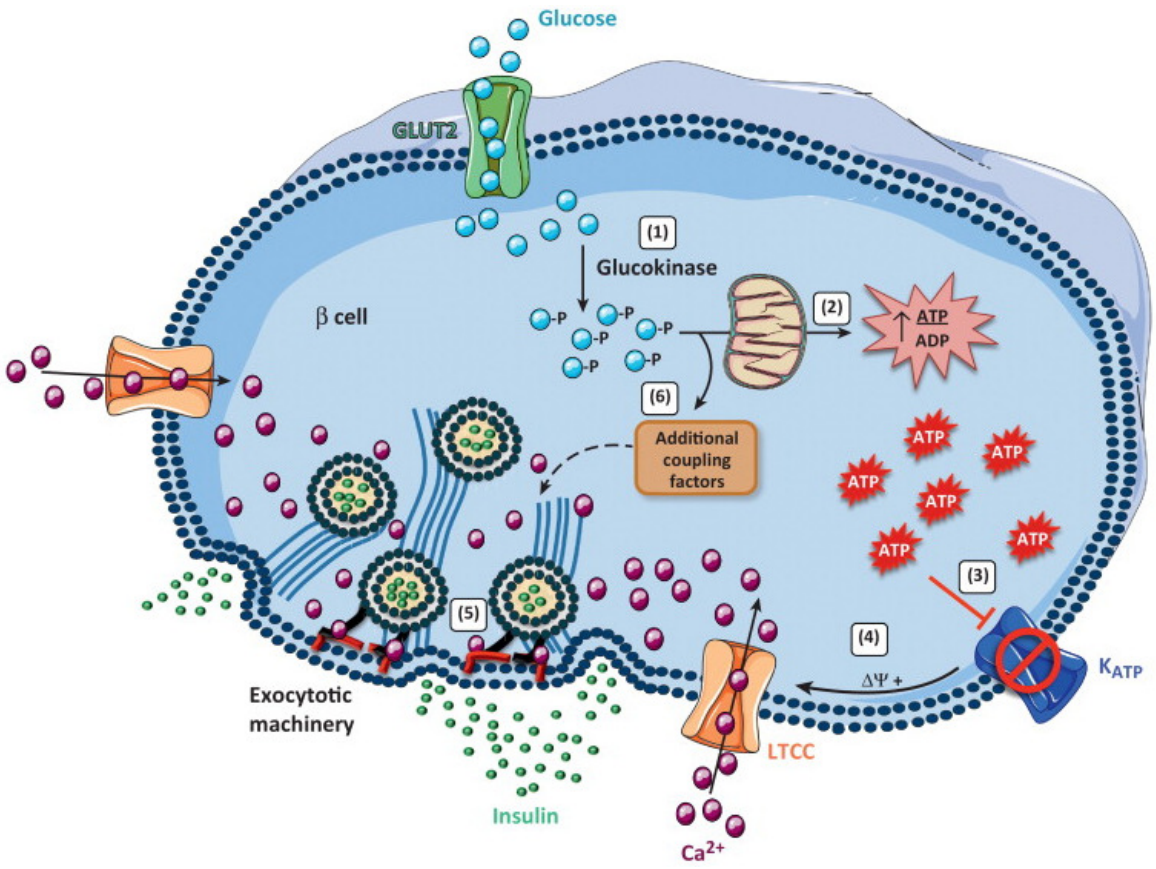


Figure 1. 7 Glucose-stimulated insulin secretion.

Pancreatic β cells express GLUT2 transporter protein that facilitates entry of glucose into β cells. Upon glucose metabolism through glycolysis and oxidative phosphorylation (steps 1 and 2), ATP levels increase inducing closure of ATP-sensitive potassium channels (K_{ATP}) (step 3). Closing of the K_{ATP} channels stimulates opening of calcium channels due to plasma membrane depolarization (step 4). Ca^{2+} entry into cells stimulates fusion of insulin granules with the plasma membrane, thereby releasing insulin into the extracellular environment (step 5). Additional coupling factors derived from the cooperative action of glucose and fatty acid metabolism also contribute to insulin secretion (step 6). Figure taken from (Mancini & Poitout, 2013).

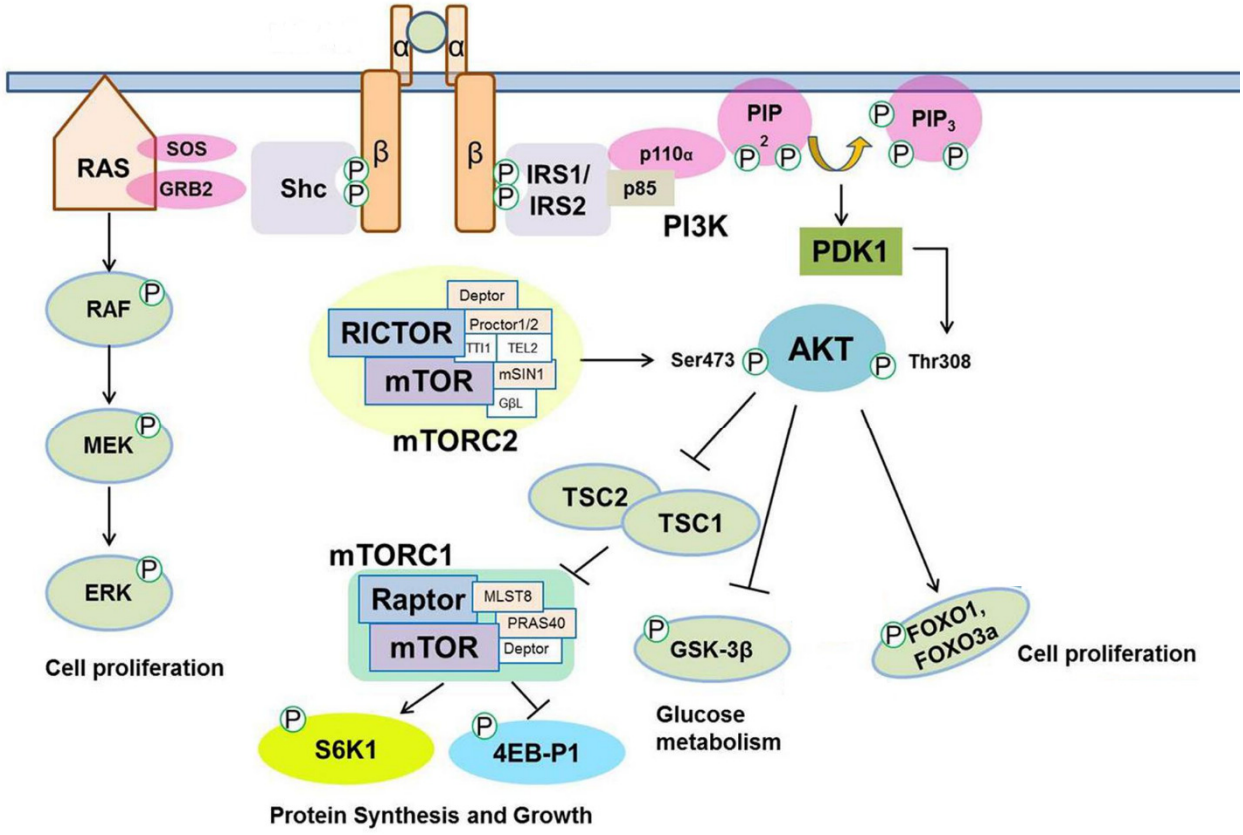


Figure 1. 8 Insulin signaling pathways.

Binding of insulin to the insulin receptor (IR) induces IR activation through a conformational change that triggers autophosphorylation of the IR on tyrosine residues. IR activation triggers different signaling pathway, the most important being the IRS-PI3K-Akt, as well as the IRS- or Shc-Ras-MAPK. Depicted on the right side, activated IR recruits and phosphorylates IRS proteins on tyrosine residues, creating docking sites for effectors proteins. Interaction of tyrosine phosphorylated IRS with the regulatory p85 subunit of PI3K leads to activation of the p110 catalytic subunit, which generates PIP3 from PIP2. PIP3 recruits PDK1 and Akt at the plasma membrane, resulting in PDK1-mediated activation of Akt through phosphorylation on Thr^{308}, and further activation of Akt is achieved by the mTORC2 complex which phosphorylates Akt on

Ser^{473}. Akt then results in mTORC1 complex formation and activation through inhibition of TSC1/2 complex activity. In summary, Akt activation triggers processes involved in protein synthesis and growth. On the other hand, IR activation also recruits the adaptor protein Shc, which gets phosphorylated on tyrosine residues, and creates binding sites for the Grb2-Sos complex. Upon recruitment and binding, Grb2-Sos binds and activates Ras, which then initiates a cascade of activation/phosphorylation events starting at Raf1, and going through MEK and ERK proteins. This pathway activates cell proliferation processes. Figure adapted from (Jung & Suh, 2014).

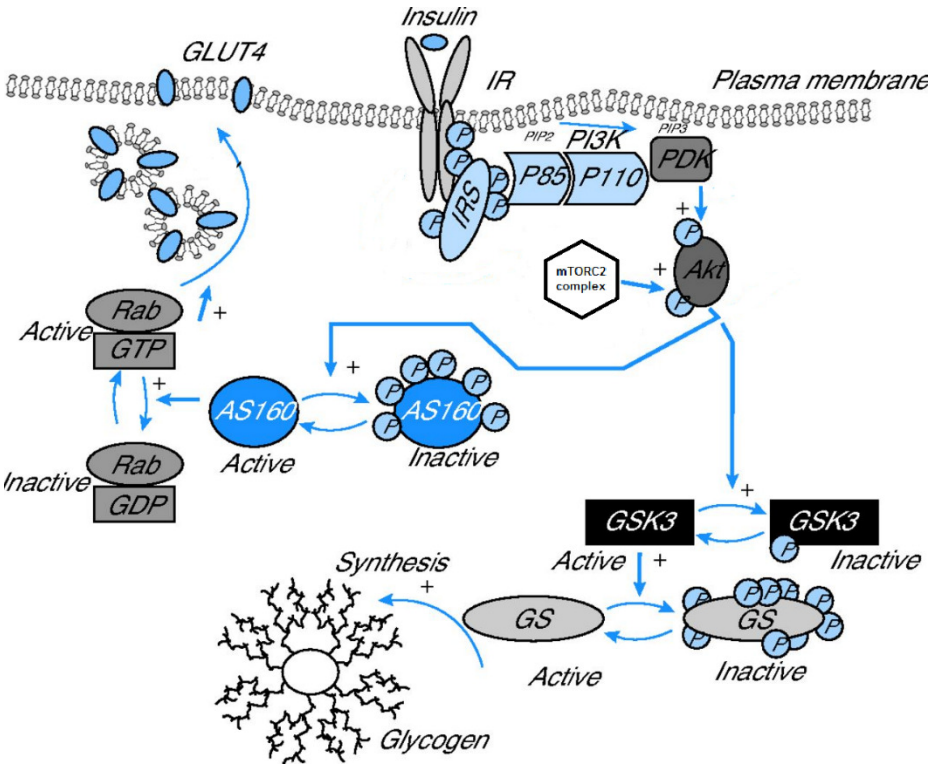


Figure 1. 9 Insulin signaling in peripheral tissue.

IR activation upon insulin binding allows recruitment and phosphorylation of IRS proteins on tyrosine residues, creating docking sites for the regulatory p85 subunit of PI3K, which activates PI3K. PIP3, generated by PI3K recruits PDK1 and Akt. Akt is activated by PDK1 and mTORC2 through phosphorylation. Only two Akt targets are depicted in this figure: Akt phosphorylates and inactivates GSK3, releasing its inhibition of glycogen synthase, thus increases glycogen formation. Akt also phosphorylates and inactivates AS160, thus activating Rab, a small GTPase involved in translocation of GLUT4 to the plasma membrane and glucose uptake. Adapted from (Wojtaszewski & Richter, 2006).

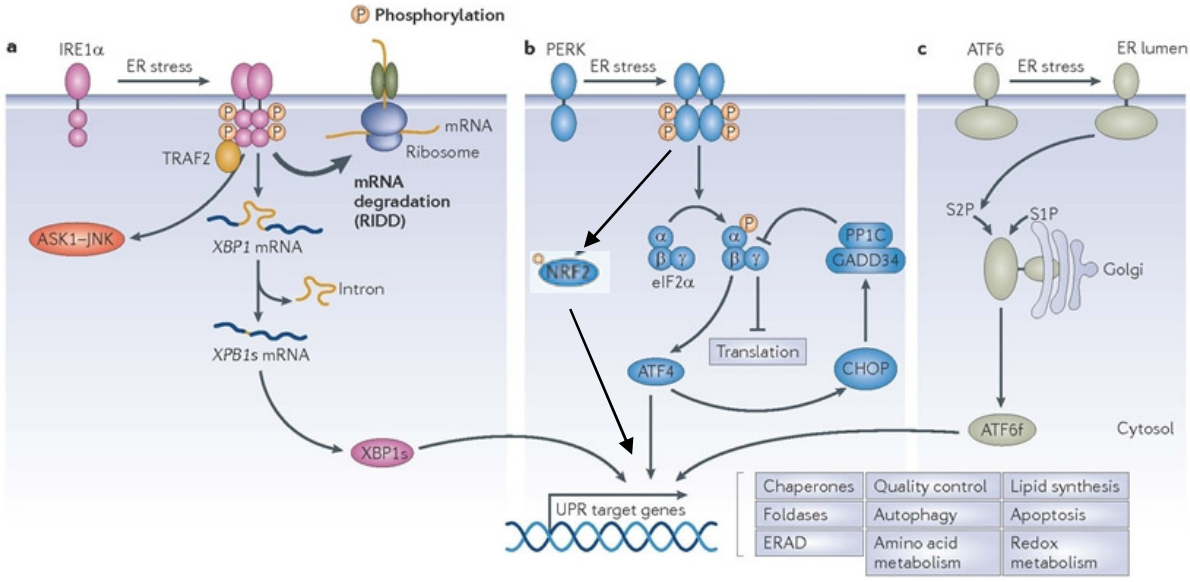


Figure 1. 10 Unfolded protein response (UPR) signaling.

Accumulation of improperly folded proteins induces the activation of ER stress sensors transmembrane proteins: IRE1α (a), PERK (b) and ATF6 (c). Activation of these proteins assembles a signaling network resulting in attenuation of global protein synthesis and activation of a wide selection of UPR target genes involved in ER protein folding and quality control processes. IRE1α activation leads to mRNA XBP1 splicing, selective mRNA degradation (RIDD) and JNK activation. PERK induces phosphorylation of eIF2αSer^{51}, associated with attenuation of general protein synthesis, and phosphorylation of Nrf2 to activate antioxidant gene expression. ATF6 activation involves its translocation to the Golgi, where it gets processed to release an active ATF6 transcription factor that translocates to the nucleus and induces gene expression. Adapted from (Hetz et al, 2013).

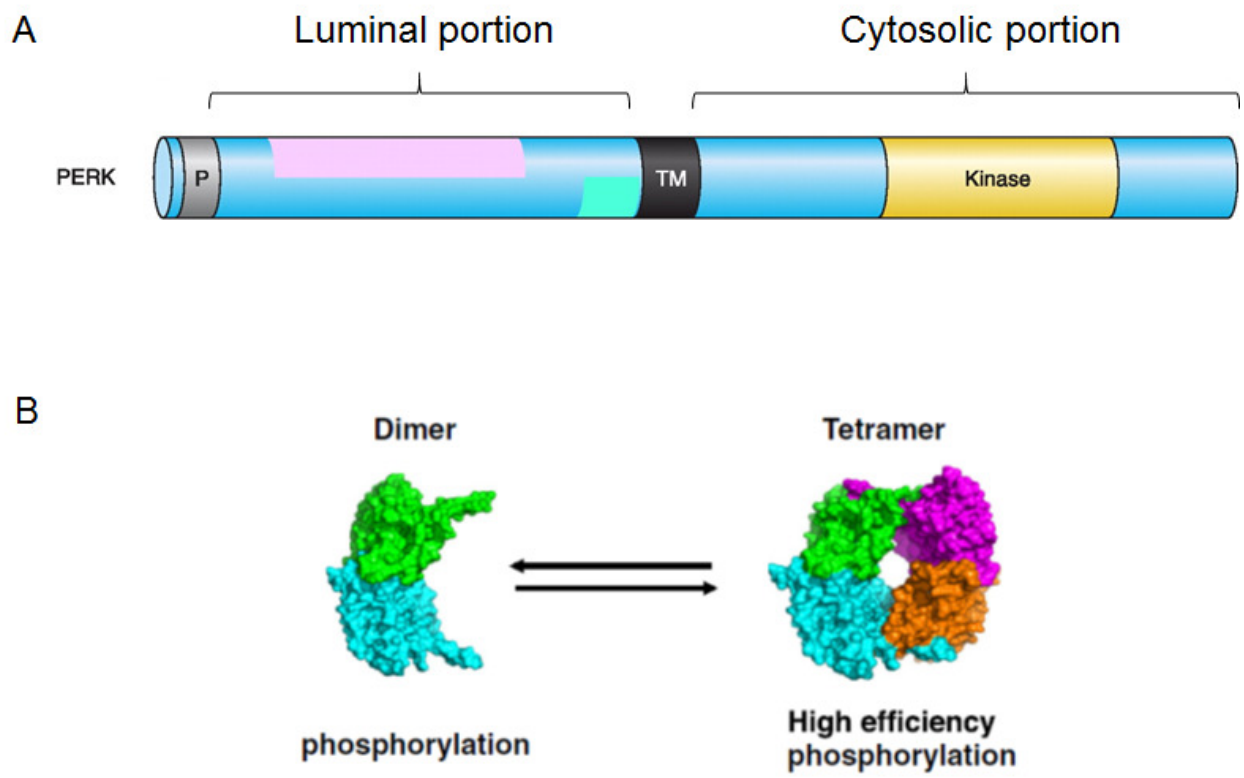


Figure 1. 11 PERK structure.

(A) PERK monomer structure consists of an N-terminal luminal domain, which harbors a BiP binding site (pink rectangle), a transmembrane (TM) domain, and a cytosolic domain containing a protein kinase domain. (B) Model representation of dimer and tetramer formation of PERK with different phosphorylation efficiencies that could also represent different PERK activation states. Adapted from (Hetz et al, 2011) for (A), and (Carrara et al, 2015) for (B).

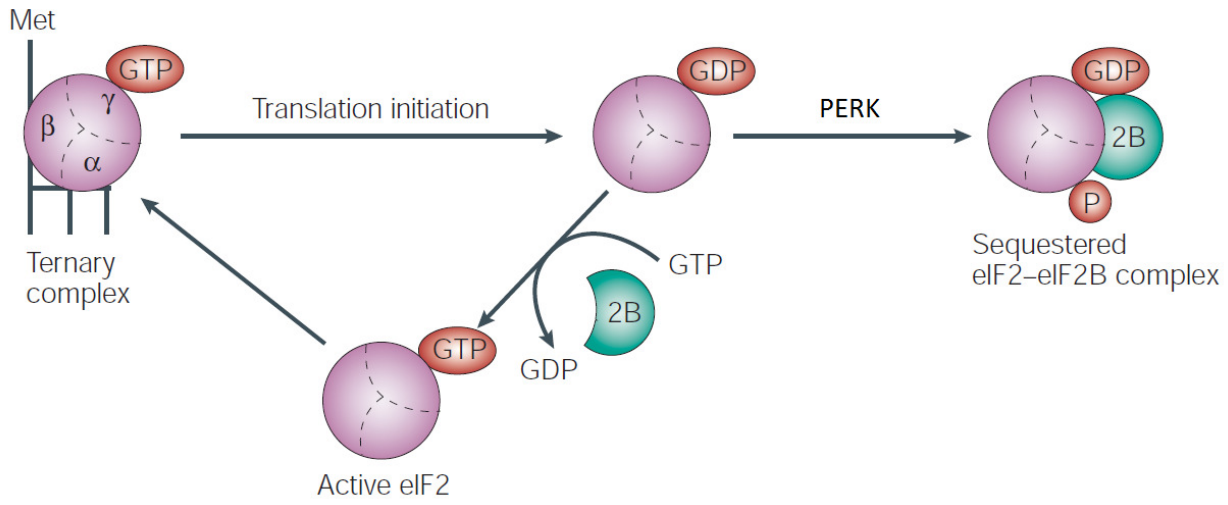


Figure 1. 12 eIF2α regulation.

eIF2 activity is regulated by guanine nucleotide exchange and phosphorylation. Active eIF2-GTP-bound structure forms a ternary complex with the methionine initiator tRNA (Met-tRNA) and the small 40S ribosomal subunit (not shown), in order to initiate translation. Once this step is completed, eIF2-GTP is hydrolyzed to eIF2-GDP, which could undergo a novel cycle of activation by interacting with the guanine nucleotide exchange complex eIF2B. In ER stress conditions, eIF2α phosphorylated on Ser^{51} by PERK binds with higher affinity to eIF2B and sequesters eIF2B, thereby decreasing the availability of active eIF2-GTP. Figure adapted from (Gebauer & Hentze, 2004).

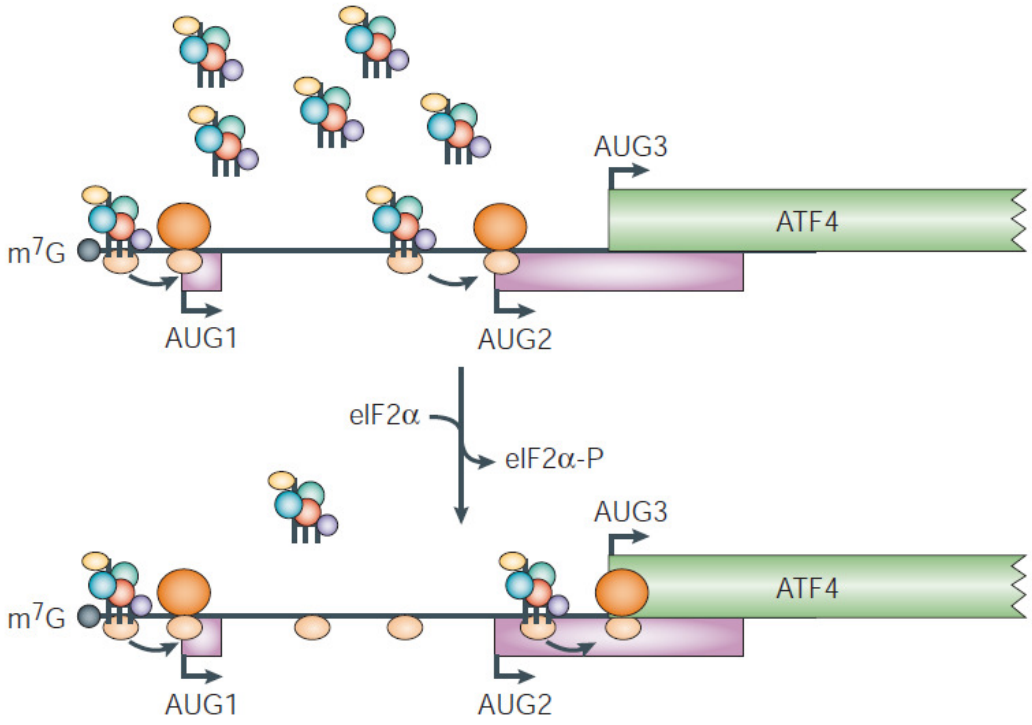


Figure 1. 13 Translational regulation of ATF4.

ATF4 mRNA translation is regulated by two upstream open reading frames (uORFs) in its 5' untranslated region (UTR) (purple rectangles). Under physiological conditions, the ternary complex of eIF2-tRNA-40S is present abundantly allowing for ribosomes to initiate translation at uORF1 (AUG1). Because the ternary complex is highly available, the ribosomes remain associated to the mRNA and often initiate back at uORF2 (AUG2), an inhibitory site of ATF4 translation due to its overlapping with the coding region of ATF4 (green rectangle), thereby suppressing ATF4 translation. However, under ER stress leading to eIF2αSer^{51} phosphorylation, availability of the ternary complex is low, allowing the ribosomes to scan through the 5' untranslated region and skip uORF2 start codon (AUG2), thus enabling translation of ATF4 mRNA at uORF3 (AUG3). Taken from (Holcik & Sonenberg, 2005).

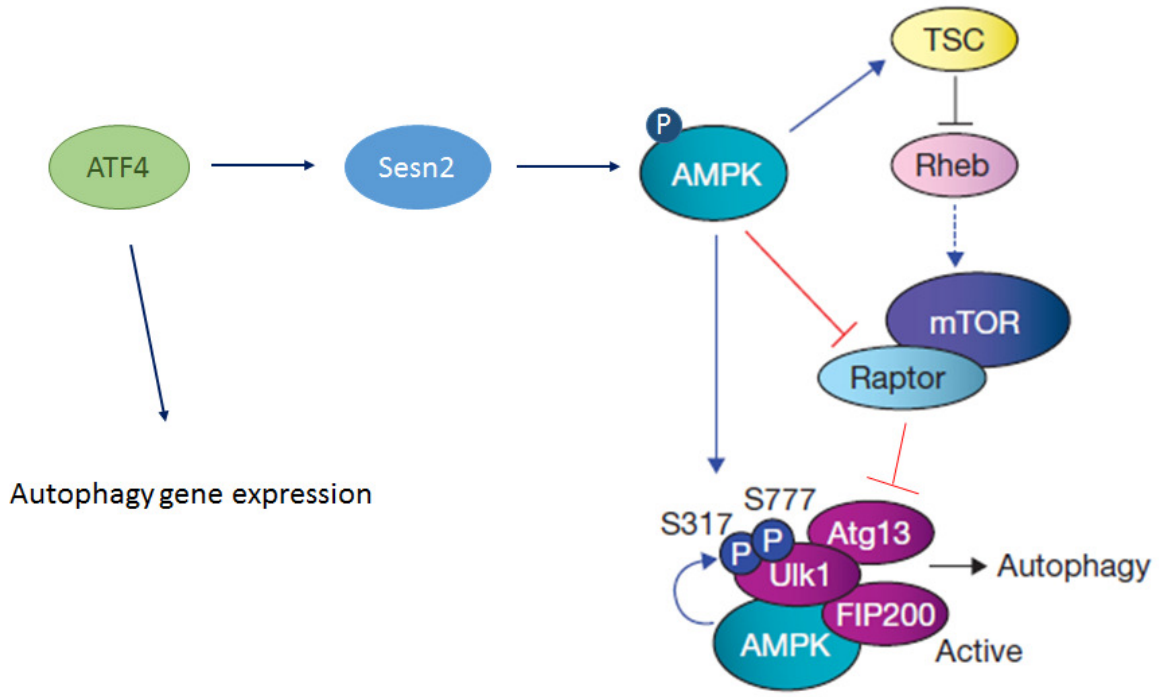


Figure 1. 14 ATF4 direct and indirect activation of autophagy.

ATF4 activates autophagy through either direct upregulation of autophagy genes or induction of Sesn2, which contributes to autophagy by activating AMPK. Activated AMPK attenuates mTORC1 activity by activating TSC1/2 heterodimer and inhibiting the mTOR subunit Raptor. Attenuation of mTORC1 activity releases the inhibition imposed on autophagy. In addition, AMPK acts directly on autophagy through activating phosphorylation of autophagy initiating kinase (Ulk1). Figure adapted from (Kim et al, 2011).

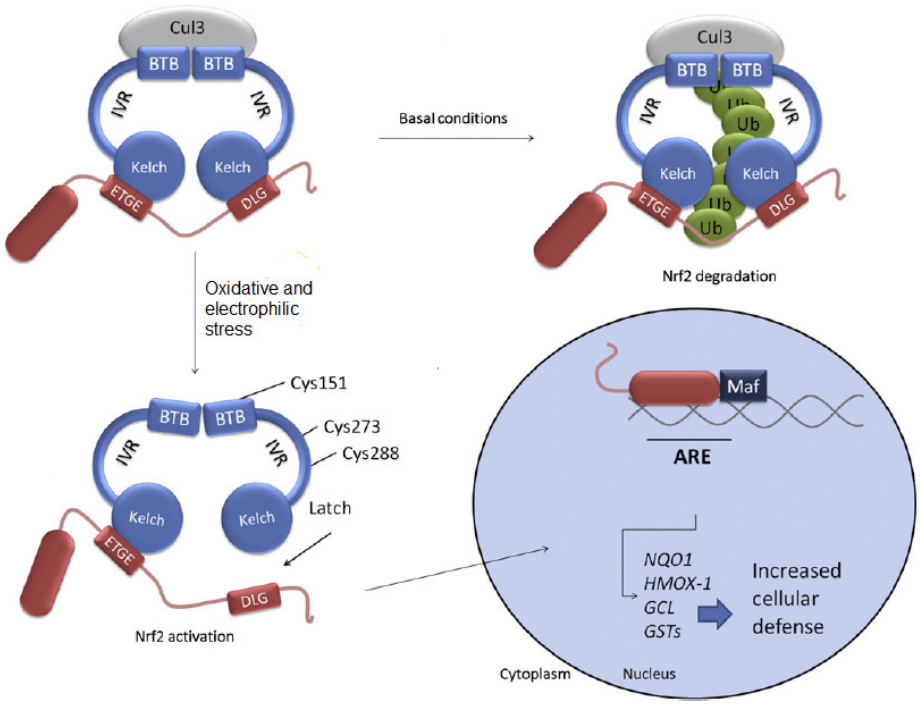


Figure 1. 15 Nrf2 regulation.

Nrf2 is regulated by the Keap1-Cullin 3 complex. Keap1 associates with Cullin 3 (Cul 3) through its N-terminal BTB domain. On the other side, Keap1 interacts through its C-terminal Kelch motif to either ETGE or DLG motifs found in the Neh2 domain of Nrf2. Under basal conditions, formation of the Cul3-Keap1-Nrf2 complex leads to ubiquitination of Nrf2 by the action of Cul3 E3 ligase, and subsequent degradation of Nrf2. Under oxidative and electrophilic stress, cysteine residues on Keap1 BTB and intervening region (IVR) domains are attacked causing a conformational change leading to disruption of the Keap1-Nrf2 interaction, thus allowing Nrf2 to escape degradation and undergo translocation to the nucleus in order to heterodimerize with Maf (a transcription factor) and bind to antioxidant response elements, thereby activating antioxidant gene expression. Adapted from (Kansanen et al, 2012).

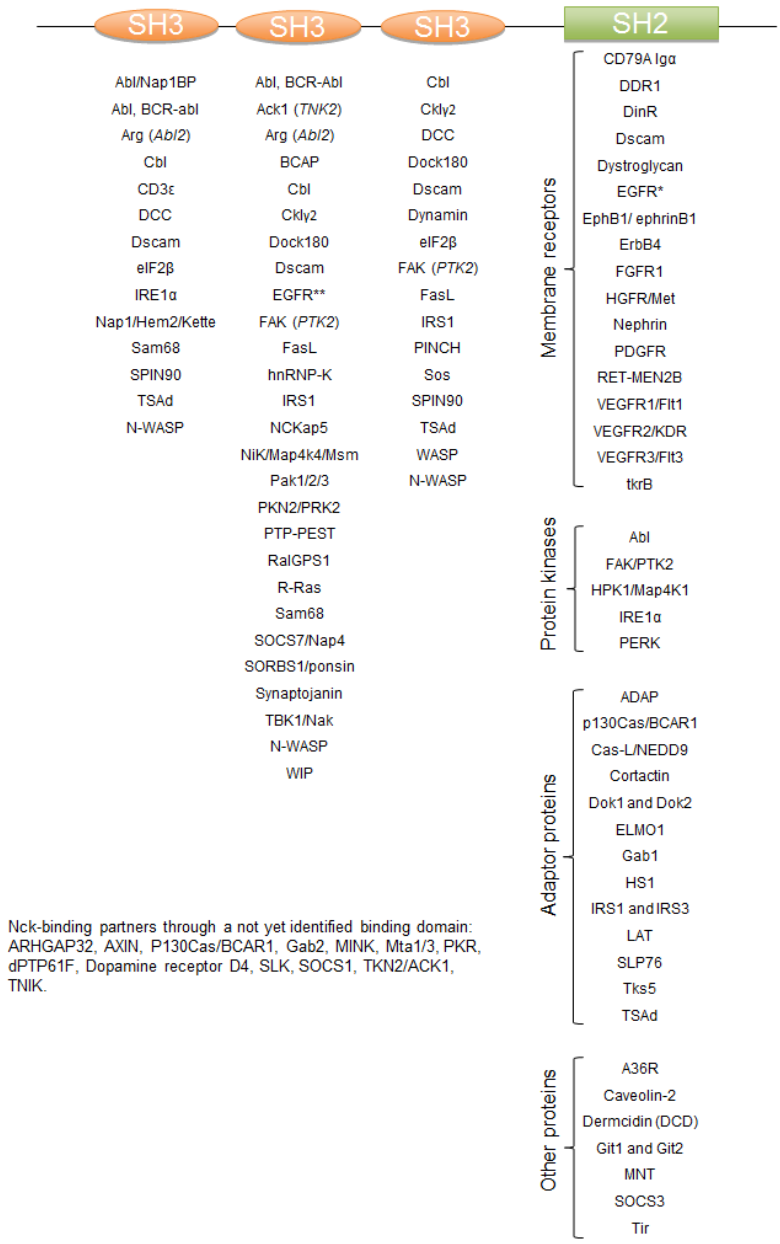


Figure 1. 16 Nck linear structure and identified binding partners.

Nck is composed of 3 N-terminal SH3 domains and 1 C-terminal SH2 domain. Known Nck-binding proteins are reported under their corresponding domain of interaction. * (Li et al, 1992), ** (Hake et al, 2008). Figure adapted from (Labelle-Cote & Larose, 2011) and updated from recent literature.

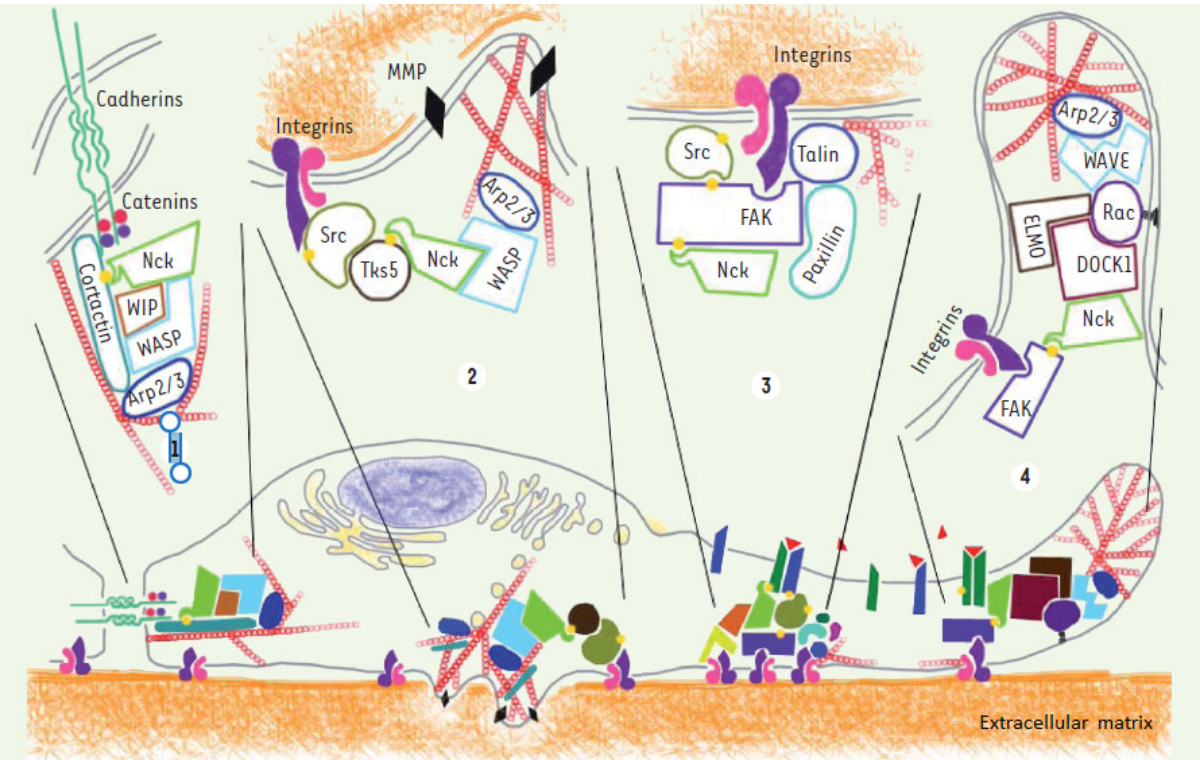


Figure 1. 17 Nck mediates membrane receptor signaling to effector proteins regulating actin cytoskeletal rearrangement.

Schematic representation of some of the molecular complexes associated with Nck and involved in regulation of actin cytoskeletal rearrangement. (1) Represents adherens junction formation. Cadherin activation upon intercellular contact allows Src kinase to phosphorylate cortactin on tyrosine residues. Nck binds through its SH2 domain to cortactin, and recruits to the complex effector proteins involved in actin rearrangement such as WIP and WASP. (2) Represents invadopodia formation. Upon integrin activation, Src phosphorylates Tks5. This induces Tks5 binding to Nck, which then recruits actin machinery including WASP and Arp2/3. (3) Represents adhesion complex forming downstream of integrin, including activation of focal adhesion kinase (FAK) that binds Nck. (4) Lamellipodia formation requires actin cytoskeleton reorganization. This

involves Rac1 GTPase, GEF Dock1 and Elmo adaptor protein complex formation, which contributes to the activation of Rac1. Nck2, by associating with FAK and Dock1, helps to direct the molecular complex Dock1-Elmo near Rac1 to promote actin polymerization. Figure adapted from (Labelle-Cote & Larose, 2011).

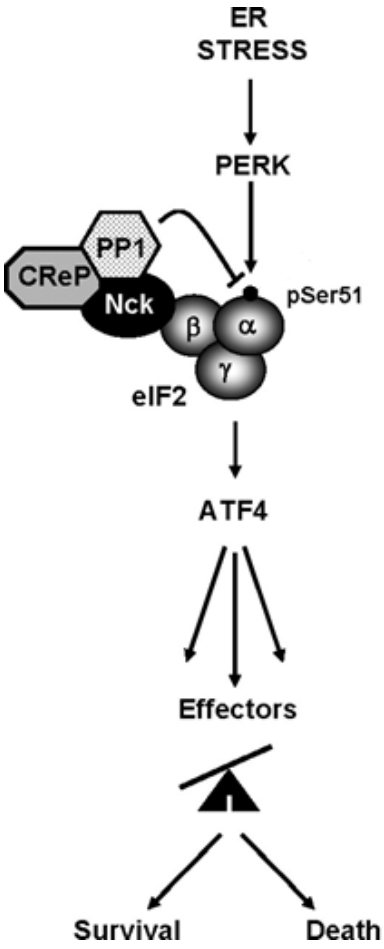


Figure 1. 18 Nck modulation of eIF2α downstream signaling and protein translation.

Nck bound to the β subunit of eIF2 recruits a holophosphatase complex containing the catalytic subunit of Ser/Thr protein phosphatase 1 (PP1) and the PP1c adaptor protein CReP, thereby regulating dephosphorylation of eIF2αSer^{51}, downstream signaling and protein translation. Adapted from (Latreille & Larose, 2006).

1.8 Rationale and Objectives of the study

Our laboratory previously demonstrated that Nck1 modulates ER stress-induced phosphorylation of eIF2αSer^{51} (Kebache et al, 2004). We also showed that genetic inactivation of *Nck* in mouse embryonic fibroblasts (MEFs) leads to spontaneous induction of eIF2αSer^{51} phosphorylation and downstream signaling targets including ATF4 and GADD34, as well as impacts survival to prolonged ER stress (Latreille & Larose, 2006). Although this study provided an interesting mechanism underlying the regulation of eIF2αSer^{51} phosphorylation by Nck1 (Latreille & Larose, 2006), it did not address whether Nck1 also modulates activity of PERK, the important eIF2α kinase contributing to the UPR activated upon ER stress and which is known to play a major role in regulating pancreatic β cell function in physiological and pathological conditions (Shi et al, 1998; Delepine et al, 2000; Harding et al, 2001). Therefore, the main objective of my study was to address whether Nck1 modulates PERK activity. Having determined that Nck1 regulates PERK activation, I further investigated the underlying mechanism and the effects of silencing Nck1 on pancreatic β cell function and survival to diabetes-related stresses.

CHAPTER 2. Interaction of Nck1 and PERK phosphorylated at Y^{561} negatively modulates PERK activity and PERK regulation of pancreatic β-cell proinsulin content

Lama Yamani, Mathieu Latreille, and Louise Larose

Reproduced with permission from *Molecular Biology of the Cell*. 2014, Vol. 25 (5), 702-711

2.1 Preface

Previous work in Dr. Larose's laboratory revealed that Nck1 interacts with eIF2β and regulates the phosphorylation of the eIF2α (Kebache 2002, 2004; Latreille, 2006). Interestingly, upon ER stress, PERK activity (as marked by $pThr^{980}$), was decreased in *Nck*$^{-/-}$ MEFs with a rescued NCK1 expression compared to *Nck*$^{-/-}$ MEFs (Kebache et al, 2004). This suggested that Nck, in addition to its regulatory role of eIF2α phosphorylation modulates PERK activity. Therefore, I monitored PERK activity in *Nck*$^{-/-}$ MEFs under unstressed and ER stress conditions. I showed that genetic deletion of Nck enhanced PERK activity under ER stress conditions, as well as basal conditions. Based on these results, I investigated the mechanisms by which Nck regulates PERK. We revealed that Nck1 directly interacts with PERK through its SH2 domain on the PERK juxtamembrane domain, and identified pY^{561} PERK as the binding site, and that mutating this site to phe (F), a nonphosphorylatable residue, abolishes the interaction. To elucidate the role of pY^{561} on PERK *in vivo*, I studied the interaction in a relevant biological system where PERK activity is essential. Based on that, I used mouse insulinoma pancreatic β cells MIN6. Increased PERK pY^{561} levels in MIN6 cells delayed PERK activation upon ER stress. Similarly, we showed *in vitro* that Y561F PERK mutant was catalytically more active than wild-type PERK, indicating that the phosphorylation of PERK on Y^{561} limits PERK activity. Then, to study the role of Nck1 in MIN6 cells and PERK activity, I stably expressed shRNA against Nck1, which efficiently reduced Nck1 expression, and showed that PERK activity and signaling increased under basal conditions. In addition, I showed that Nck1 downregulation prompts pY^{561} dephosphorylation in MIN6 cells, which correlates with a faster activation of PERK. More importantly, Nck1 reduction in MIN6 cells enhanced insulin levels in a PERK-dependent manner. The increase in insulin levels was

shown to be due to increased insulin synthesis. These findings also correlated with increased insulin content in isolated islets from *Nck1*$^{-/-}$ mice compared to wild-type counterparts. This is the first study to report a role for Nck1 in pancreatic β cell function through regulation of PERK activity, and identifies a novel phosphorylated site on PERK as well as a PERK binding partner.

2.2 Abstract

PERK, the PKR-like endoplasmic reticulum (ER) kinase, is an ER transmembrane serine/threonine protein kinase activated during ER stress. In this study, we provide evidence that the Src-homology domain-containing adaptor Nck1 negatively regulates PERK. We show that Nck directly binds to phosphorylated Y^{561} in the PERK juxtamembrane domain through its SH2 domain. We demonstrate that mutation of Y^{561} to a nonphosphorylatable residue (Y561F) promotes PERK activity, suggesting that PERK phosphorylation at Y^{561} (pY^{561}PERK) negatively regulates PERK. In agreement, we show that pY^{561}PERK delays PERK activation and signaling during ER stress. Compatible with a role for PERK in pancreatic β cells, we provide strong evidence that Nck1 contributes to PERK regulation of pancreatic β cell proteostasis. In fact, we demonstrated that down-regulation of Nck1 in mouse insulinoma MIN6 cells results in faster dephosphorylation of pY^{561}PERK, which correlates with enhanced PERK activation, increased insulin biosynthesis, and PERK-dependent increase in proinsulin content. Furthermore, we report that pancreatic islets in whole-body Nck1-knockout mice contain more insulin than control littermates. Together our data strongly suggest that Nck1 negatively regulates PERK by interacting with PERK and protecting PERK from being dephosphorylated at its inhibitory site pY^{561} and in this way affects pancreatic β cell proinsulin biogenesis.

2.3 Introduction

The endoplasmic reticulum (ER) is a subcellular organelle responsible for the synthesis, processing, and quality control of transmembrane and secretory proteins. Perturbation of ER homeostasis leads to accumulation of misfolded proteins and causes ER stress. This triggers the activation of three ER transmembrane proteins: inositol-requiring enzyme 1α (IRE1α), PKR-like endoplasmic reticulum kinase (PERK), and activating transcription factor 6 (ATF6), which together initiate a cytoplasmic signaling network defined as the unfolded protein response (UPR) (Ron & Walter, 2007). The UPR encompasses a translational and transcriptional program that helps cells alleviate ER stress. However, chronic or irreversible ER stress initiates a UPR-mediated apoptotic pathway that induces cell death (Szegezdi et al, 2006).

PERK, a type I ER transmembrane Ser/Thr protein kinase, is activated after dimerization and autophosphorylation induced by ER stress (Shi et al, 1998; Harding et al, 1999). Activated PERK phosphorylates the α-subunit of the eukaryotic initiation factor 2 (eIF2α) at Ser-51, which results in translation attenuation but paradoxically promotes translation of the activating transcription factor 4 (ATF4), which controls an important part of the UPR transcriptional program (Harding et al, 2000b; Vattem & Wek, 2004). Of interest, a role for PERK in pancreatic β cells was highlighted after the discovery of the Wolcott-Rallison syndrome (WRS), a neonatal/early infancy form of diabetes caused by mutations in the human PERK gene resulting in PERK loss of function (Delepine et al, 2000). Furthermore, *PERK*$^{-/-}$ mice, which phenocopy WRS dysfunctions, also display postnatal hyperglycemia, which was initially attributed to the loss of pancreatic β cell mass associated with a dramatic increase in β cell apoptosis (Harding et al, 2001; Zhang et al, 2002a). However, it was later demonstrated that the loss of β cell mass in *PERK*$^{-/-}$ mice is not due

to apoptosis. It instead results from reduction in β cell proliferation and differentiation during the neonatal period, which impedes postnatal gain of pancreatic β cell mass (Zhang et al, 2006). Finally, conditional deletion of PERK in young adult or mature mice significantly increased β cell death, revealing that PERK also contributes to the maintenance of β cell function in adults. However, β cell proliferation was dramatically increased, highlighting that PERK regulates β cell function in adults through a different mechanism than that during early postnatal development (Gao et al, 2012). A direct role for PERK in β cell proliferation and proinsulin/insulin content was further supported by the fact that acute inhibition of PERK generated by adenoviral vector-mediated expression of a dominant-negative Perk mutant lacking the kinase domain in INS 832/13 rat insulin-secreting β cells (AdDN-Perk INS 832/13 β-cells) led to reduced proliferation and insulin content (Feng et al, 2009). Unexpectedly, this study also showed that although insulin content was reduced, proinsulin was abnormally retained in the ER, suggesting an additional role for PERK in trafficking and maturation of secretory proteins. In agreement, *shPerk*-expressing INS 832/13 β-cells in which PERK protein level was reduced by 56–66% decreased insulin synthesis, and defective ER-Golgi anterograde proinsulin trafficking was also observed (Gupta et al, 2010). In contrast, acute inhibition of PERK using a specific PERK pharmacological inhibitor (PERKi) in mouse insulinoma MIN6 cells increased proinsulin synthesis (Harding et al, 2012). However, PERKi still induced rapid buildup of insoluble proinsulin associated with a subtle defect in proinsulin maturation, further supporting the concept that PERK is required for proper ER quality control of proinsulin folding and trafficking. The discrepancy regarding the effects of PERK inhibition on insulin synthesis might be explained by the different degree of PERK inhibition reached using short hairpin RNA (shRNA) or pharmacological inhibitor. We previously reported that Nck regulates PERK-mediated eIF2αS^{51} phosphorylation (Kebache et al, 2004;

Latreille & Larose, 2006). Nck is a Src-homology (SH) domain-containing adaptor protein, consisting of three N-terminal SH3 domains and one C-terminal SH2 domain (Chen et al, 1998). Nck is well known to couple activated receptor tyrosine kinases (RTKs) through its SH2 domain to SH3 domain-bound effectors involved in actin cytoskeleton remodeling (Rivera et al, 2006; Abella et al, 2010). In mammals, two different genes encode highly identical Nck isoforms, Nck1 and Nck2, which are believed to be functionally redundant (Vorobieva et al, 1995; Chen et al, 1998; Braverman & Quilliam, 1999). Recently PERK was shown to be phosphorylated at Tyr-615 in its kinase domain, and this modification was found to be essential for maximal PERK activation upon ER stress (Su et al, 2008). In this study, we report that PERK phosphorylation at Tyr-561 (Y^{561}) in the juxtamembrane creates a specific binding site for Nck and limits PERK activation. Furthermore, we provide strong evidence that this interaction regulates the ability of pancreatic β cells to synthesize proinsulin.

2.4 Materials and methods

2.4.1 Cells

Cos-1 cells and MEFs were cultured in high-glucose Dulbecco's modified eagle medium (DMEM; Invitrogen, Burlington, ON, Canada) supplemented with 10% fetal bovine serum (FBS; Invitrogen), 0.75 mg/ml penicillin, and 0.1 mg/ml streptomycin and kept at 37°C in a 95% O2–5% CO2 environment. Mouse insulinoma (MIN6) cells were cultured in high-glucose DMEM as reported but with 0.55 μM β-mercaptoethanol and 15% FBS.

2.4.2 Cell treatments

Thapsigargin (Tg; Sigma, St. Louis, MO), dithiothreitol (DTT; Roche, Mississauga, ON, Canada), and protein tyrosine phosphatase inhibitors (peroxide derivative of Na_3VO_4 [Sigma] and bpVPhen) were used as indicated in figure legends. Cos-1, MIN6, and *PERK*$^{-/-}$ MEF cells transfected with indicated plasmids using Lipofectamine Plus (Invitrogen) were lysed 48 h posttransfection in lysis buffer (Latreille & Larose, 2006) and total cell lysates subjected to either immunoblotting or immunoprecipitation.

2.4.3 Antibodies

PERK polyclonal antiserum was obtained after rabbit immunization with a GST chimera of the PERK cytoplasmic segment (amino acids [aa] 537–1114) (Harding et al, 1999). PERK phosphospecific Y^{561} (pY^{561} PERK) polyclonal antibody was generated by GenScript (Genscript USA, Piscataway, NJ) using a synthetic phosphopeptide derived from PERK juxtamembrane domain (QTESKpYDSVSADVS). Pan Nck (Nck) antibody recognizes both Nck isoforms (Lussier & Larose, 1997). Nck1 polyclonal antibody was generated as previously reported (Latreille et al, 2011). Human peIF2αSer^{52} (mouse peIF2αSer-51) antibody was from BioSource International (44-728G; Medicorp, Montreal, QC, Canada). pThr^{981} PERK (Sc-32577), eIF2α (Fl-315), pTyr (PY99), GST (B-14), GFP (B-2), RasGAP, insulin (H-86), and GLUT2 (H-67) antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). pSer/Thr antibodies were from BD Transduction Laboratories (Lexington, KY), and β-actin antibody (AC-74) was from Sigma. All other chemicals were from standard commercial sources.

2.4.4 Constructs

Plasmids encoding GST chimera of the PERK cytoplasmic segment (GST-cPERK, aa 537–1114) WT, kinase dead (K618A), Myc-PERK fulllength WT, and K618A were provided by David Ron (University of Cambridge, Cambridge, United Kingdom). GST-cPERK mutated in Y561F was generated by overlapping PCR using the primers 5′-ACTGACATCGGCACTCACGGAGTCGAATTTACTTTCAGTCTGGCACTG-3′ and 5′-GACTCCGTGAGTGCCGATGTCAGT-3′. MycPERK full-length Y561F mutant was obtained by site-directed mutagenesis (Mutagenex). Recombinant Nck proteins were produced by thrombin cleavage of purified bacterially produced GST-Nck. GFP-Nck1 full length, the three SH3 domains (3SH3, aa 1–267), and the SH2 domain (SH2, aa 268–377) were generated by subcloning corresponding cDNAs into pEGFP-C1 (Clontech, Mountain View, CA). All constructs were confirmed by DNA sequencing.

2.4.5 In vitro binding assays

GST and GST-cPERK WT or K618A (100 ng) bound on glutathione beads were incubated with recombinant Nck1 or Nck2 (100 ng) for 3 h at 4°C in the binding buffer (Latreille & Larose, 2006). Beads were washed with binding buffer, and proteins were recovered in Laemmli buffer and subjected to SDS-PAGE. Nck was revealed by immunoblot using Nck antibody and enhanced chemiluminescence as recommended by the manufacturer (Amersham GE Healthcare, Piscataway, NJ). Transfected Cos-1 cells were washed with phosphate-buffered saline (PBS) and lysed in binding buffer. GST-cPERK (5–10 μg) was used in pull-down experiments performed at 4°C for 3 h. Bound proteins were resolved by SDS-PAGE and subjected to immunoblotting.

2.4.6 In vitro kinase assays

In vitro eIF2α phosphorylation assays were performed by incubating recombinant His-eIF2α (300 ng) and GST-cPERK (200 ng) in kinase buffer (10 mM Tris-HCl, pH 7.4, 50 mM KCl, 2 mM $MgCl_2$, 1 mM DTT, 0.2 mM phenylmethylsulfonyl fluoride, 2 μg/ml leupeptin, 4 μg/ml aprotinin). Reactions were initiated by adding [γ-^{32}P]ATP (10 μCi) in a final volume of 25 μl, incubated for 30 min at 30°C, and then stopped with Laemmli buffer. Samples were subjected to SDS-PAGE and dried gels exposed for autoradiography.

2.4.7 Phosphoamino acid analysis

In vitro ^{32}P-labeled GST-cPERK WT (200 ng) produced as described was subjected to SDS-PAGE and transferred onto polyvinylidene fluoride (PVDF) membrane, and upon autoradiography the corresponding PERK band was excised and washed with H2O. Hydrolysis was performed for 1 h at 100°C in 6 N HCl. Lyophilized supernatants were resuspended in buffer at pH 1.9 containing 1 mg/ml phosphoserine, phosphothreonine, and phosphotyrosine as internal standards. Phosphoamino acids were separated by twodimensional electrophoresis (pH 1.9 and 3.5) on TLC plates (EM Sciences), visualized by autoradiography, and identified according to ninhydrin-stained standards.

2.4.8 shNck1 MIN6 cells

A mouse shRNA Nck1 was designed according to RNA interference oligoRetriever using the sequence of a mouse small interfering RNA (siRNA; Nck1 (Integrated DNA Technologies, Coralville, IA) efficient in decreasing Nck1 expression in transient transfection. This shNck1 was

subcloned into MSCV-LMP retroviral vector (Open Biosystems, Fisher Scientific, Ottawa, ON, Canada) to obtain mouse shNck1 flanked by microRNA-30 sequence and confirmed by sequencing. Retroviral productions of the empty LMP and the shNck1-containing LMP were achieved according to classic procedures. MIN6 cells infections were established by adding the appropriate virus preparation and medium at a ratio of 1:1 in the presence of Polybrene (8 μg/ml). Stable pools of control and shNck1 MIN6 cells were selected in medium containing puromycin.

2.4.9 Isolated pancreatic islets

Isolated islets (110–200) obtained as described (Peyot et al., 2004) were subjected to acid-ethanol extraction (0.2 M HCl in 75% ethanol) to determine insulin content using radioimmunoassay (Linco Research, St. Charles, MO). In parallel, isolated islets were lysed in Tris-EDTA containing 0.5% Triton X-100 to quantify protein using Bio-Rad assay (Bio-Rad, Mississauga, ON, Canada) and DNA using SYBR green.

2.4.10 Insulin biosynthesis

Control and shNck1 MIN6 cells were incubated for 1 h in MIN6 cell medium lacking methionine and cysteine and supplemented with 15% dialyzed FBS. Cells were then labeled using [^{35}S]Met/Cyst labeling protein mix (Perkin Elmer, Woodbridge, ON, Canada) at 100 μCi/ml for 30 min. Cells were then washed with ice-cold PBS and lysed, and 400 μg of protein from cell lysates was used to immunoprecipitate insulin. Samples were run on a 6 M urea/16% tricine gel and transferred onto a PVDF membrane before being dried and exposed for detection of ^{35}S label incorporated into insulin using a phosphoimager (Typhoon FLA 9500; GE Canada, Mississauga, ON, Canada).

2.4.11 Statistics

Statistical significance was determined using Student's t test with $p \leq 0.05$. In all tests, two groups with only one changed parameter were compared. Mann-Whitney test was used for the indicated experiments.

2.5 Results

2.5.1 Nck regulates PERK

We previously showed that mouse embryonic fibroblasts (MEFs) lacking Nck (*Nck1*$^{-/-}$;*Nck2*$^{-/-}$) display increased basal levels of eIF2α phosphorylated at Ser-51 (peIF2αS^{51}), concomitant with up-regulated expression of UPR markers, including ATF4, Gadd34, Grp94, and BiP (Latreille & Larose, 2006). However, the effect of Nck deficiency on PERK activity was not addressed. To investigate PERK activation in *Nck1*$^{-/-}$;*Nck2*$^{-/-}$ MEFs, we measured the levels of PERK phosphorylated at Thr-980. MEFs lacking Nck spontaneously displayed significantly increased levels of PERK phosphorylated at Thr^{980} (pThr-980) compared with wild-type (Figure 2.1A). In addition, upon thapsigargin (Tg)-induced ER stress, *Nck1*$^{-/-}$;*Nck2*$^{-/-}$ MEFs also displayed increased levels of pThr-980 PERK compared with wild-type (Figure 2.1B). In unstressed cells, PERK migrates as a doublet of 130-150 kDa that is proposed to represent differential species of phosphorylation states of the protein (Harding et al, 1999). Conversely, ER stress results in PERK migration toward 170 kDa, representing hyperphosphorylation of the protein. The shift in PERK migration toward 170 kDa was more pronounced in *Nck1*$^{-/-}$;*Nck2*$^{-/-}$ MEFs compared with controls (Figure 2.1B, PERK blot), supporting PERK hyperactivation in cells depleted of Nck. In agreement, ER stress-induced peIF2αS^{51} levels also significantly

increased in MEFs lacking Nck regardless of an apparent increase in eIF2α compared with controls (Figure 2.1B). These results reveal that Nck negatively controls PERK activation in unstressed and ER stress conditions.

2.5.2 Nck and PERK interact *in vitro*

To determine whether Nck interacts with PERK, we performed *in vitro* binding assays using glutathione S-transferase (GST) chimera of the cytoplasmic segment of PERK (GST-cPERK) and recombinant Nck proteins. Using this approach, we observed that both Nck1 and Nck2 directly interact with GST-cPERK but not with GST alone (Figure 2.2A). In this setting, the GST moiety of GST-cPERK acts as a dimerizing module causing activation of the PERK kinase activity (Harding et al, 1999). In agreement, GST-cPERK wild-type (WT) migrates slowly on SDS-PAGE compared with the GST-cPERK catalytically inactive K618A mutant (Figures 2.2B). Using Cos-1 cell lysate, we demonstrated that GST-cPERK WT interacts with endogenous Nck, whereas GST-cPERK K618A fails to bind Nck (Figure 2.2B). These results indicate that the interaction of Nck with PERK is direct and dependent on PERK kinase activity. Nck contains three SH3 domains known to recognize proline-rich sequences, followed by one SH2 domain engaging phosphotyrosine residues in target proteins (Figure 2.2C) (Chen et al, 1998; Braverman & Quilliam, 1999). To define the molecular determinant(s) mediating Nck and PERK interaction, we expressed GFP-Nck1 WT and deletion mutants in Cos-1 cells and performed pull-down assays using GST-cPERK. Deletion of the Nck1 SH2 domain (3SH3) abrogated binding to PERK, whereas deletion of the three SH3 domains (SH2) did not impair this interaction (Figure 2.2C). Therefore we conclude that the SH2 domain of Nck1 mediates the interaction with PERK. PERK is highly phosphorylated on serine and threonine residues upon activation, but studies established

that PERK is also phosphorylated on tyrosine, which is essential for optimal PERK activation (Su et al, 2008). Our finding that Nck interacts with PERK through its SH2 domain strongly suggests that GST-cPERK is indeed phosphorylated on tyrosine. Therefore we examined phosphorylation of GST-cPERK by immunoblotting using appropriate antibodies. In contrast to the catalytically inactive GST-cPERK K618A, we found that GST-cPERK WT is phosphorylated on serine and threonine (pSer/Thr), including Thr-980, and on tyrosine, as reported previously (Figure 2.2D) (Su et al, 2008). Furthermore, phosphoamino acid analysis of GST-cPERK revealed the presence of phosphoserine, phosphothreonine, and phosphotyrosine residues (Figure 2.2E). These results demonstrate that PERK activity correlates with PERK autophosphorylation on serine/threonine and tyrosine residues *in vitro* and strongly suggest that PERK tyrosine phosphorylation might mediate direct interaction with Nck.

2.5.3 Identification of pY^{561} in the PERK juxtamembrane domain as the Nck-binding site

We screened PERK cytoplasmic amino acid sequence and found that mouse PERK Y^{561} (human Y^{565}) perfectly matches the Nck SH2 domain consensus binding motif pYDXV(S/A/T/Y)X(D/E) (Figure 2.3A) (Frese et al, 2006). This motif is highly conserved among mammalian PERK orthologues but is absent in *Caenorhabditis elegans* and *Drosophila melanogaster* (unpublished data). In addition, the PERK Y^{561} surrounding amino acid sequence revealed a high degree of homology with Nck SH2 domain-binding sequences identified in enteropathogenic *Escherichia coli* Tir, nephrin, and Git-1 proteins (Gruenheid et al, 2001; Frese et al, 2006), suggesting that phosphorylation of PERK at Y^{561} could be a potential binding site for Nck. To address this, we generated a PERK mutant in which Y^{561} is replaced by a nonphosphorylatable phenylalanine (Y561F) and tested its ability to interact with Nck. GST-

cPERK Y561F failed to bind Nck in pull-down experiments (Figure 2.3B). However, GST-cPERK Y561F migrates like the active GST-cPERK WT on SDS-PAGE (Figure 2.3B, Coomassie staining), suggesting that it is catalytically active. This was confirmed by detection of pThr-980 on GST-cPERK Y561F, although GST-cPERK Y561F displayed a significant decrease in overall tyrosine phosphorylation compared with WT (Figure 2.3C). Together these results demonstrate that pY^{561}, which markedly contributes to PERK global tyrosine autophosphorylation, mediates PERK interaction with Nck.

2.5.4 Phosphorylation of PERK at Y^{561} modulates PERK activity

To address whether phosphorylation of PERK at Y^{561} affects PERK activity, we carried out a comparative analysis of GST-cPERK WT and Y561F ability to phosphorylate recombinant His-eIF2α in vitro in the presence of [γ-^{32}P]ATP. We observed that incorporation of radioactivity into His-eIF2α was significantly increased in reactions performed with GST-cPERK Y561F compared with WT (Figure 2.4A), suggesting that phosphorylation at Y^{561} negatively regulates PERK activity. This was further confirmed in *PERK*$^{-/-}$ MEFs transiently overexpressing full-length PERK WT, Y561F, or K618A, in which we assessed peIF2αS^{51} in response to ER stress. In agreement with our in vitro observations, we found that in response to dithiothreitol (DTT) treatment, peIF2αS^{51} levels were increased in *PERK*$^{-/-}$ MEFs expressing PERK Y561F compared with WT (Figure 2.4B and Supplementary Figure S2.1). In contrast, *PERK*$^{-/-}$ MEFs expressing the catalytically inactive PERK K618A mutant failed to induce peIF2αS^{51} upon DTT treatment, demonstrating that DTT-induced peIF2αS^{51} is specific to PERK in these conditions. These results demonstrate that PERK Y561F displays enhanced ER stress-induced peIF2αS^{51}, further supporting

a negative regulatory role for PERK Y^{561} phosphorylation in regulating PERK activation and signaling.

2.5.5 PERK is phosphorylated at Y^{561} in MIN6 cells

To detect PERK phosphorylation at Y^{561}, we generated a specific antibody against a synthetic phosphopeptide encompassing the amino acid sequence surrounding mouse PERK Y^{561}. We hypothesized that phosphorylation of PERK at Y^{561} (pY^{561}) would occur upon ER stress, given that previous studies showed that PERK phosphorylation on specific tyrosine residues was detected in this condition (Su et al, 2008; Krishnan et al, 2011; Bettaieb et al, 2012). However, we could not detect pY^{561} PERK in control or Tg-treated MIN6 cells (unpublished data). The difficulty in detecting pY^{561} PERK could be due to its low levels or fast cycling between phosphorylated and unphosphorylated states *in vivo*. Therefore, to stabilize tyrosine phosphorylation of PERK, we treated MIN6 cells with pervanadate (PV), a nonspecific tyrosine phosphatase inhibitor, and subsequently assessed pY^{561} PERK by immunoblotting. Of interest, we detected high levels of pY^{561} PERK and a new PERK species with distinct mobility in PV-treated cells (Figure 2.5A, left). It is noteworthy that PV treatment did not activate PERK, as neither pThr-^{980} PERK nor peIF2αS^{51} levels were increased in this condition. Treating MIN6 cells with bpVPhen, another pY phosphatase inhibitor (Posner et al, 1994), also led to PERK phosphorylation at Y^{561} without altering PERK activation and signaling (Figure 2.5A, right). To demonstrate that the immunoreactive band detected with the pY^{561} PERK antibody is specific, we transfected MIN6 cells with PERK WT, Y561F, or K618A and treated them with PV. As expected, immunoblotting of total cell lysates with pY^{561} PERK antibody showed a positive signal only in cells overexpressing PERK WT (Figure 2.5B). Collectively these data provide strong evidence that

PERK is phosphorylated at Y^{561} in unstressed cells. Supporting the labile nature of pY^{561} PERK, we observed rapid loss of PERK phosphorylation at Y^{561} upon washing out PV (Figure 2.5C, lanes 1–5). Of interest, treating cells with thapsigargin upon PV removal greatly accelerated dephosphorylation of PERK at Y^{561} (Figure 2.5C, lanes 6–10). We obtained similar findings using DTT to induce ER stress (Supplementary Figure S2.2A). In agreement, the PERK species that migrate faster in PV-pretreated MIN6 cells disappeared concomitantly with the loss of pY^{561} PERK upon PV removal, and this was accelerated by ER stress (Figure 2.5C and Supplementary Figure S2.2A). Together these results demonstrate that ER stress promotes pY^{561} PERK dephosphorylation.

To determine whether PERK phosphorylation at Y^{561} modulates ER stress-induced PERK activation and signaling, we followed pThr-980 PERK and peIF2αS^{51} in ER-stressed MIN6 cells pretreated or not with PV (Figure 2.5D). As expected, pThr-980 PERK increased upon ER stress, in agreement with PERK activation in cells not pretreated with PV (Figure 2.5D, lanes 1–5). Conversely, pY^{561} PERK decreased upon thapsigargin treatment in PV-pretreated MIN6 cells and inversely correlated with increased levels of peIF2αS^{51} (Figure 2.5D, lanes 6–10). Of interest, PV pretreatment resulted in lower levels of Tg-induced pThr-980 PERK and peIF2αS^{51}, demonstrating that phosphorylation of PERK at Y^{561} correlates with reduced PERK activation and signaling. Similar results were observed in PV-pretreated MIN6 cells exposed to DTT (Supplementary Figure S2.2B) and bpVPhen-pretreated Cos-1 cells exposed to Tg or DTT (Supplementary Figure S2.2C). Collectively these data suggest that pY^{561} PERK impairs ER stress-induced PERK activation and signaling.

2.5.6 Nck interacts with pY^{561} PERK in MIN6 cells

Given that PERK is phosphorylated at Y^{561} in PV-treated MIN6 cells (Figure 2.5) and that Nck interacts with pY^{561} PERK *in vitro* (Figure 2.3B), we assessed whether Nck and pY^{561} PERK interact *in vivo* by performing classic coimmunoprecipitation assays using MIN6 cells pretreated or not with PV. For this, Nck immunoprecipitates were probed for pY^{561} PERK. As shown in Figure 2.6, pY^{561} PERK was detected in Nck immunoprecipitates from PV-pretreated MIN6 cells, and this interaction decreased over time upon Tg exposure (Figure 2.6 and Supplementary Figure S2.3A). These results show that the interaction of Nck with PERK occurs in unstressed cells and is rapidly lost when levels of pY^{561} PERK decrease upon ER stress.

2.5.7 Nck1 modulates PERK and proinsulin levels in pancreatic β cells

To assess the importance of Nck1 in regulating PERK, we generated MIN6 cells stably expressing a specific shRNA against Nck1 (shNck1), which show >80% down-regulation of Nck1 (Figure 2.7A). We then demonstrated that down-regulation of Nck1 in MIN6 cells significantly increased PERK activation (pThr-980) and signaling (peIF2αS^{51}) in unstressed conditions (Figure 2.7A). Moreover, we observed that down-regulation of Nck1 in PV-pretreated cells increased the rate of Tg-induced pY^{561} PERK dephosphorylation and correlated with increased levels of peIF2αS^{51} (Figure 2.7B and Supplementary Figure S2.3B). These results suggest that Nck1 protects pY^{561} PERK from being dephosphorylated during ER stress and thereby limits PERK activation and signaling. Given that PERK physiological activity controls proinsulin synthesis and processing in pancreatic β cells (Gupta et al, 2010; Harding et al, 2012), we assessed proinsulin levels in shNck1 MIN6 cells that display increased physiological PERK activity. Of interest, we

found that together with increased PERK activity (Figure 2.7A), proinsulin levels were significantly up-regulated in shNck1 MIN6 cells (Figure 2.8A). Increased proinsulin appears to be specific since the levels of GLUT2 and RasGAP were not changed in shNck1 MIN6 cells. In addition, we demonstrated that increased proinsulin levels correlate with increased proinsulin synthesis, as supported by increased [^{35}S]Met/ Cyst incorporation into insulin in shNck1 MIN6 cells compared with control cells (Figure 2.8B). In agreement with PERK-mediated modulation of proinsulin levels, we demonstrated that overexpression of PERK at low levels (transfection of 0.5–2.0 μg) in MIN6 cells slightly enhanced PERK activation and proinsulin content (Supplementary Figure S2.4). However, consistent with PERK activation inducing attenuation of general translation, we found that higher levels of PERK overexpression (transfection of 5 and 10 μg) led to marked PERK activation and reduced proinsulin content. To demonstrate that PERK mediates the effect of downregulating Nck1 on proinsulin content, we overexpressed the catalytically inactive PERK K618A in shNck1 MIN6 cells. We expect that overexpression of PERK K618A will counteract PERK physiological activity by dimerizing with endogenous PERK. Accordingly, we demonstrated that PERK K618A significantly reverses the effect of downregulating Nck1 on proinsulin levels in MIN6 cells (Figure 2.8C). We extended the significance of these findings by measuring insulin levels in isolated pancreatic islets of *Nck1*$^{-/-}$ mice. We observed that *Nck1*$^{-/-}$ islets displayed increased insulin content compared with *Nck1*$^{+/+}$ islets (Figure 2.8D). Collectively our results provide strong evidence that in absence of ER stress, Nck1 binding to PERK protects PERK from being dephosphorylated at its negative regulatory site pY^{561} and thereby limits PERK activation and signaling. Furthermore, our findings indicate that Nck1 is a negative regulator of PERK activation and PERK-mediated proinsulin biosynthesis in pancreatic β cells.

2.6 Discussion

PERK, a member of the UPR, is activated in response to stress impairing ER homeostasis. In these conditions, it induces phosphorylation of eIF2α at Ser-51, which attenuates mRNA translation initiation, thereby decreasing general protein synthesis (Harding et al, 2000b). In the present study, we provide strong evidence that the SH2/SH3 domain-containing adaptor protein Nck1 is a negative regulator of PERK, which affects β cell proinsulin synthesis in physiological conditions. Indeed, we show that stable depletion of Nck1 in MIN6 cells results in enhanced physiological PERK activity, accompanied by increased proinsulin synthesis. As reported by others (Gupta et al, 2010), these results confirm that physiological PERK activity positively regulates proinsulin biosynthesis, as opposed to its well-established role as a negative regulator of general protein synthesis through phosphorylation of eIF2αSer-51 under ER stress conditions (Harding et al, 2000a). However, whereas partial PERK depletion in INS832/13 cells was reported to specifically decrease proinsulin biosynthesis (Gupta et al, 2010), acute pharmacological inhibition of PERK in MIN6 cells increased proinsulin biosynthesis (Harding et al, 2012). This discrepancy on the role of PERK on proinsulin biosynthesis can be due to the degree of PERK inhibition achieved by the different approaches used to modulate PERK activity and its regulation of general protein synthesis. Indeed, partial decrease in PERK activity by shPerk down-regulates proinsulin biosynthesis without affecting β cell general protein synthesis (Gupta et al, 2010), whereas the robustness of the effect of acute and complete inhibition of PERK activity by small molecules (PERKi) could increase proinsulin biosynthesis through nonspecific increase of global protein synthesis (Harding et al, 2012).

2.6.1 Nck directly interacts with and negatively regulates PERK

Nck couples activated receptor tyrosine kinases (RTKs) to effectors that promote RTK intracellular signaling (Chen et al, 1998). Of interest, our study demonstrates an alternative role for Nck in regulating PERK signal transduction. In fact, as for RTKs, Nck, through its SH2 domain, directly interacts with tyrosine-phosphorylated PERK, but in contrast to mediating RTK signaling, it dampens PERK activation and signaling. Supporting this, we showed that Nck directly interacts with PERK phosphorylated at Y^{561} and that PERK mutation Y561F, which abolished Nck binding, promotes PERK activation and signaling. Furthermore, we showed that in response to ER stress, Nck1 down-regulation resulted in faster PERK pY^{561} dephosphorylation and activation. These results suggest that phosphorylation of PERK at Y^{561} regulates Nck1 and PERK interaction, whereas Nck1 binding to PERK delays PERK pY^{561} dephosphorylation and activation upon ER stress. Taken together, our results also suggest that the interaction between Nck1 and PERK is dynamic because it requires PERK phosphorylation at Y^{561}, which is dependent on PERK kinase activity, as demonstrated by the inability of the PERK-inactive mutant K618A to be tyrosine phosphorylated at Y^{561} and to interact with Nck1. However, it remains to be determined whether Nck1 participates in a feedback mechanism to restore PERK inactivation when ER stress is resolved or inactivating PERK after reaching a threshold level of PERK activation during ER stress.

Previous reports identified several proteins regulating PERK activity through direct interaction with PERK luminal or cytoplasmic domains. Among these, GRP78/BiP, an ER chaperone that binds to PERK luminal domain, prevents PERK dimerization (Bertolotti et al, 2000; Hendershot, 2004). Protein kinase inhibitor of 58 kDa (p58IPK), which acts as a co-chaperone for

BiP in the ER (Rutkowski et al, 2007), is also part of a negative feedback loop through its direct interaction with PERK luminal domain, leading to inhibition of PERK kinase activity during late phases of the UPR (Yan et al, 2002b; van Huizen et al, 2003). In addition, calcineurin, a cytoplasmic Ca^{2+}-dependent phosphatase that is upregulated and activated upon ER stress, interacts with the cytoplasmic domain of preactivated PERK to further promote PERK activation (Bollo et al, 2010). Finally, PARP16, an ER-anchored protein member of the poly(ADP-ribose) polymerase family facing the cytoplasm, interacts with PERK and is required for PERK activation upon ER stress, as well as being sufficient for PERK activation in the absence of ER stress (Jwa & Chang, 2012). We demonstrate for the first time that Nck1, which is detected at the ER (Latreille & Larose, 2006), directly interacts with PERK cytoplasmic domain through the phosphorylation of Y^{561} in the PERK juxtamembrane region and that this interaction is PERK kinase and Nck1 SH2 dependent. We provide evidence that PERK is phosphorylated at Y^{561} in vivo and demonstrate that substitution of Y^{561} with a phenylalanine enhances PERK catalytic kinase activity, as monitored by phosphorylation of eIF2αS^{51}. We show that phosphorylation of PERK at Y^{561} delays PERK activation and signaling upon ER stress and that PERK phosphorylation at Y^{561} is rapidly lost in response to ER stress. Collectively these novel findings strongly suggest a negative regulatory role for PERK phosphorylation at Y^{561} on PERK activation. Previous studies reported that PERK is phosphorylated on tyrosine residues (Ma et al, 2001; Su et al, 2008). In particular, PERK tyrosine phosphorylation in the kinase domain at Y^{615} was associated with optimal activation of PERK under ER stress (Su et al, 2008), but our findings reveal that PERK phosphorylation at Y^{561} negatively regulates PERK activation. Of interest, Nck, which directly binds to pY^{561}PERK, dissociates from PERK upon ER stress, correlating with PERK pY^{561} dephosphorylation and activation. In addition, we show that Nck1 protects pY^{561}PERK from

dephosphorylation. Together these data support a role for Nck1 in negatively regulating PERK activation by interacting with and protecting pY^{561}PERK from being dephosphorylated.

Nck1 could negatively regulate PERK through various mechanisms. In the absence of stress, Nck1 bound to PERK juxtamembrane domain prevents spontaneous PERK dimerization (i.e., through an allosteric or steric mechanism). During ER stress, PERK reaches a threshold level of functional activity (i.e., PERK Thr-980, eIF2αS^{51}), whereas Nck1 re-association to the juxtamembrane domain of PERK contributes to restoring inactive PERK monomeric states. Alternatively, Nck1 binding to PERK juxtamembrane domain allosterically regulates BiP interaction with PERK to control both PERK basal activity and activation upon ER stress. On the other hand, binding of Nck1 to the PERK juxtamembrane domain induces changes in PERK conformation (e.g., allosteric modulation or sterical hindrance), which disfavors its cytoplasmic domain interaction with activators such as calcineurin during the early phase of UPR activation.

Association of Nck1 with PERK phosphorylated at Y^{561} could hinder recognition by tyrosine phosphatase(s) dephosphorylating PERK at pY^{561}, thereby limiting its activation. Conceptually, the latter mechanism is supported by our findings that PERK mutation at Y^{561} for a phenylalanine promotes PERK activation and down-regulation of Nck1 in MIN6 cells accelerates dephosphorylation of pY^{561}PERK and PERK activation upon ER stress. Protein tyrosine phosphatase 1B (PTP1B) and T-cell protein tyrosine phosphatase (TCPTP) are highly related ER tyrosine phosphatases (Andersen et al, 2001) known to modulate PERK activation and signaling in MIN6 cells (Bettaieb et al, 2011; Bettaieb et al, 2012). Although down-regulation of PTP1B results in enhanced PERK activation and signaling, TCPTP down-regulation has the opposite effect (Bettaieb et al, 2011). Because increased PERK phosphorylation at Y^{561} correlates with

decreased PERK activation during ER stress, we suggest that TCPTP, as opposed to PTP1B, can mediate dephosphorylation of PERK at Y^{561} to facilitate PERK activation. On the other hand, PTP1B deficiency enhances PERK activation and signaling in brown adipose tissue, possibly through increased PERK phosphorylation at Y^{615} (Bettaieb et al, 2012). Given that Nck interacts with PTP1B through its SH3 domains (Clemens et al, 1996), Nck bound to PERK can recruit PTP1B to dephosphorylate PERK at Y^{615}, thereby promoting PERK inactivation. Whether TCPTP dephosphorylates pY^{561}PERK or Nck hampers dephosphorylation of pY^{561}PERK by TCPTP or recruits PTP1B and PERK in a common complex requires further investigation.

Finally, as an adaptor protein, Nck1 bound to PERK through its SH2 domain could couple PERK to PERK negative regulator(s) bound to its SH3 domains. In fact, we previously showed that Nck1 overexpression decreases both basal and ER stress-induced eIF2αS^{51} phosphorylation, whereas MEFs lacking Nck spontaneously display high levels of peIF2αS^{51} and PERK downstream signaling markers (Kebache et al, 2004; Latreille & Larose, 2006). In addition, we provided evidence that Nck1 assembles a holophosphatase complex containing the Ser/Thr phosphatase PP1c and eIF2, which could be responsible for the decrease of peIF2αS^{51} in cells overexpressing Nck1 (Kebache et al, 2004; Latreille & Larose, 2006). Here we show that MEFs lacking Nck and MIN6 cells with low levels of Nck1 display enhanced PERK activation, revealing that Nck1 contributes to negative regulation of PERK. Therefore consistent with our previous findings (Kebache et al, 2004; Latreille & Larose, 2006), it is possible that Nck1, while bound to PERK, brings PP1c in close proximity, maintaining PERK dephosphorylated on critical Ser/ Thr residues involved in PERK activation.

2.6.2 Nck1 modulates proinsulin biosynthesis and content in pancreatic β cells by regulating PERK

We discovered that proinsulin levels are significantly increased in Nck1-depleted MIN6 cells as well as insulin levels in pancreatic isolated islets of *Nck1*$^{-/-}$ mice. We provide strong evidence that Nck1, by regulating physiological PERK activity, affects proinsulin biosynthesis. However, how PERK, known to attenuate translation during ER stress, promotes proinsulin biosynthesis in physiological conditions needs further investigation. Our study provides new insights into the dynamic regulation of PERK and further supports an important physiological function for PERK in pancreatic β cells. Overall it relies on original findings that provide new perspectives on understanding β cell function and dysfunction.

2.7 Acknowledgements

We thank David Ron for providing PERK-related reagents. *Nck*$^{+/+}$ and *Nck*$^{-/-}$ MEFs and *Nck1*$^{+/+}$ and *Nck1*$^{-/-}$ mice were from Tony Pawson (Mount Sinai Hospital Research Institute, Toronto, Canada). We thank Genevieve Bourret (McGill University, Montreal, Canada) for help in generating the PERK antibody. We thank Peter Siegel (McGill University) for providing the MSCV-LMP vector and Jason Northey (McGill University) for help and advice on retroviral production and stable infection of MIN6 cells. This work was supported by grants to L.L. from the Canadian Diabetes Association in honor of the late Lilian I. Dale and the Canadian Institutes of Health (MOP115045). L.Y. is supported by a doctoral award from the Canadian Diabetes Association.

2.8 Figures for Chapter 2

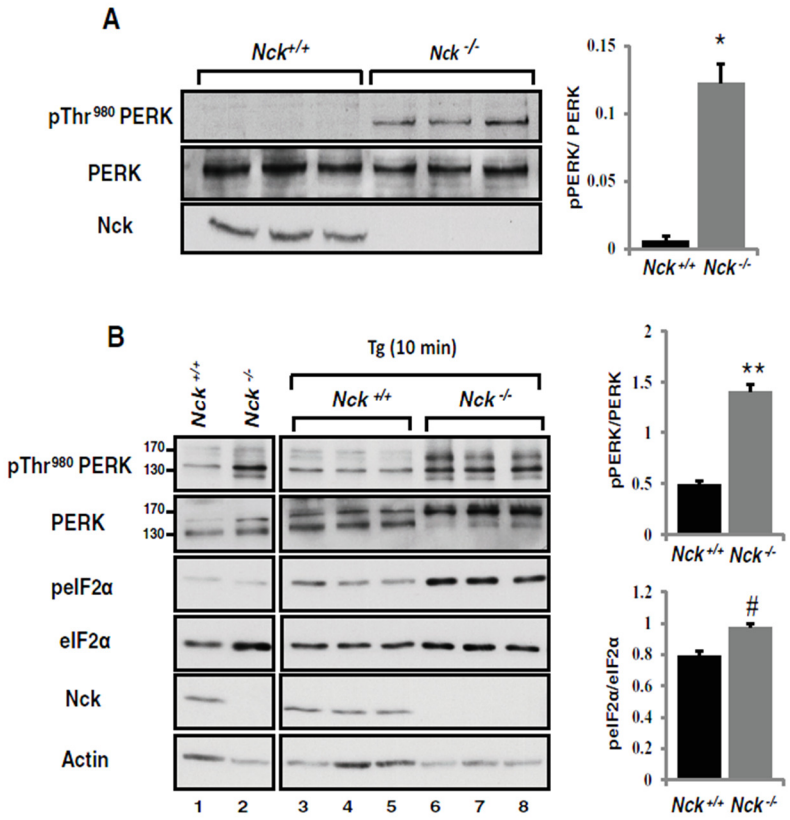


Figure 2. 1 Lack of Nck enhances PERK activity.

(A) PERK activity (pThr-980) in *Nck*$^{+/+}$ and *Nck*$^{-/-}$ MEFs. Equivalent amount of proteins (50 µg) from total cell lysates prepared from three independent culture dishes for each genotype were subjected to immunoblotting with indicated antibodies. (B) *Nck*$^{+/+}$ and *Nck*$^{-/-}$ MEFs were left untreated (lanes 1 and 2) or treated with 1 µM Tg for 10 min (lanes 3–8). Cell lysates (50 µg of protein) from independent culture dishes were analyzed by immunoblotting with the indicated antibodies. Bar charts show ratio of pThr-980 PERK to total PERK in unstressed cells (A) and pThr-980 PERK to total PERK and peIF2αS^{51} to total eIF2α upon Tg treatment as determined by densitometry (B). Data are means ± SEM (n = 3; *$p = 0.004$, **$p < 0.001$, #$p = 0.01$).

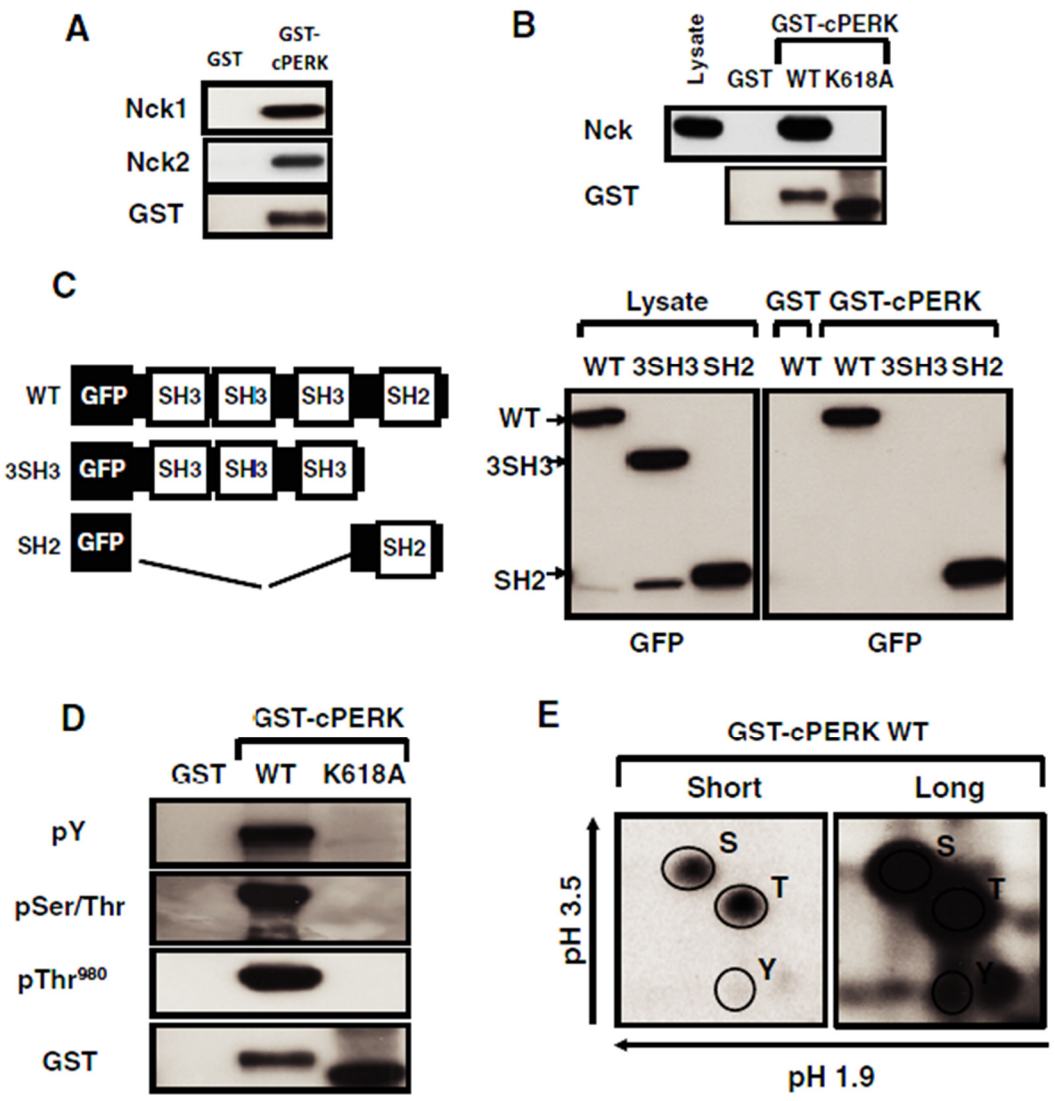


Figure 2. 2 Nck and PERK interaction.

(A) *In vitro* binding of recombinant GST-cPERK WT and Nck. Nck1 or Nck2 bound to GST-cPERK was revealed by immunoblotting using a panNck antibody, and GST-cPERK was revealed using a GST antibody. (B) Pull-down assays using GST and GST-cPERK WT or catalytically inactive (K618A) incubated with Cos-1 cell lysates. Bound endogenous Nck was revealed by immunoblotting using a panNck antibody, and a GST antibody was used to show GST proteins. (C) Schematic representation of GFP-Nck1 full length and deletion mutants (left). Pull-down

assays using GST and GST-cPERK WT incubated with Cos-1 cell lysates expressing indicated GFP-Nck1 constructs. GFP-Nck1– related proteins in cell lysates and pull-down assays were revealed by immunoblotting using a GFP antibody. (D) Phosphorylation of GST-cPERK WT and K618A (100 ng) was monitored using indicated antibodies. GST-proteins were revealed by immunoblotting using a GST antibody. (E) Two-dimensional phosphoamino acid analysis of GST-cPERK WT subjected to in vitro autophosphorylation in the presence of [γ-^{32}P]ATP. Phosphoserine, phosphothreonine, and phosphotyrosine are indicated. Data are typical of three independent experiments.

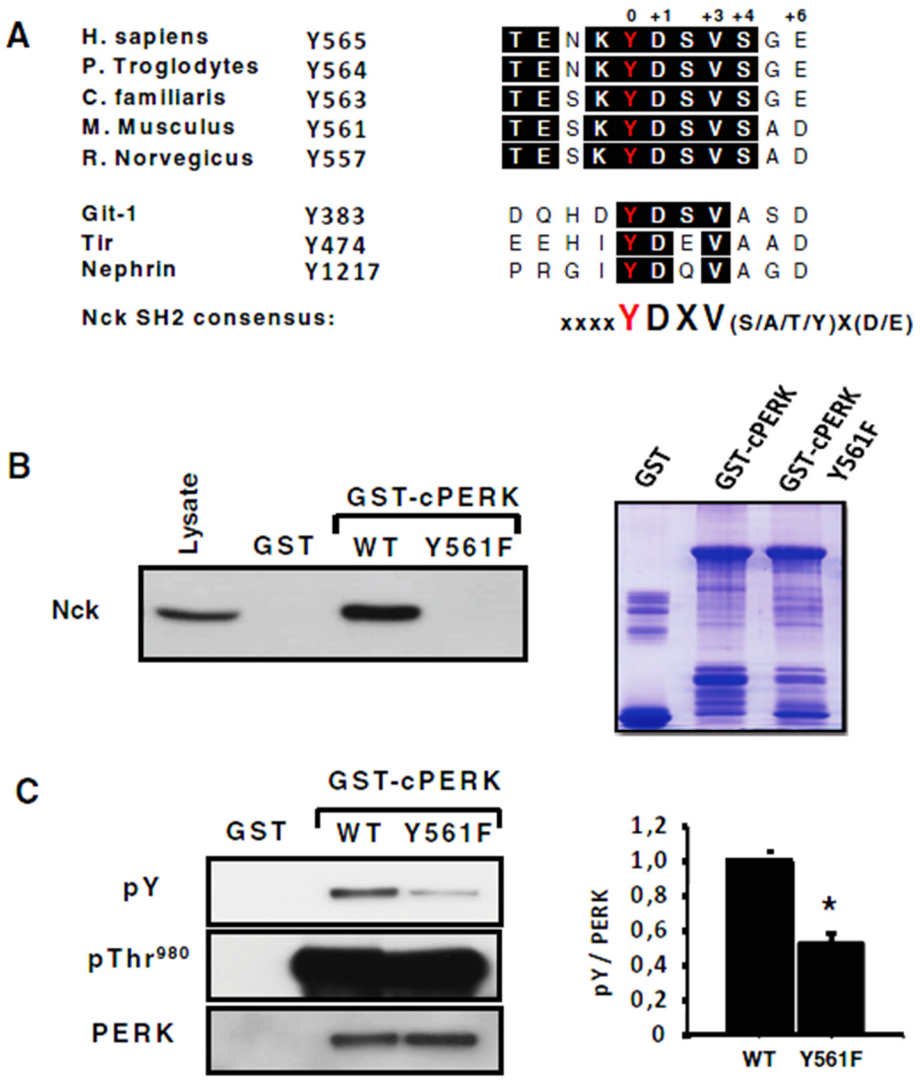


Figure 2. 3 PERK juxtamembrane pY^{561} is the Nck-binding site.

(A) Alignment of amino acids in the PERK orthologues juxtamembrane domain showing a conserved tyrosine residue (mouse Y^{561}) matching the Nck SH2 domain consensus binding motif (Frese et al, 2006) found in enteropathogenic *E. coli* Tir, Git-1, and nephrin proteins (Gruenheid et al, 2001; Frese et al, 2006). (B) Left, pull-down assay using GST and indicated GST-cPERK proteins incubated with Cos-1 cell lysates. Bound endogenous Nck was revealed by immunoblotting using a panNck antibody (n = 3). Right, purified, bacterially expressed GST-

cPERK proteins (1 μg) were resolved on SDS–PAGE and stained with Coomassie blue. (C) Global phosphorylation on tyrosine and at Thr-980 of GST-cPERK WT and Y561F mutant (100 ng) shown by immunoblotting with the indicated antibodies. Bar chart represents the ratio of global tyrosine phosphorylation of respective GST-cPERK over total GST-cPERK determined by densitometry. Data are mean ± SEM (n = 3; $*p < 0.001$).

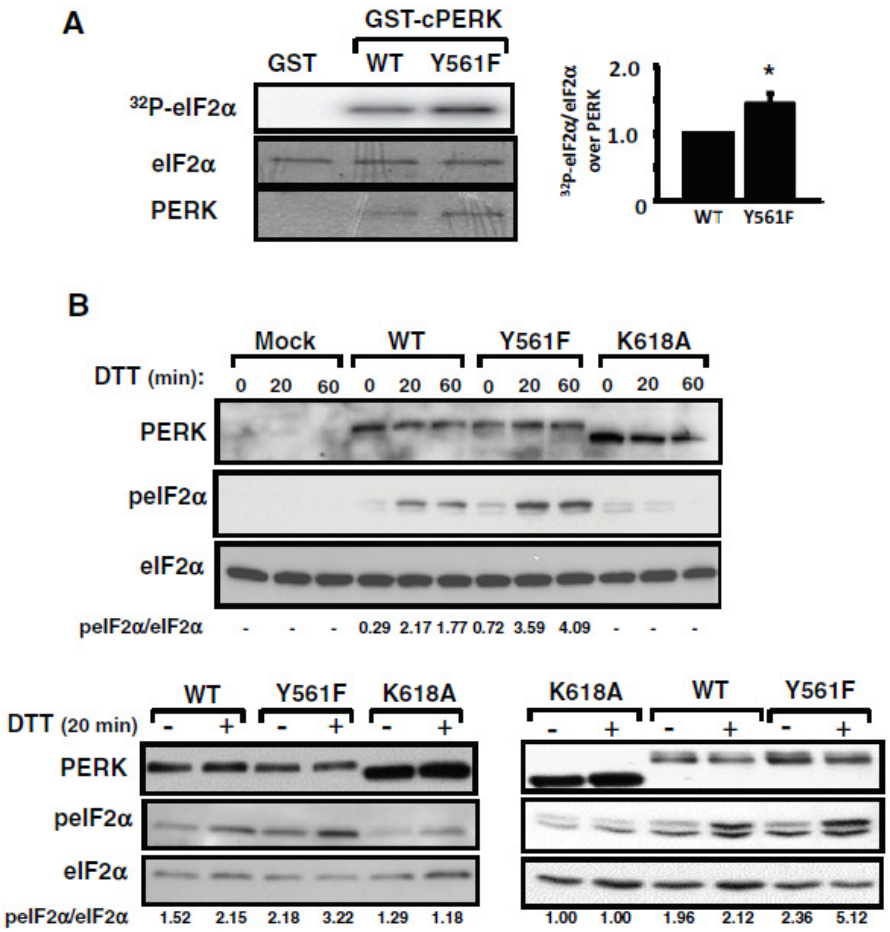


Figure 2. 4 PERK Y561F mutation enhances PERK catalytic activity.

(A) Phosphorylation of recombinant His-eIF2α by GST-cPERK WT and Y561F mutant in *in vitro* kinase assay. Bar chart shows quantification by densitometry of His-eIF2α phosphorylation normalized for the amounts of eIF2α and GST-cPERK present in the reactions. Data are mean ± SEM (n = 3; $*p < 0.01$). (B) *PERK*$^{-/-}$ MEFs transiently transfected with empty vector (Mock), full-length PERK WT, Y561F, or K618A cDNAs were treated 48 h later with 1 mM DTT for 0, 20, or 60 min. Cell lysates normalized for protein content (50 µg of protein) were subjected to immunoblotting with indicated antibodies. Shown are three independent experiments, with peIF2αS^{51}/total eIF2α ratio, as determined by densitometry, reported under the blots.

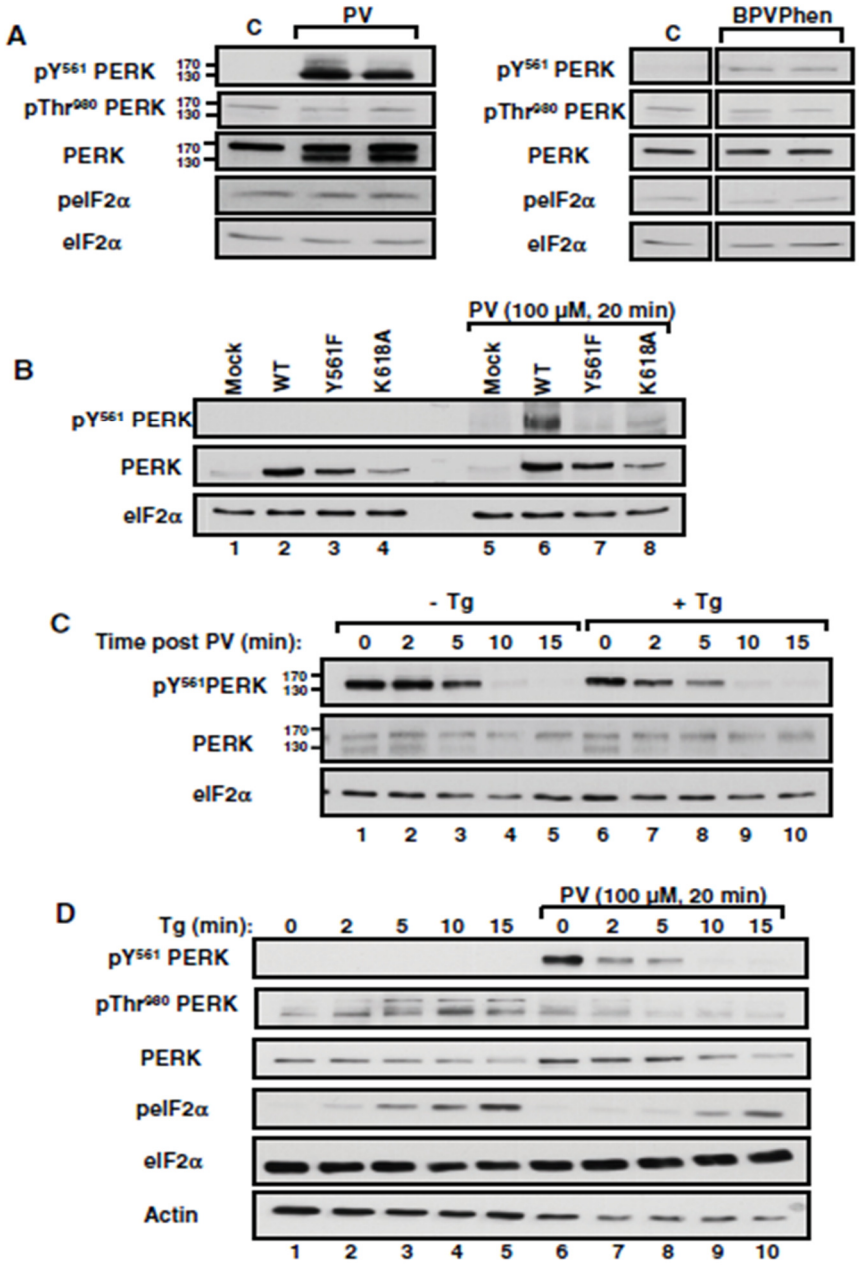


Figure 2. 5 PERK phosphorylation at Y^{561} delays PERK activation and signaling in MIN6 cells.

(A) Left, MIN6 cells were left untreated (lane 1) or treated with 100 μM PV for 20 min (lanes 2 and 3). Cell lysates (50 μg of protein) were subjected to immunoblotting with indicated antibodies.

Right, MIN6 cells were left untreated (–) or treated (+) with bpVPhen (200 μM, 10 min). Total cell lysates (50 μg of protein) from independent culture dishes were simultaneously subjected to immunoblotting using indicated antibodies (n = 3). (B) Specificity of the pY^{561} PERK antibody. MIN6 cells transiently transfected with empty vector (Mock), PERK WT, Y561F, and K618A cDNA-containing vectors were, 24 h later, left untreated (lanes 1–4) or treated with 100 μM PV for 20 min (lanes 5–8). Total cell lysates (50 μg of protein) were subjected to immunoblotting with indicated antibodies (n = 3). (C) MIN6 cells were pretreated with 100 μM PV for 20 min (lanes 1–10) and then washed with PBS and either left untreated (lanes 1–5) or treated with 1 μM Tg (lanes 6–10) for indicated times. Cell lysates (50 μg of protein) were subjected to immunoblotting using indicated antibodies. (D) MIN6 cells were left untreated (lanes 1–5) or pretreated with 100 μM PV for 20 min (lanes 6–10). Then cells were washed with PBS and treated with Tg at 1 μM for indicated times. Cell lysates (50 μg of protein) were subjected to immunoblotting using indicated antibodies. Data are representative of three independent experiments.

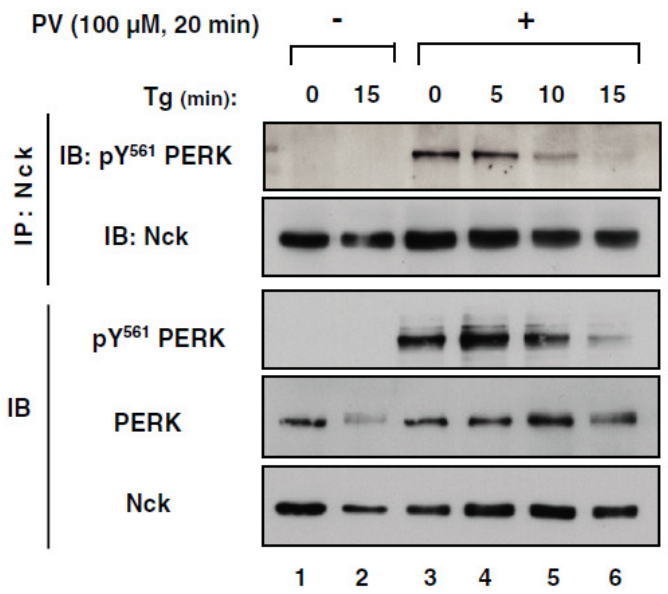


Figure 2. 6 Nck interacts with PERK phosphorylated at Y^{561} in MIN6 cells.

MIN6 cells were left untreated (lanes 1 and 2) or pretreated with 100 μM PV for 20 min (lanes 3–6) and then washed with PBS and treated with Tg (1 μM) for indicated times (lanes 3–6). Cell lysates (500 μg of protein) were subjected to Nck immunoprecipitation (IP: Nck), and Nck immunoprecipitates were subjected to immunoblotting with anti– phospho-Y561 PERK antibody. Total cell lysates (50 μg of protein) were subjected to immunoblotting with indicated antibodies (IB).

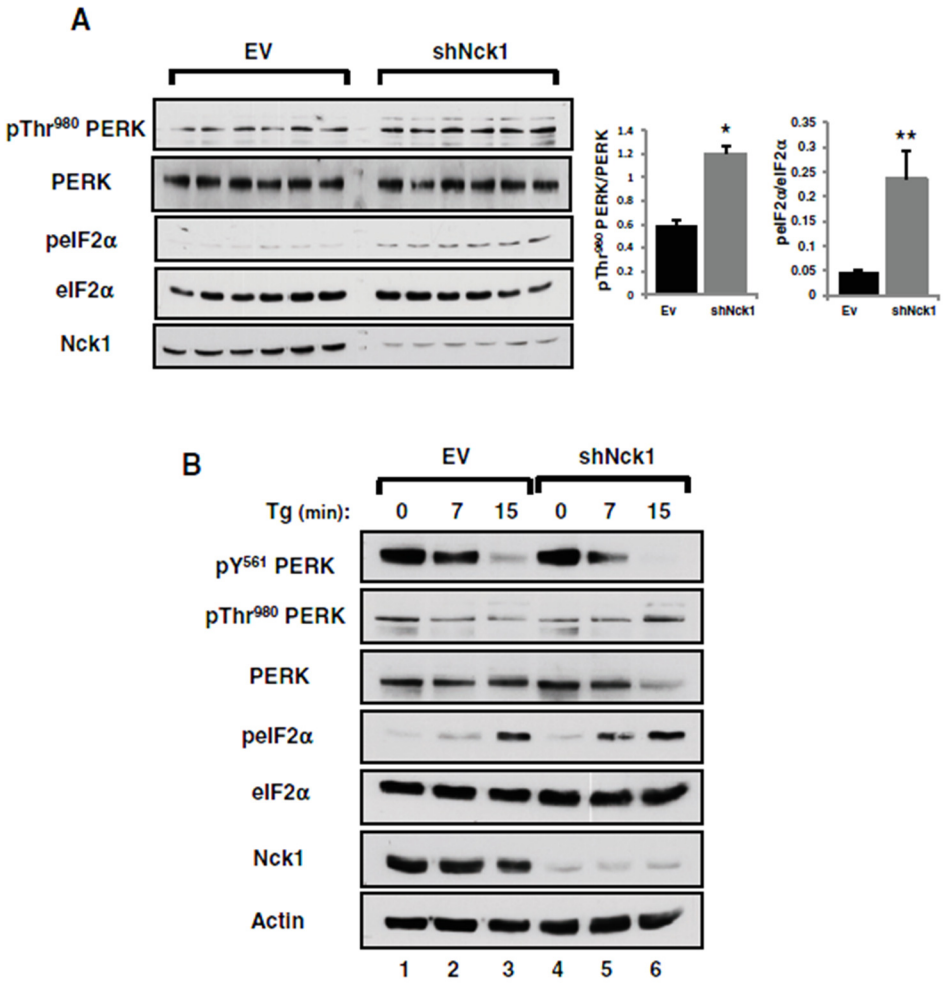


Figure 2. 7 Nck1 modulates PERK activation and phosphorylation at Y^{561} in MIN6 cells.

(A) LMP-EV and LMP-shNck1 MIN6 cell lysates (50 µg of protein) from six independent culture dishes were subjected to immunoblotting with indicated antibodies. Bar charts show the ratio of pThr-980 PERK to total PERK and peIF2αS^{51} to total eIF2α determined by densitometry. Data are mean ± SEM (n = 3; *$P < 0.001$, **$P = 0.002$, Mann–Whitney). (B) LMP-EV and LMP-shNck1 MIN6 cells were pretreated with 100 µM PV for 20 min and then washed with PBS and further treated with 1 µM Tg for 0, 7, and 15 min. Cell lysates (50 µg of protein) were subjected to immunoblotting with indicated antibodies (n = 3).

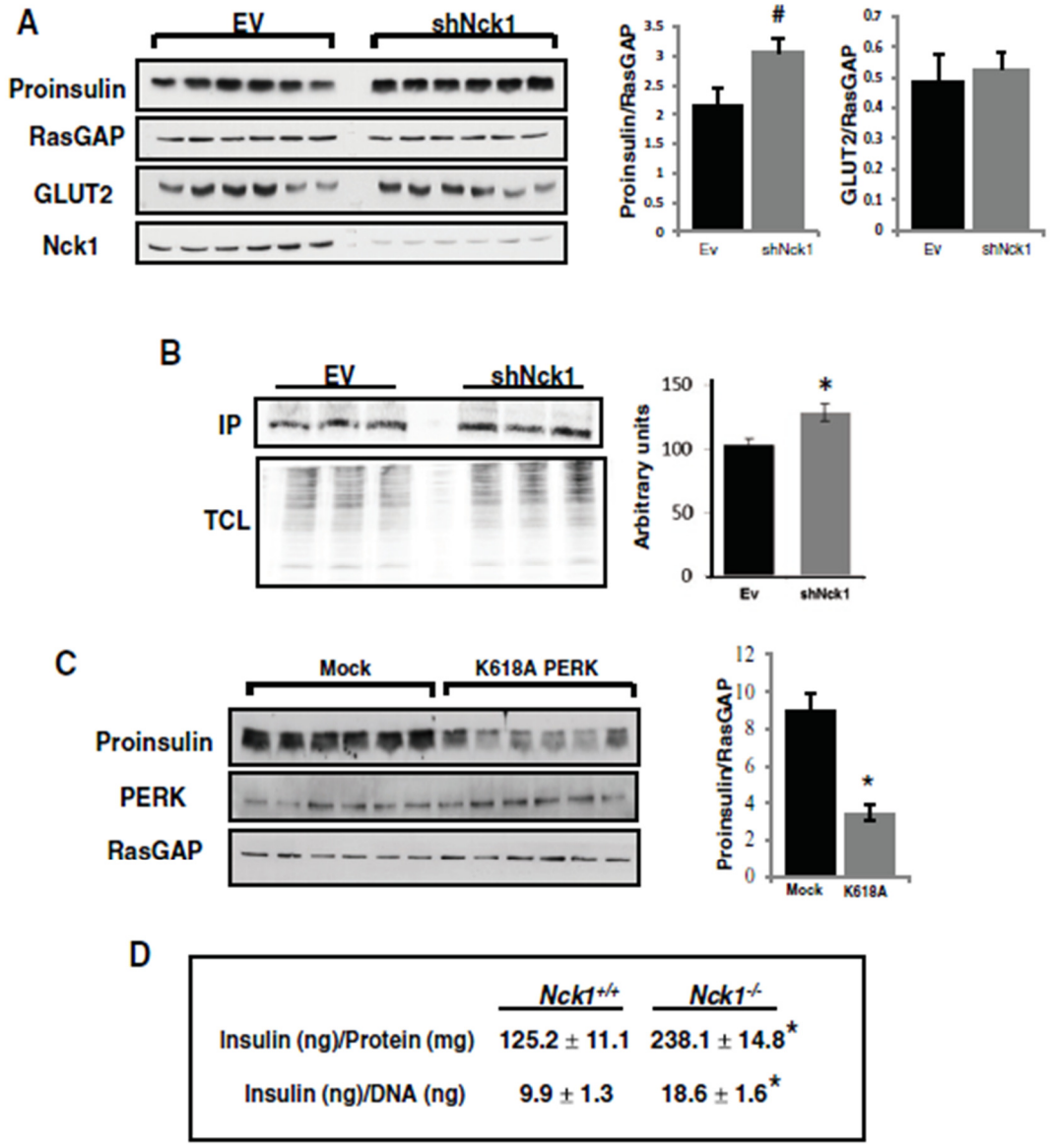
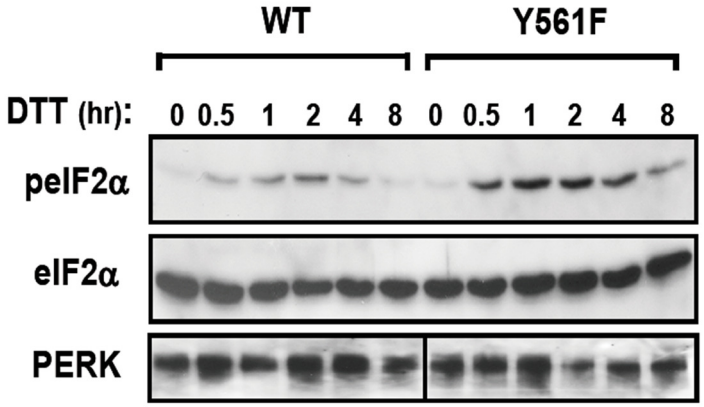


Figure 2. 8 Nck1 modulates proinsulin levels and insulin biosynthesis in MIN6 cells and insulin content in isolated pancreatic islets.

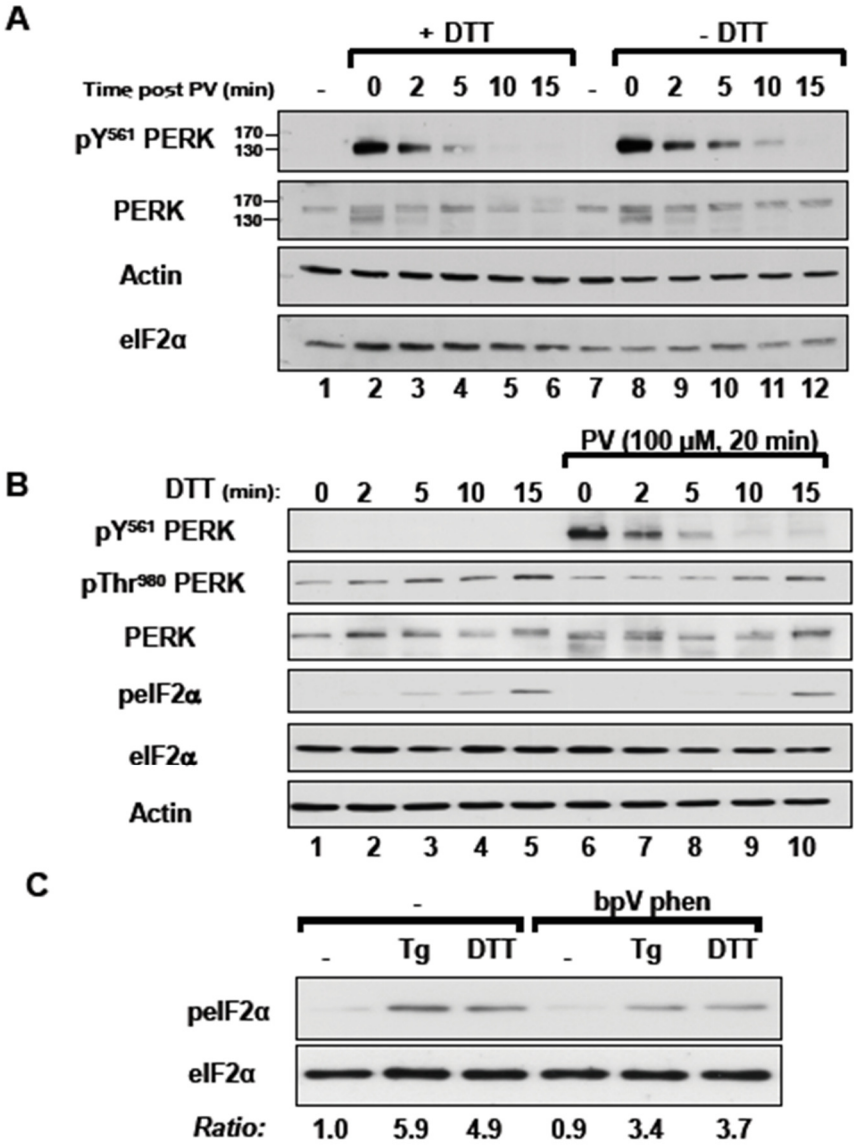
(A) LMP-EV and LMP-shNck1 MIN6 cell lysates (50 μg of protein) from independent culture dishes were subjected to immunoblotting with indicated antibodies. Bar charts show quantification

by densitometry of the indicated protein levels. Data are mean ± SEM (n = 3; *#p = 0.036*). (B) [^{35}S]Met/Cyst incorporation into insulin immunoprecipitates (IP; top) and total cell lysate proteins (TCL) from LMP-EV and LMP-shNck1 MIN6 cells. Bar chart represents quantification in arbitrary units of [^{35}S]Met/Cyst incorporation into insulin IPs. Data are mean ± SEM (n = 4; **p = 0.047*). (C) Total cell lysates (50 μg of protein) prepared from independent culture dishes of shNck1 MIN6 cells transiently transfected with pcDNA3.1 (Mock) or pcDNA3.1-PERK K618A (K618A) were subjected to immunoblotting using the indicated antibodies. Bar chart shows the ratio of proinsulin to RasGAP as determined by densitometry. Data are mean ± SEM (n = 3; **p < 0.001*). (D) Insulin levels in *Nck1*$^{+/+}$ and *Nck1*$^{-/-}$ isolated pancreatic islets. Data are mean ± SEM (n = 6; **p < 0.05*).



Supplementary figure S2. 1 PERK Y561F mutation enhances ER stress-induced PERK-mediated peIF2αS^{51}.

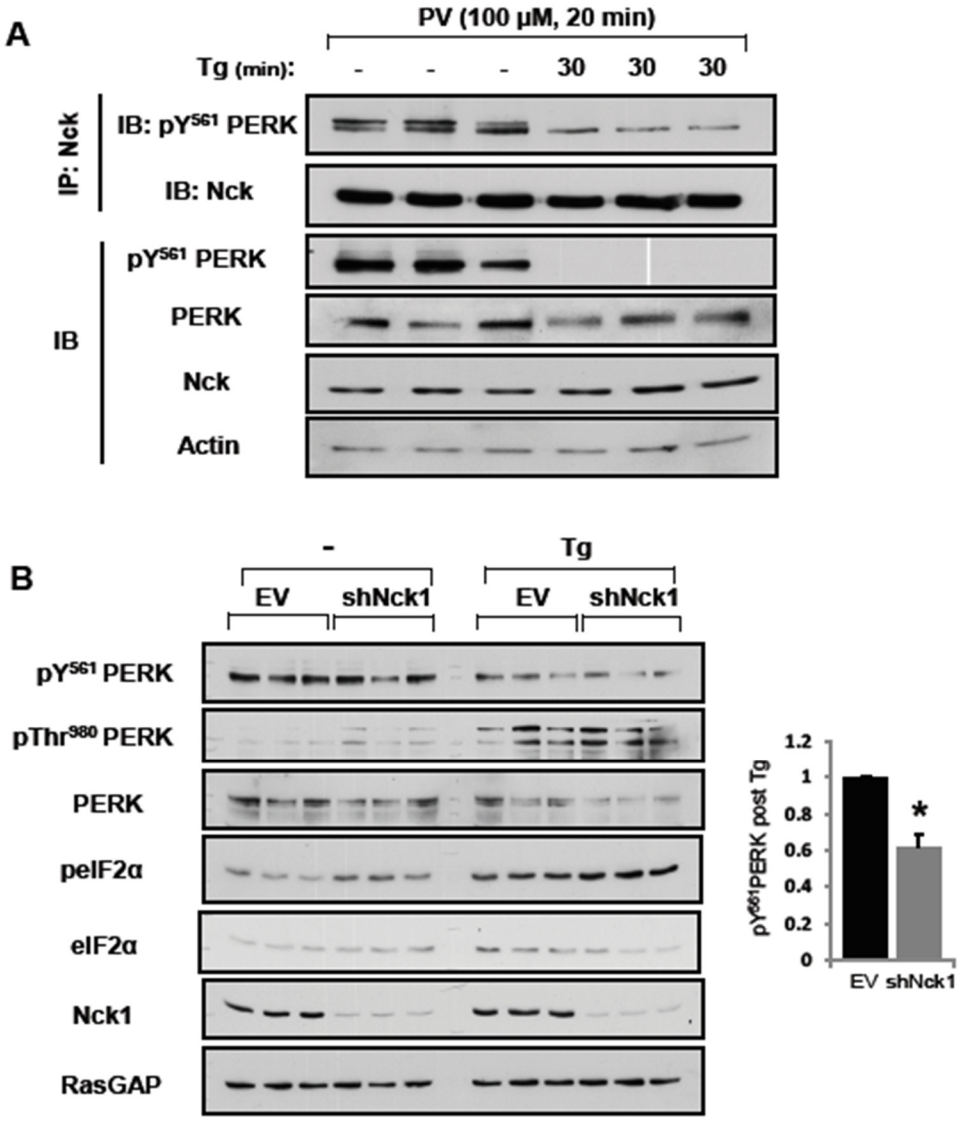
PERK$^{-/-}$ MEFs transiently expressing PERK WT or Y561F mutant were treated 48 hr later with 1 mM DTT for indicated times. Total cell lysates (50 µg protein) were subjected to immunoblotting using indicated antibodies. An inserted black line on PERK immunoblot specifies that PERK WT and Y561F samples were simultaneously processed, but from two different gels (n=3).



Supplementary figure S2. 2 PERK phosphorylation at Y^{561} delays PERK activation and signaling in MIN6 cells.

(A) MIN6 cells untreated (lanes 1 and 7) or pretreated with 100 μM PV for 20 min (lanes 2-6 and 8-12) were washed with PBS and then treated with 10 mM DTT (lanes 2-6) or not (lanes 8-12) for indicated times. Total cell lysates (50 μg protein) were subjected to immunoblotting with indicated

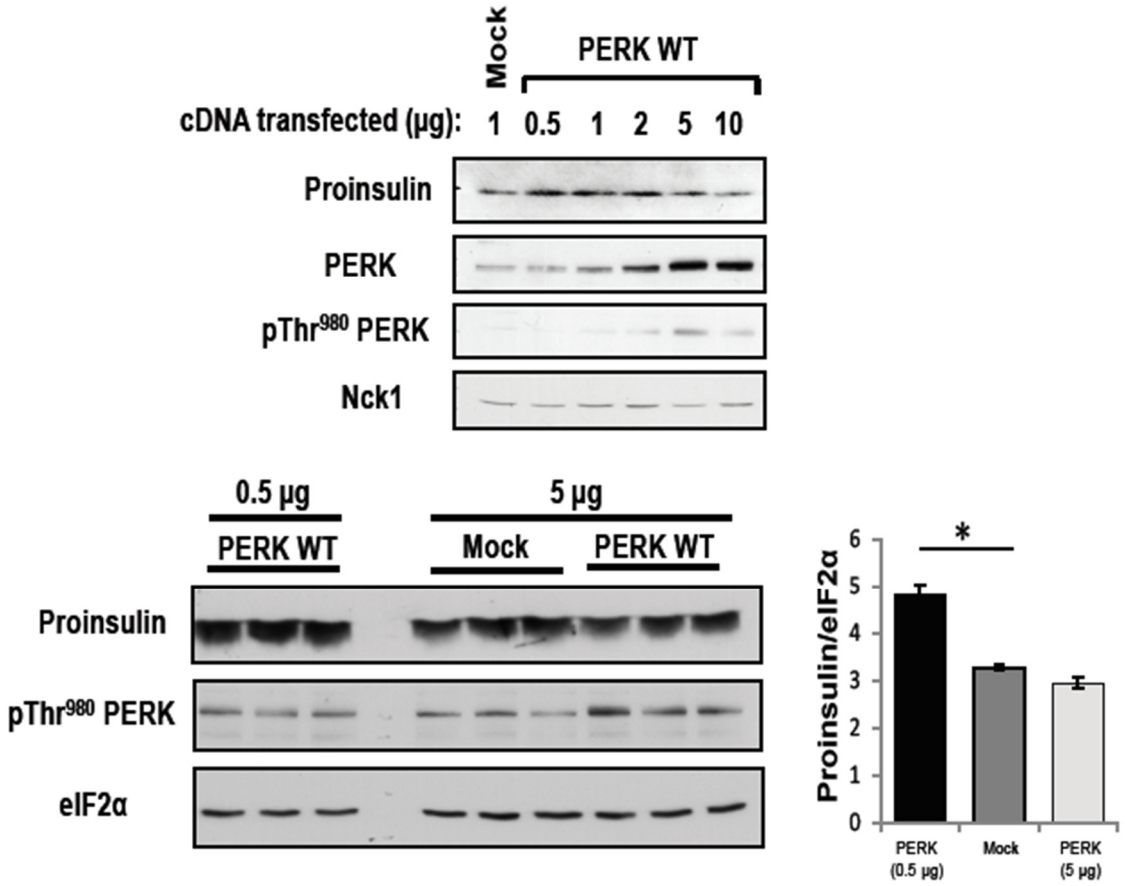
antibodies. (B) MIN6 cells untreated (lanes 1-5) or pretreated with 100 µM PV for 20 min (lanes 6-10) were washed with PBS and then treated with 10 mM DTT for indicated times. Total cell lysates (50 µg protein) were subjected to immunoblotting with indicated antibodies. (C) Cos-1 cells untreated (-) or pretreated with pbVphen (100 µM, 10 min) were washed with PBS and then untreated (-) or treated with Tg (0.5 µM) or DTT (0.5 mM) for 20 min. Total cell lysates (50 µg protein) were subjected to immunoblotting using indicated antibodies. Ratios of peIF2αS^{51}/eIF2α established by densitometry quantification upon normalisation to untreated cells are reported. Data are representative of 3 independent experiments.



Supplementary figure S2. 3 Nck interacts with PERK and modulates PERK activity.

(A) MIN6 cells were pretreated with 100 µM PV for 20 min, then washed and either left untreated (-) or treated with 1 µM Tg for 30 min. Total cell lysates (500 µg protein) were subjected to Nck immunoprecipitation and Nck immunoprecipitates were subjected to immunoblotting with indicated antibodies. Total cell lysates (50 µg protein) were probed by immunoblotting with indicated antibodies. (B) LMP-EV and LMP-shNck1 MIN6 cells were pretreated with 100 µM PV

for 20 min, then washed with PBS and either left untreated (-) or treated with 1 μM Tg for 10 min. Total cell lysates (50 μg protein) were subjected to immunoblotting using indicated antibodies. Bar chart shows the ratio of pY^{561} PERK determined by densitometry in Tg-treated compared to untreated cells. Data are mean ± SEM (*$P<0.007$) and are representative of 3 independent experiments.



Supplementary figure S2. 4 Overexpression of PERK modulates proinsulin levels in MIN6 cells.

Total cell lysates (50 µg protein) from MIN6 cells transiently transfected with empty vector (Mock) or increasing amounts of plasmid encoding PERK WT were subjected to immunoblotting with indicated antibodies. Bar chart shows densitometry of proinsulin levels. Data are mean ± SEM (**P<0.001*). Data are representative of 3 independent experiments.

CHAPTER 3. Nck1 deficiency improves pancreatic β cell survival to diabetes-relevant stresses by modulating PERK activation and signaling

Lama Yamani, Bing Li and Louise Larose[†]

Department of Medicine, McGill University and McGill University Health Centre-Research Institute, Montreal, Quebec, H4A 3J1 Canada.

Under revision to Cellular Signalling

3.1 Preface

In the previous chapter, we identified Nck as a binding partner and a regulator of PERK activity and signaling. We also demonstrated that Nck1 silencing or deletion increases insulin levels in pancreatic β cells and determined that it was PERK-dependent in the case of Nck1-silenced MIN6 cells. In chapter 3, I further investigate the role of Nck1 silencing in MIN6 cells. Here, I show that in agreement with increased insulin content, Nck1-silenced MIN6 cells display increased number of mature secretory granules, without impairment of general cell or ER morphology. Interestingly, we observed that Nck1-silenced MIN6 cells were protected against diabetes-related stresses. Correlating with that, Nck1-silenced MIN6 cells were more resistant to PERK activation in response to diabetes-related stresses. However, pharmacological induction of ER stress induced similar levels of PERK activation and cell death in both control and Nck1-depleted MIN6 cells. I then investigated cellular mechanisms triggered by the spontaneous induction of PERK activity in unstressed cells, as they might contribute towards cell survival. I revealed that Nck1-silenced MIN6 cells display enhanced autophagy in a PERK-dependent manner. In addition, the direct PERK substrate Nrf2, responsible for activating the antioxidant gene program in cells, was upregulated. Finally, Akt activation, which is considered a prosurvival mechanism, was also enhanced. Altogether, these adaptive mechanisms contribute to generate robust pancreatic β cells that produce more insulin and are protected against diabetes-related stresses.

3.2 Abstract

Increasing evidence strongly support a critical role for PERK in regulating pancreatic β cell function. In agreement, we previously reported that enhancing PERK basal activity, by silencing the SH domain-containing adaptor protein Nck1 in pancreatic β cells, increased insulin content in a PERK-dependent manner. Here we report that Nck1-deficient MIN6 cells display normal overall morphology while as expected increased number of secretory granules. Furthermore, we demonstrate that cell survival to diabetes-relevant stresses is increased, while cell viability in response to chemical endoplasmic reticulum (ER) stress inducers is not changed. In agreement, PERK activation in Nck1-depleted MIN6 cells exposed to palmitate was significantly reduced while it remained strongly induced by the ER stress inducer thapsigargin. Interestingly, silencing Nck1 in MIN6 cells results in increased PERK basal activity and expression of the PERK downstream target sestrin2, which promotes autophagy by attenuating mTORC1 activation through AMPK-dependent and -independent mechanisms. Accordingly, activated AMPK was increased, mTORC1 signaling decreased, and autophagy markers increased in Nck1-silenced MIN6 cells. Increased autophagy was recapitulated in *Nck1*$^{-/-}$ mice pancreatic β cells. In addition, basal levels of the PERK substrate Nrf2 and its antioxidant gene targets (HO-1 and Nqo1) were upregulated in Nck1-silenced MIN6 cells, revealing an active PERK-Nrf2 signaling in these cells. Finally, Akt activation was increased in Nck1-silenced MIN6 cells. Altogether, this study demonstrates that Nck1 silencing in pancreatic β cells promotes PERK activation and signaling to protect β cells against pathological stresses. These findings further provide new perspectives to advance our understanding of molecular mechanisms and signaling systems regulating pancreatic β cell fates.

3.3 Introduction

Increasing evidence from studies in humans, and findings from cell cultures and animal models strongly support an important role for the PKR-like endoplasmic reticulum kinase (PERK) in pancreatic β cell function and insulin biogenesis. Indeed, a role for PERK in pancreatic β cells was first highlighted by the discovery that the Wolcott-Rallison Syndrome (WRS) in humans, a neonatal/early infancy form of diabetes characterized by a critical reduction in β cell mass and function is caused by loss of function mutations in the *PERK* gene (Delepine et al, 2000). Furthermore, *PERK*$^{-/-}$ mice phenocopy WRS dysfunctions, and display diminished β cell mass due to reduced proliferation and differentiation during the neonatal period, consequently inhibiting postnatal gain of β cell mass (Harding et al, 2001; Zhang et al, 2002a; Zhang et al, 2006). In addition, acute inhibition of PERK through adenovirus-mediated expression of a dominant negative PERK mutant lacking its kinase domain in rat insulin-secreting β cells (INS 832/13) led to reduced cell proliferation and insulin content (Feng et al, 2009). Surprisingly, proinsulin was abnormally retained in the ER of these cells, which implicates PERK in maturation and trafficking of proinsulin. Of interest, conditional deletion of *PERK* in young or mature adult mice significantly increased β cell death even though β cell proliferation was increased (Gao et al, 2012). This reveals that PERK contributes to maintain β cell function also in adults, but apparently through a different mechanism than during early postnatal development.

PERK was initially defined as a serine/threonine protein kinase activated in conditions altering the endoplasmic reticulum (ER) homeostasis (Shi et al, 1998; Harding et al, 1999). However, recent studies revealed that PERK also displays tyrosine kinase activity that mediates its autophosphorylation on tyrosine residues and contributes to regulate its activation (Su et al,

2006; Yamani et al, 2014). ER stress-induced activated PERK phosphorylates the α-subunit of the eukaryotic initiation factor 2 (eIF2α) on Ser^{51}, resulting in attenuation of general translation, which helps to recover ER homeostasis by reducing the biosynthetic burden (Harding et al, 2000b; Vattem & Wek, 2004; Ron & Walter, 2007). Paradoxically, this promotes translation of ATF4 (Activating Transcription Factor 4), which controls an important transcriptional program with complex outputs (Vattem & Wek, 2004). Under physiological conditions inducing transient PERK activation, ATF4 is protective by regulating expression of genes that contribute to the adaptation of cells to stress conditions created by short-lived oxygen and nutrient deprivation, and ER/oxidative stress (Harding et al, 2003; Ameri et al, 2004; Blais et al, 2004). Concomitantly, PERK also directly phosphorylates the transcription factor nuclear factor (erythroid-derived 2)-like 2 (Nrf2), facilitating its nuclear translocation and subsequent transcription of target genes, whose protein products contribute to redox homeostasis and cell survival to stress (Cullinan et al, 2003; Cullinan & Diehl, 2004; Yagishita et al, 2014), and prevent onset of diabetes (Uruno et al, 2013). In addition, ATF4 was shown to dimerize with Nrf2 to promote the expression of anti-oxidant genes such as heme oxygenase-1 (HO-1) (He et al, 2001; Cullinan & Diehl, 2006), suggesting cooperation between PERK downstream signaling events in physiological conditions. In contrast, in pathologic settings created by the persistence of stress conditions, sustained PERK-peIF2α-ATF4 signaling induces prolonged expression of C/EBPα-homologous protein GADD153 (CHOP) (Harding et al, 2000b; Vattem & Wek, 2004), which triggers expression of genes that guide cells towards apoptosis (Tabas & Ron, 2011). Altogether these studies provide evidence supporting that cellular response to stress can adopt diametrically different directions determined according to the intensity and duration of activation of the PERK-peIF2α-ATF4 signaling pathway (Matsumoto et al, 2013).

Autophagy is a cellular process involving the formation of autophagosomes, small double layer membrane vesicles that sequestrate intracellular components to be targeted to bulk lysosomal degradation. Under physiological settings, basal levels of autophagy mediate clearance of extra or dysfunctional organelles or macromolecules, ensuring quality control essential to cellular homeostasis (Xie & Klionsky, 2007; Levine & Kroemer, 2008). However, autophagy is enhanced in response to various stresses, providing crucial substrates for energy production and metabolic function engaged in protecting cells against deleterious conditions like nutrient-scarce conditions (Klionsky & Emr, 2000). Interestingly, numerous studies highlighted the importance and protective role of autophagy in pancreatic β cell function and resistance to stress, and demonstrated that impairment of autophagy leads to pancreatic β cell dysfunction and death (Ebato et al, 2008; Jung et al, 2008; Choi et al, 2009; Quan et al, 2012).

We previously reported that silencing Nck1 in pancreatic β cells MIN6 enhances PERK basal activity, leading to PERK-dependent increased insulin biosynthesis and content (Yamani et al, 2014). Nck (non-catalytic region of tyrosine kinase) are 47 kDa adaptor proteins containing three Src Homology (SH) 3 domains and one SH2 domain (Lehmann et al, 1990; Braverman & Quilliam, 1999). Nck family, composed of the highly homologous Nck1 and Nck2 proteins encoded by two different genes (Chen et al, 1998; Braverman & Quilliam, 1999), mediate intracellular signal transduction by coupling cell surface receptors to specific downstream effectors (McCarty, 1998). Nck1 and Nck2 have been involved in critical biological processes, including embryonic development (Bladt et al, 2003), actin cytoskeletal reorganization (Bokoch et al, 1996; Galisteo et al, 1996), axonal guidance (Garrity et al, 1996), proliferation (Li et al, 1992) and the unfolded protein response (UPR) (Kebache et al, 2004; Nguyen et al, 2004; Latreille & Larose,

2006). In the present study, we report that stably silencing Nck1 in MIN6 cells is protective against cell death induced by stress of pathological relevance to diabetes. In parallel, we demonstrated that silencing Nck1 in MIN6 cells enhances PERK basal activity, phosphorylation of eIF2αS^{51}, and ATF4 mRNA and nuclear protein levels. In accordance, we showed that in a PERK-dependent manner autophagy is enhanced, and antioxidant gene expression increased in MIN6 cells depleted of Nck1. Given autophagy and antioxidant response clearly aid β cell survival to stress (Ebato et al, 2008; Choi et al, 2009; Quan et al, 2012), our findings provide strong evidence that silencing Nck1 in MIN6 cells enables PERK activation, initiating a signaling network that contributes to improve β cell function and resistance to pathological stress relevant to diabetes.

3.4 Material and Methods

3.4.1 Cell Culture

Control and shNck1 MIN6 cells were generated as previously described (Yamani et al, 2014). Cells were cultured in high glucose Dulbecco's Modified Eagle's Medium (DMEM, Invitrogen) supplemented with 15% fetal bovine serum (FBS, Invitrogen), 0.75 mg/ml penicillin and 0.1 mg/ml streptomycin (Invitrogen), 0.55 μM β-Mercaptoethanol (Sigma) and kept at 37°C in a 5% CO_2 environment.

3.4.2 Transmission Electron Microscopy

Control and shNck1 MIN6 cells were plated in 4-well plates under regular medium conditions. Cells were washed twice with 1X PBS and then fixed with 2.5% glutaraldehyde (Electron Microscopy Science (EMS)) in 0.1 M sodium cacodylate buffer (EMS) overnight at 4°C. Samples were washed 3 times with 0.1 M sodium cacodylate (washing buffer) for a total of 1 h, then post-fixed with 1% aqueous osmium tetroxide (Mecalab) and 1.5% aqueous potassium

ferrocyanide (Sigma) for 2 h, followed by washing three times with washing buffer for a total of 15 min. Samples were dehydrated with acetone (Fisher Scientific) in increasing concentrations: 30%, 50%, 70%, 80%, 90% and 3X with 100% each for 8-15 min. Infiltration was performed with Epon (Mecalab)/acetone: 1:1 overnight, 2:1 all day, 3:1 overnight and pure Epon next day for 4 h. Embedding was done with appropriate labels then polymerized in 60°C oven 48 h. Samples were trimmed and cut in 90-100 nm thick sections with UltraCut E ultramicrotome (Reichert-Jung) and placed onto a 200 mesh copper grid (EMS). Sections were stained with Uranyl acetate (EMS) for 8 min, then Reynold's lead (EMS) for 5 min. For morphology, cells were analyzed using FEI Tecnai 12 120 kV transmission electron microscope (TEM) equipped with an AMT XR80C 8 megapixel CCD camera.

3.4.3 Cell Treatments

Thapsigargin (Tg, Sigma) and dithiothreitol (DTT, Roche) treatments were as indicated in figure legends. Palmitate (PA, Sigma) was prepared in 3 mM stock solution conjugated with 5% fatty acid free BSA (Roche) in Hepes-balanced Kreb's-Ringer bicarbonate buffer (119 mM NaCl, 4.74 mM KCl, 2.54 mM $CaCl_2$, 1.19 mM $MgCl_2$, 1.19 mM KH_2PO_4, 25 mM $NaHCO_3$, 10 mM Hepes, pH 7.4) by shaking overnight at 37°C. The stock solution was then diluted at the desired concentration in culture medium. D-Glucose (Sigma) stock solution was 2 M and diluted to desired concentration in culture medium. Thiazolyl Blue Tetrazolium Bromide (MTT, Sigma) and Bafilomycin A1 (BafA1, Sigma) were used as indicated in figure legends. Cell transient transfection with empty or myc-tagged kinase dead PERK (K618A) plasmids was performed using Lipofectamine 2000 (Invitrogen) as recommended by the manufacturer. Cells were lysed 48 h post transfection in lysis buffer as previously described (Latreille & Larose, 2006). Total cell lysate

was subjected to Western blotting using antibodies described below. For mCherry-GFP-LC3 transient transfection, lipofectamine 3000 was used and cells were visualized 24 h post transfection.

3.4.4 MTT viability assay

Cell viability was analyzed using MTT assay. Cells were plated at $2x10^5$ cells/well in 24-well plates and the next day exposed to indicated compounds for 24 h. MTT solution prepared fresh at 5 mg/ml in PBS was diluted 1:6 in regular medium and added to each well (600 µl) at the end of the treatment. Incubation was pursued at 37°C for 3.5 h. MTT solution was then removed and 500µl/well of DMSO was then added. Absorbance was measured at 570 nm using EnSpire Multimode plate reader (Perkin Elmer).

3.4.5 Western blot analysis and antibodies

Cells were washed in cold PBS and lysed on ice using lysis buffer as reported above. Cell lysates were then centrifuged and supernatant used for Western blot analysis. Where indicated, RIPA buffer (50 mM Hepes pH 7.4, 1% Triton X-100, 1% Sodium deoxycholate, 0.1% SDS, 150 mM NaCl, 10% Glycerol, 1.5 mM $MgCl_2$, 1 mM EGTA, 1 mM sodium orthovanadate, 10 mM Sodium pyrophosphate, 100 mM Sodium fluoride) supplemented with protease inhibitors was used to lyse cells. Where indicated, crude nuclear extracts were generated as previously described (Meury et al, 2010). PERK and Nck1-specific polyclonal antibodies were previously described (Yamani et al, 2014). ATF4 (H-290), CHOP/GADD153, Nrf2 (C-20), $pThr^{981}$ PERK, and RasGAP (B4F8) antibodies were purchased from Santa Cruz. Actin, $pThr^{308}$ Akt, Akt, pAMPK, AMPK, Caspase 3, Cyclin D1, eIF2α, FOXO1, PARP, PERK (C33E10), SQSTM1 (p62), pp70S6K, p70S6K, p4EBP1 and 4EBP1 were from Cell Signaling while $pSer^{52}$ eIF2α, Alexa Fluor 488 dye,

and Alexa Fluor 568 dye were purchased from Life Technologies. Antibodies against Sens2 and LC3B were purchased from Proteintech and Sigma respectively.

3.4.6 mRNA expression analysis by qRT-PCR

From control and shNck1 MIN6 cells, total RNA was extracted using TRIzol (Invitrogen) following the manufacturer recommendations. Reverse-transcription to cDNA was performed using High Capacity cDNA Reverse Transcription kit (Applied Biosystems). PCR reactions were prepared using Power SYBR Green PCR Master Mix (Applied Biosystems). qRT-PCR data were normalized to Cyclophilin b mRNA expression levels. All primers used for PCR reactions are listed in supplementary Table S3.1.

3.4.7 Confocal microscopy

For cell immunofluorescence analysis, cells were plated on cover slips in 4-well plates and next morning fixed using 4% formaldehyde solution in PBS for 15 min at room temperature. Fixed cells were blocked and permeabilized in 5% serum and 0.3% Triton X-100 solution for 1 h incubation at RT. Then, cells were incubated overnight at 4°C in a primary antibody solution containing the Nrf2 antibody (1:200) in 1% BSA/0.3% Triton X-100, and followed by 1 h incubation at RT with red Alexa-fluor 568 as a secondary antibody. Finally, cells were briefly stained with DAPI (4', 6-diamidino-2-phenylindole). Coverslips were mounted on slides for image capturing. Images were taken using LSM780 confocal microscope and analyzed using ImageJ.

For tissue immunofluorescence staining, three pairs of *Nck1*$^{+/+}$ and *Nck1*$^{-/-}$ male mice generated in house from *Nck1*$^{+/-}$ mice previously obtained from Dr. Tony Pawson (Toronto, Canada) were used to provide pancreatic tissue to analyze LC3 distribution in β cells. Briefly, pancreatic tissue sections (4 μm) were dewaxed, then rehydrated with successive washes in 100%,

90%, 80%, 70%, and 50% ethanol. Sections were permeabilized using 0.1% Triton X-100 in PBS and rinsed with 1% H_2O_2 in PBS before to be blocked in 10% serum containing 0.1% Triton X-100 in PBS for 1 h incubation at RT. Primary antibody was prepared in PBS with 1% serum/0.1% Triton X-100 containing LC3 antibody. Alexa Fluor 488 (2-3 h in the dark at RT) was used as secondary antibody. Tissue sections were DAPI-stained, washed with PBS and coverslips mounted on slides. Pictures were taken using LSM780 confocal microscope and analyzed with ImageJ.

3.4.8 Statistical analysis

Results are expressed as mean ± standard error of mean (SEM). Significance was assessed using unpaired two-tailed Student's *t*-test, with $p<0.05$ considered as statistically significant. Bar charts represent mean of at least 3 independent experiments, unless otherwise indicated.

3.5 Results

3.5.1 Increased number of insulin secretory granules in Nck1-silenced MIN6 cells

We previously reported that Nck1-silenced (shNck1) MIN6 cells display increased insulin biosynthesis and content compared to control MIN6 cells (Yamani et al, 2014). In accordance, we showed that isolated pancreatic islets from *Nck1*$^{-/-}$ mice also contain more insulin than control (*Nck1*$^{+/+}$) mice islets. In Nck1-deficient MIN6 cells, increased insulin biosynthesis may overload the ER folding capacity, creating ER distension. To assess whether insulin-related changes impact MIN6 cell morphology, we performed electron microscopy analysis on control and shNck1 MIN6 cells (Figure 3.1A). Overall, we did not detect striking morphological differences between both cell subtypes, except that shNck1 MIN6 cells display more insulin secretory granules. Following close examination, we determined that the number of mature insulin secretory granules, defined as membrane-bound electron-dense cores surrounded by electron-transparent halos, were

significantly higher (~1.6 fold) in shNck1 MIN6 cells compared to control (Figure 3.1B). These findings demonstrate that increased insulin content in Nck1-depleted MIN6 cells (Yamani et al, 2014) results from a real increase in mature insulin secretory granules rather than accumulation of insulin in intracellular processing structures such as the endoplasmic reticulum (ER) or the Golgi.

3.5.2 Nck1-silenced MIN6 cells are protected against diabetes-relevant stresses

Given that silencing Nck1 in MIN6 cells has a positive effect on insulin synthesis and processing, we proposed that it may influence survival of pancreatic β cells to stress. To test this hypothesis, we compared control and shNck1 MIN6 cells abilities to survive diabetes-relevant stresses. Both cell subtypes grown in regular MIN6 cell culture medium were left untreated or exposed to various stresses for 24 h and cell viability was assessed by determining the number of living cells using the colorimetric MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay. Following oxidative stress induced by 100 µM H_2O_2, shNck1 MIN6 cells already displayed higher cell viability compared to control cells, but the protective effect of silencing Nck1 was statistically significant when cell death is greater in response to a higher concentration of H_2O_2 (200 µM) (Figure 3.2A). Similarly, when MIN6 cells were exposed to the saturated lipid palmitate (PA) known to induce lipotoxicity, Nck1-silenced cells displayed enhanced viability (Figure 3.2B). Similar to H_2O_2, the positive effect of silencing Nck1 on MIN6 cells survival is more important at higher concentration of PA (1mM vs 0.5mM). Finally, silencing Nck1 in MIN6 cells also significantly decreased cell death in response to high glucose (30 mM) (Figure 3.2C). Therefore, silencing Nck1 in MIN6 cells appears to be protective against cell death induced by oxidative stress, lipotoxicity and glucotoxicity, all of which are relevant to the onset of diabetes.

To rule out that increased cell proliferation in shNck1 MIN6 cells could contribute to increased viability as measured using MTT, we compared the number of viable cells using this assay in control and shNck1 MIN6 cells over 4 days of culture in regular growth condition. As reported in Figure S3.1A, using MTT assay we observed similar proliferation rates in both cell subtypes. Further confirming that silencing Nck1 does not affect MIN6 cell proliferation, we demonstrated that the levels of the proliferation marker cyclin D1 were comparable in control and shNck1 MIN6 cells (Figure S3.1B).

To demonstrate further that silencing Nck1 protects MIN6 cells against apoptosis-mediated cell death, we measured proapoptotic markers in PA-treated cells. Western blot analysis showed that after PA exposure, shNck1 MIN6 cells significantly display lower levels of both of the classical apoptotic markers, cleaved PARP and caspase 3 (Figure 3.2D), confirming that shNck1 MIN6 cells are protected against lipotoxicity-induced apoptosis.

To explore whether silencing Nck1 in MIN6 cells also protects against cell death induced by chemical agents creating ER stress that activates the UPR, a condition contributing to β cell dysfunction and apoptosis (Oyadomari et al, 2002), we evaluated cell survival following 24 h exposure to thapsigargin (Tg) and dithiothreitol (DTT), both agents known to induce ER stress. Surprisingly, silencing Nck1 in MIN6 cells failed to protect against cell death induced by the strong ER stress inducers Tg (1 μM) or DTT (10 mM) (Figure 3.2E). These results indicate that silencing Nck1 protects pancreatic β cells against pathological stresses, but not from strong agents that induce ER stress. These findings suggest that according to the intensity and the type of stress, differential mechanisms could determine pancreatic β cell fate.

3.5.3 Silencing Nck1 in MIN6 cells attenuates PA-induced PERK activation and signaling

We previously reported that silencing Nck1 in MIN6 cells enhances basal PERK activity and signaling (Yamani et al, 2014). To determine whether PERK contributes to cell survival to diabetes-relevant stresses in Nck1-silenced MIN6 cells, in preliminary experiments we compared PERK activation and signaling in control and shNck1 MIN6 cells up to 24 h following either PA or Tg exposure (Figure S3.2). As we previously reported (Yamani et al, 2014), untreated shNck1 MIN6 cells present higher basal levels of PERK activation as shown by increased phosphorylated PERK (pPERK) immunoreactive signal in untreated cells (Figure S3.2, time 0). However, during PA exposure, control cells displayed a gradual increase in PERK activity peaking between 4 to 8 h (Figure S3.2A) while in shNck1 MIN6 cells, PA-induced PERK phosphorylation, which also was maximal between 4 to 8 h, appeared to be lower. In contrast, both control and shNck1 MIN6 cells displayed similar levels of PERK phosphorylation during the time course of Tg exposure (Figure S3.2B). These data suggest that the intensity of PERK activation during PA exposure differs between control and shNck1 MIN6 cells while in response to Tg, both cell subtypes show similar levels of PERK activation. To further demonstrate this, we analyzed PERK phosphorylation in control and shNck1 MIN6 cells after 8 h exposure to either PA or Tg (Figure 3.3). Our findings confirmed that exposure of control cells to PA or Tg resulted in robust PERK activation as indicated by marked increased PERK phosphorylation. In contrast, shNck1 MIN6 cells displayed attenuated PERK phosphorylation upon PA exposure (Figure 3.3A) even though Tg-induced PERK phosphorylation was comparable in both cell subtypes (Figure 3.3B). In addition, we followed PERK signaling by measuring the levels of phosphorylated eIF2α on Ser^{51} in control and shNck1 MIN6 cells upon PA and Tg exposure. Similarly to what we observed for PERK phosphorylation, peIF2αSer^{51} in shNck1 MIN6 cells was significantly lower upon PA

exposure compared to control cells (Figure 3.3A) while in response to Tg, peIF2αSer^{51} was induced to the same extend in both cell subtypes (Figure 3.3B). Altogether, these results indicate that Nck1-silenced MIN6 cells, which display increased PERK basal activity, are more resistant to PA-induced PERK activation. In agreement with the ability of these cells to survive better to PA exposure, we propose that increased PERK basal activity and signaling following Nck1 silencing in MIN6 cells likely launches an adaptive pathway that promotes survival to stresses relevant to diabetes.

3.5.4 Silencing Nck1 in MIN6 cells promotes ATF4 expression without significantly inducing CHOP expression

PERK is recognized to influence cell fate in response to stress. Indeed, transient low levels of PERK activation are believed to promote cell survival while sustained PERK hyperactivation and signaling is associated with apoptosis (Rutkowski et al, 2006). Therefore, we hypothesize that the slight increase in PERK basal activation in shNck1 MIN6 cells initiates an adaptive response that protects pancreatic β cells to stresses inducing mild activation of PERK. To further support this hypothesis, we determined ATF4 and CHOP mRNA and nuclear protein levels, two PERK signaling downstream targets that contribute to establish the balance between survival and apoptosis upon PERK activation. Correlating with increased PERK basal activity and signaling in shNck1 MIN6 cells, we found that both ATF4 mRNA and nuclear protein levels were significantly increased compared to control cells (Figure 3.4A-B). CHOP mRNA levels followed the same trend although never reached significant levels in shNck1 MIN6 cells (Figure 3.4A). More importantly, CHOP nuclear protein levels were not detected in both control and shNck1 MIN6 cells, indicating that PERK basal activity in shNck1 MIN6 cells does not induce CHOP protein expression (Figure

3.4B). Altogether, these findings support that Nck1-depleted MIN6 cells are inclined towards a PERK-mediated prosurvival adaptive state associated with increased ATF4 expression, which is however insufficient to lead to the activation of the apoptotic CHOP pathway.

3.5.5 Depletion of Nck1 in pancreatic β cells enhances autophagy

PERK was previously shown to play a role in inducing autophagy (Kouroku et al, 2007) and increasing evidence support a protective role for autophagy in pancreatic β cell function and survival (Ebato et al, 2008; Choi et al, 2009; Quan et al, 2012). Therefore, we assessed autophagy in control and shNck1 MIN6 cells by monitoring the levels of LC3II and p62, two well-known markers of autophagy. Interestingly, we detected increased levels of the lipidated form of LC3 (LC3II) and p62 in shNck1 MIN6 cells compared to control cells, suggesting increased autophagy in shNck1 MIN6 cells (Figure 3.5A). To visualize enhanced autophagy in Nck1 silenced MIN6 cells, we transiently transfected control and shNck1 MIN6 cells with a plasmid encoding mCherry-GFP tagged LC3, and given autophagosomes are LC3-positive cytoplasmic punctate structures, we followed mCherry signal. As shown in Figure 3.5B, shNck1 MIN6 cells displayed more mCherry-positive punctate structures representing autophagosomes compared to the diffuse cytoplasmic mCherry signal in control cells. We then determined whether increased levels of autophagy markers observed in shNck1 MIN6 cells results from an increase in autophagosomes formation or a decrease in lysosomal degradation. Serum-starved MIN6 cells were treated or not with Bafilomycin A1 (BafA1), a lysosomal autophagosomes fusion inhibitor and as expected, BafA1 treatment induced LC3II accumulation in both cell subtypes (Figure S3.3). However, the levels of LC3II were higher in shNck1 MIN6 cells compared to control cells at both 2 and 4 h of BafA1 treatment, demonstrating that silencing Nck1 in MIN6 cells promotes autophagy. To

determine whether increased autophagy is PERK-dependent, shNck1 MIN6 cells were transiently transfected with a plasmid encoding a kinase inactive PERK (K618A) 48 h before assessing LC3II and p62 levels. As shown in Figure 3.5C, Western blots showed that expressing a kinase inactive PERK decreased both LC3II and p62 levels, demonstrating that the positive effect of silencing Nck1 in MIN6 cells on autophagy is PERK-mediated (Figure 3.5C).

To confirm that silencing Nck1 promotes autophagy in pancreatic β cells in an *in vivo* context, we performed LC3 immunofluorescent staining using an LC3-specific antibody on pancreatic tissue sections from 22 weeks old *Nck1*$^{+/+}$ and *Nck1*$^{-/-}$ mice fed a normal chow diet (Figure 3.5D). DAPI staining in blue indicates the nuclei, while the gray to white range signal represents the intensity of LC3 staining diffusely distributed in the cytosol or concentrated in punctate structures indicative of autophagosomes. Confocal images showing different islet regions of 3 pairs of pancreatic tissue from *Nck1*$^{+/+}$ and *Nck1*$^{-/-}$ mice show increased LC3 positive punctate structures in β cells of *Nck1*$^{-/-}$ mice compared to *Nck1*$^{+/+}$ mice. These observations recapitulate our findings using MIN6 cells and further support a role for Nck1 in regulating autophagy in pancreatic β cells.

3.5.6 Silencing Nck1 in MIN6 cells increases sestrin2-pAMPK-mTORC1 signaling and Akt activation

To delineate the mechanism by which silencing Nck1 enhances autophagy in pancreatic β cells, we focused on the recent discovery that through ATF4, PERK increases the expression of the stress-inducible protein sestrin2 (Sesn2) (Bruning et al, 2013), which is reported to promote autophagy by attenuating mTORC1 activation through AMPK-dependent and -independent

mechanisms (Budanov & Karin, 2008; Maiuri et al, 2009; Lee et al, 2010; Parmigiani et al, 2014; Peng et al, 2014). Interestingly, we found that shNck1 MIN6 cells display increased Sesn2 levels compared to control cells (Figure 3.6A). In accordance, activation of AMPK (pAMPK) was increased while mTORC1 activity, as monitored by the levels of phosphorylated p70S6K and 4EBP1, was decreased in shNck1 MIN6 (Figure 3.6B). As Sens2, through inhibiting mTORC1 or regulating PTEN, was reported to promote Akt activation (Lee et al, 2012a; Zhao et al, 2014), we determined that silencing Nck1 in MIN6 cells resulted in increased Akt activation (Figure 3.6C, pAktThr^{308}). Further supporting Akt activation, FOXO1, whose nuclear expression is decreased by Akt activation, was found to be reduced in crude nuclear extracts from Nck1-silenced MIN6 cells (Figure 3.6D). These findings strongly support that silencing Nck1 in MIN6 cells promotes Sens2-mediated signaling that regulates mTORC1 and Akt activity.

3.5.7 Silencing Nck1 in MIN6 cells increases Nrf2 nuclear localization and expression of antioxidant genes

Nrf2, a previously identified direct PERK substrate, transcriptionally activates antioxidant genes to counteract oxidative stress (Cullinan et al, 2003; Cullinan & Diehl, 2004). Interestingly, Nrf2 is regulated by Sens2 (Bae et al, 2013), which is increased in shNck1 MIN6 cells. Therefore, we compared Nrf2 levels in control and shNck1 MIN6 cells, and observed that the levels of Nrf2 were higher in shNck1 MIN6 cells (Figure 3.7A). In addition, we investigated Nrf2 distribution using immunofluorescence staining of Nrf2 and confocal imaging. Nrf2 signal intensity was determined in nuclear regions of both control and shNck1 MIN6 cells, and suggested increased nuclear Nrf2 localization in shNck1 MIN6 cells (Figure 3.7B). Finally, we evaluated Nrf2 activity by analyzing mRNA expression levels of the antioxidant genes Nqo-1 and HO-1, and found that

these genes were upregulated in Nck1-silenced MIN6 cells (Figure 3.7C), demonstrating that silencing Nck1 in MIN6 cells increases Nrf2 transactivation of antioxidant genes.

3.6 Discussion

Previous work from our group demonstrated that by interacting with PERK, Nck1 contributes to the regulation of insulin biosynthesis and insulin content in pancreatic β cells MIN6 in culture as well as in mouse pancreatic islets (Yamani et al, 2014). In this study, we now report that silencing Nck1 in MIN6 cells promotes cell survival to high glucose, palmitate and oxidative stress while it attenuates palmitate-induced PERK activation and signaling. Alongside, we found that PERK basal activity and signaling associated with autophagy and expression of antioxidant genes were increased upon silencing Nck1 in MIN6 cells. Furthermore, we showed that enhanced autophagy was recapitulated in *Nck1*$^{-/-}$ mice pancreatic β cells, suggesting that the effects of silencing Nck1 in MIN6 cells *in vitro* are relevant *in vivo*. Altogether our findings strongly suggest that silencing Nck1 in pancreatic β cells, by slightly activating PERK, implemented an adaptive signaling network that is protective against insults relevant to β cell pathology (Figure 3.8). However, this adaptive state, established following silencing Nck1 failed to protect β cells against chemical ER stress inducers such as thapsigargin and DTT, which strongly activate PERK. As proposed by others (Ma et al, 2002; Carrara et al, 2015), under these conditions the intensity of PERK activation and signaling probably reach an irreversible threshold altering the balance between pathways that support adaptation and survival, and those guide cells towards apoptosis. Thus, we propose that Nck1 in MIN6 cells contributes to determine the intensity of PERK activation and signaling that underlies differential survival rates to various types of stresses (Figure 3.8).

Mechanistically, how Nck1 exactly modulates PERK activity still remains to be elucidated. Crystal structure of mammalian PERK luminal domains recently illustrated the existence of dimeric and tetrameric states (Carrara et al, 2015). Given that PERK mutations abrogating tetramer formation resulted in lower PERK activity and phosphorylation of eIF2α, it was suggested that the dimers and tetramers may represent different levels of PERK activation states with the conversion to tetramers being higher levels of PERK activation and signaling as induced by chemical ER stress agents. We previously reported that Nck1 interacts with and limits ER stress-induced PERK activation (Kebache et al, 2004; Latreille & Larose, 2006; Yamani et al, 2014). In agreement, we showed that silencing Nck1 enhances PERK basal activity and signaling, although to a lower extent compared to ER stress-induced PERK activation (Yamani et al, 2014). It is then possible that Nck1 limits PERK activation by hampering its dimerization while silencing Nck1 facilitates spontaneous PERK dimer formation (Figure 3.8), which through autophosphorylation is sufficient to slightly, but significantly activate PERK as reported by others (Ma et al, 2002).

Our study identifies Nck1 as a novel regulator of autophagy in pancreatic β cells. Increasing evidence support an essential role for autophagy in pancreatic β cell homeostasis. Indeed, pancreatic β cell-specific deletion in mice of Atg7 (Autophagy related 7), an important component of the autophagy system, led to hyperglycemia and hypoinsulinemia attributed to a decrease in β cell mass (Jung et al, 2008). They also demonstrated ER distension in autophagy-deficient pancreatic β cells, suggesting that autophagy plays an important role in quality control of protein folding. Moreover, mice with autophagy-deficient pancreatic β cells displayed greater glucose intolerance compared to wild-type mice when fed a high fat diet, further highlighting the importance of autophagy in maintaining pancreatic β cell function in stress conditions (Ebato et

al, 2008). In addition, mice with autophagy-deficient pancreatic β cells were more susceptible to develop diabetes due to a compromised unfolded protein response (Quan et al, 2012). Finally, impaired autophagy in rodent pancreatic β cells expressing the human islet amyloid polypeptide (hIAPP) resulted in hIAPP oligomer formation and amyloid accumulation in pancreatic islets, leading to increased β cell death and concomitant decreased β cell mass associated with the development of Type 2 diabetes (Kim et al, 2014a; Rivera et al, 2014; Shigihara et al, 2014). Among different mechanisms underlying autophagy, activation of PERK and phosphorylation of eIF2αSer^{51} were both involved in ER stress-induced Atg5-Atg12-Atg16 complex-dependent conversion from LC3-I to -II, an important step in the formation of autophagosomes (Kouroku et al, 2007). In addition, PERK was recently shown to protect against liver damage during obesity by stimulating autophagy through upregulation of the stress-inducible protein Sesn2 (Park et al, 2014). Expression of Sesn2, induced by the ER stress-activated transcription factors CCAAT-enhancer-binding protein β (c/EBPβ) (Park et al, 2014) and ATF4 (Bruning et al, 2013) was reported to promote autophagy by attenuating mTORC1 activation through AMPK-dependent and independent mechanisms (Lee et al, 2010; Parmigiani et al, 2014). Altogether, these findings strongly support the existence of a significant intertwining relationship between PERK signaling and autophagy. In agreement, we demonstrate that silencing Nck1 in MIN6 cells promotes Sesn2 expression and signaling that correlates with attenuation of mTORC1 signaling and enhanced PERK-induced autophagy. These findings strongly suggest that Nck1, by modulating PERK activity, plays an important role in regulating autophagy.

Increased survival to oxidative stress upon silencing Nck1 in MIN6 cells strongly suggests an efficient antioxidant response, which could reflect adaptation to higher metabolic demand to

keep up with enhanced insulin biosynthesis activity in these cells (Yamani et al, 2014). Accordingly, in MIN6 cells depleted of Nck1 we found upregulation and enhanced nuclear translocation of the transcription factor Nrf2, a direct PERK substrate and key regulator of a pathway responsible for sensing and responding to oxidative stress (Cullinan et al, 2003; Nguyen et al, 2009). Further supporting that activation of Nrf2 may mediate the protective effect of silencing Nck1 in MIN6 cells against oxidative stress a recent study reported that Nrf2 silencing in MIN6 cells, or the knockout *Nrf2* in pancreatic islets results in sensitizing cells to oxidative damage (Fu et al, 2015). Furthermore, it was shown that pre-activation of Nrf2 in MIN6 cells protects against reactive oxygen species (ROS)-mediated cell damage (Li et al, 2014b; Fu et al, 2015). Therefore, increased PERK basal activity in Nck1-deficient MIN6 cells may directly promote constitutive Nrf2 activation mediating cell survival to oxidative damage. On the other hand, silencing Nck1 may also decrease MIN6 cells susceptibility to death induced by oxidative stress through PI3K-Akt activation of Nrf2, a prosurvival mechanism in response to oxidative stress (Wang et al, 2008; Zou et al, 2013). Akt activation in MIN6 cells depleted of Nck1 may result from the inhibition of the mTORC1-dependent negative feedback loop leading to PI3K inhibition since mTORC1 activity was attenuated in these cells. Supporting this, a recent study illustrated an important role for PERK-dependent phosphorylation of eIF2αSer^{51} in dictating cell death or survival to oxidative stress by activating Akt (Rajesh et al, 2015). Indeed, it was demonstrated that peIF2αSer^{51} has a prosurvival function against oxidative stress by downregulating mTORC1 activity, which promotes Akt activation. Thus, silencing Nck1 may protect against oxidative stress-induced β cell death through PERK direct activation of Nrf2, or indirectly through peIF2αSer^{51}-induced Akt activation. Alternatively, silencing Nck1 may promote cell survival through a PERK-independent mechanism mediating Akt activation. Further

experiments are required to evaluate whether interdependence of PERK-dependent or independent activation of Akt accounts for adaptation of Nck1-depleted MIN6 cells to oxidative stress.

Our findings support a model in which Nck1 plays a significant role in regulating PERK activation and signaling that determine pancreatic β cell fates to insults relevant to diabetes (Figure 3.8). In this model, silencing Nck1 in MIN6 cells leads to a subtle increase in basal activity of PERK, sufficient to enhance ATF4 expression and Nrf2 nuclear accumulation. ATF4 is known to upregulate the expression of Sesn2, which induces AMPK activation and attenuation of mTORC1 signaling that finally leads to enhanced autophagy and activation of Akt. Concomitantly, enhanced Nrf2 nuclear translocation promotes the expression of antioxidant genes, which contribute to improve β cells ability to survive oxidative stress. Although we present ATF4- and Nrf2-mediated signaling mechanisms independently, increasing evidence support an intertwining relationship between these pathways. Indeed, Sesn2 was reported to promote the activation of Nrf2 by enhancing autophagy-dependent degradation of Keap1, a suppressor of Nrf2 (Bae et al, 2013), while AMPK activity was shown to upregulate Nrf2 levels (Onken & Driscoll, 2010; Zimmermann et al, 2015). Moreover, ATF4 was reported to act as a binding partner for Nrf2 to induce expression of antioxidant genes (He et al, 2001).

This study clearly demonstrates that silencing Nck1 in pancreatic β cells promotes PERK basal activity and dependent signaling leading to enhanced autophagy and Nrf2-dependent antioxidant genes expression, which together confer an adaptive state that improve pancreatic β cells ability to withstand and overcome various stresses. Our findings are novel, and point toward that sensitizing PERK is beneficial for pancreatic β cell function and resistance to stress.

3.7 Acknowledgements

We thank Dr. David Ron (University of Cambridge) for the kinase dead PERK (K618A) plasmid and Dr. Barry Posner (McGill University) for providing some commercial antibodies. Cell samples were processed and electron microscopy images taken in the Electron Microscopy Research (FEMR) core facility at McGill University. We thank Dr. J.J. Bergeron and Dr. L. Hermo from McGill University for providing insightful advices on electron microscopy analysis. Confocal microscopy images were taken at the McGill University Health Centre's Imaging Core Facility under the guidance of Dr. Min Fu. Pancreatic tissue sections were generated by the Goodman Cancer Research Center Histology Facility at McGill University. We thank Maria Flores Sanchez for technical help. This work was supported by a Canadian Institutes of Health Research grant (MOP-115045) to L.L.; and L.Y. was supported by a doctoral award from the Canadian Diabetes Association.

3.8 Figures for Chapter 3

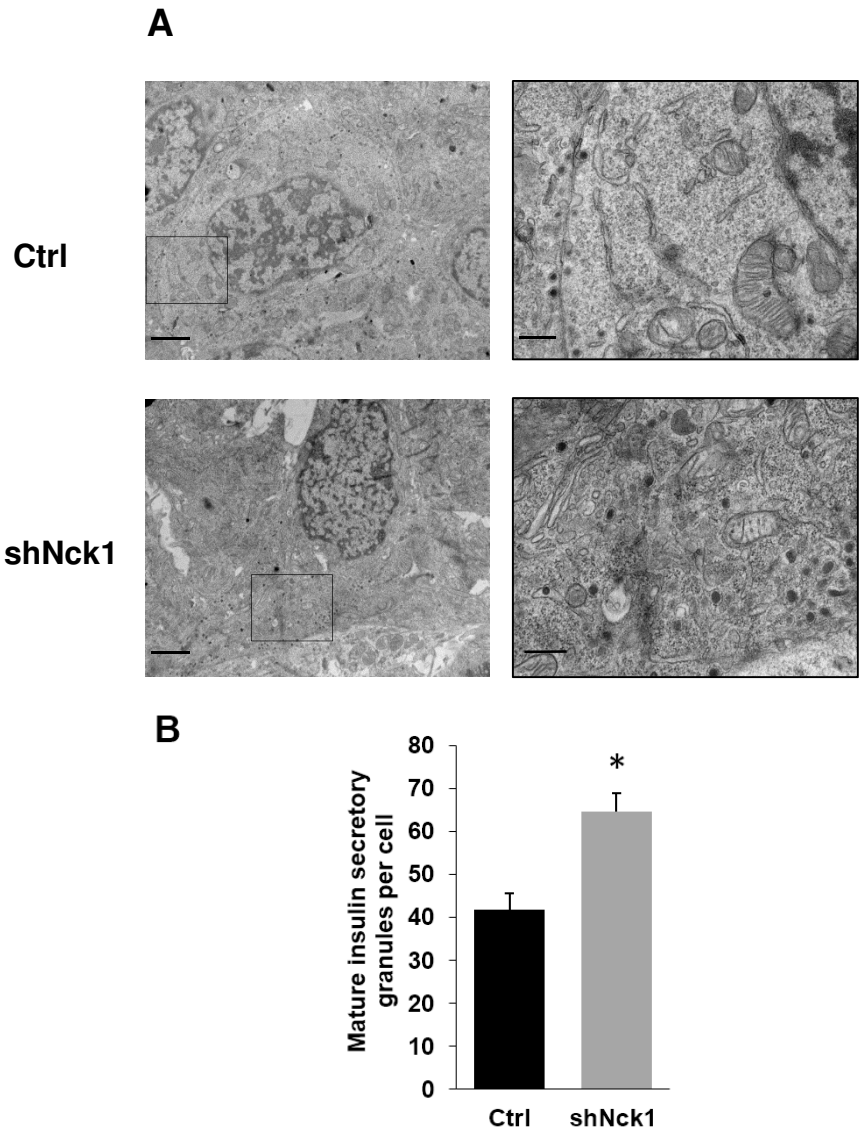


Figure 3. 1 Electron microscopy of control and Nck1-silenced MIN6 cells.

(A) Representative electron micrographs of control (Ctrl) and Nck1-silenced (shNck1) MIN6 cells (n=3 per group). Left panels: scale bar is 2 µm. Right panels represent respective magnified (4X) sections, scale bar: 500 nm. (B) Total number of mature insulin secretory granules per indicated MIN6 cell subtype. n=3, * significantly different from Ctrl ($p<0.0001$).

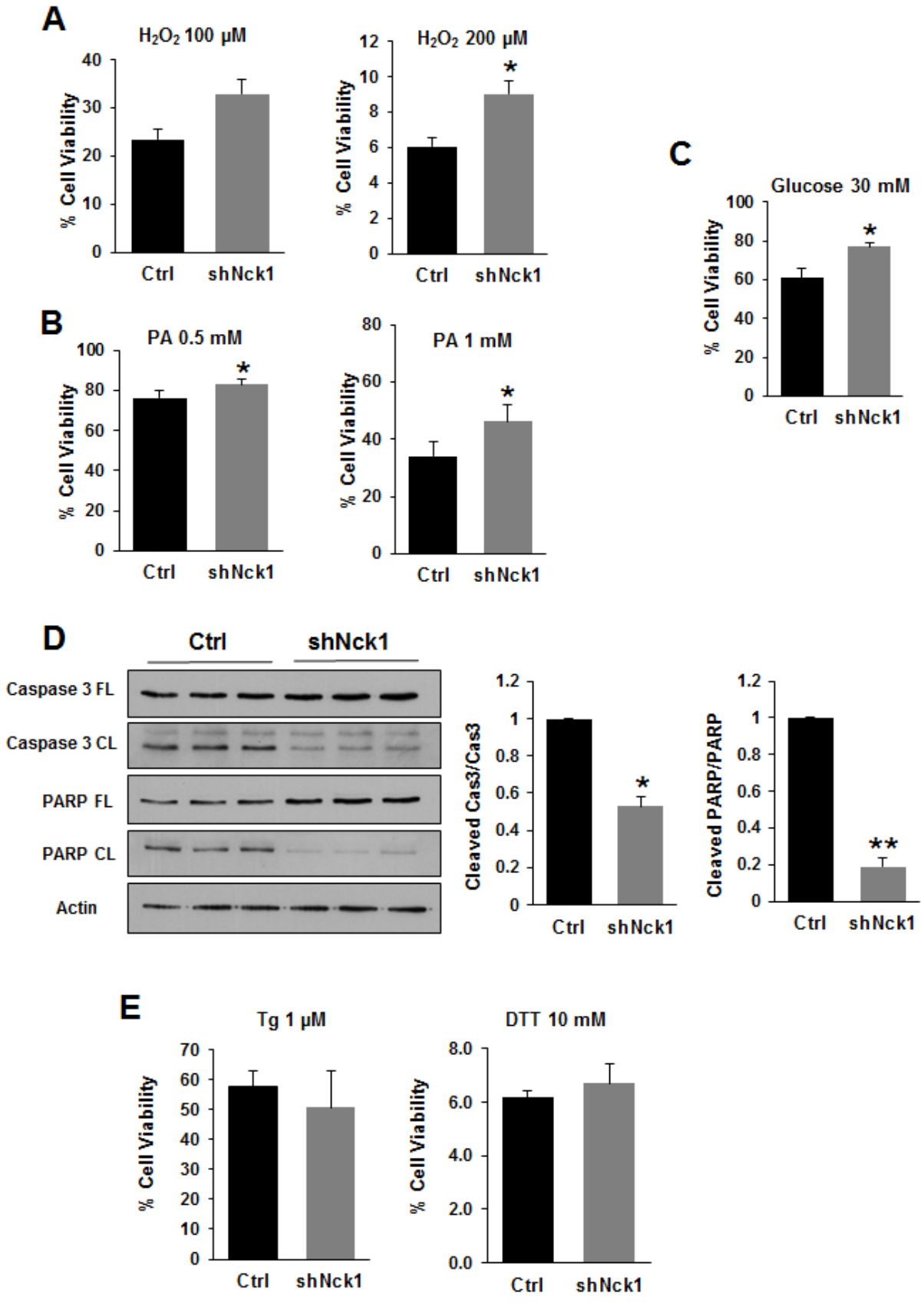

A
H2O2 100 μM
% Cell Viability
Ctrl
shNck1
H2O2 200 μM
*
B
PA 0.5 mM
PA 1 mM
C
Glucose 30 mM
D
Ctrl
shNck1
Caspase 3 FL
Caspase 3 CL
PARP FL
PARP CL
Actin
Cleaved Cas3/Cas3
Cleaved PARP/PARP
**
E
Tg 1 μM
DTT 10 mM

Figure 3. 2 Silencing Nck1 protects MIN6 cells against diabetes-related stresses.

Cells were treated with indicated agents for 24 h before to be subjected to MTT assays as described in Materials and Methods. Treatments include (A) H_2O_2 100 µM (n=2), 200 µM (n=6, *$p<0.01$), (B) Palmitate (PA) 0.5 mM (n=4, *$p<0.05$), 1 mM (n=4, *$p<0.01$), and (C) Glucose 30 mM (n=4, *$p<0.05$). Bar charts represent % cell viability in treated cells normalized to respective untreated cells. (D) Total cell lysates from cells treated 24 h with PA (1 mM) were subjected to Western blot analysis. Full length (FL) and cleaved (CL) respective proteins are indicated. Western blot images are typical representative of 3 independent experiments performed in triplicate and bar charts are quantitation of 3 experiments. *$p<0.001$, **$p<0.0001$. (E) Cells treated for 24 h with Thapsigargin (Tg 1 µM, n=6) or Dithiothreitol (DTT 10 mM, n=3) before to be subjected to MTT assays. Bar charts represent % cell viability of treated cells normalized to respective untreated cells.

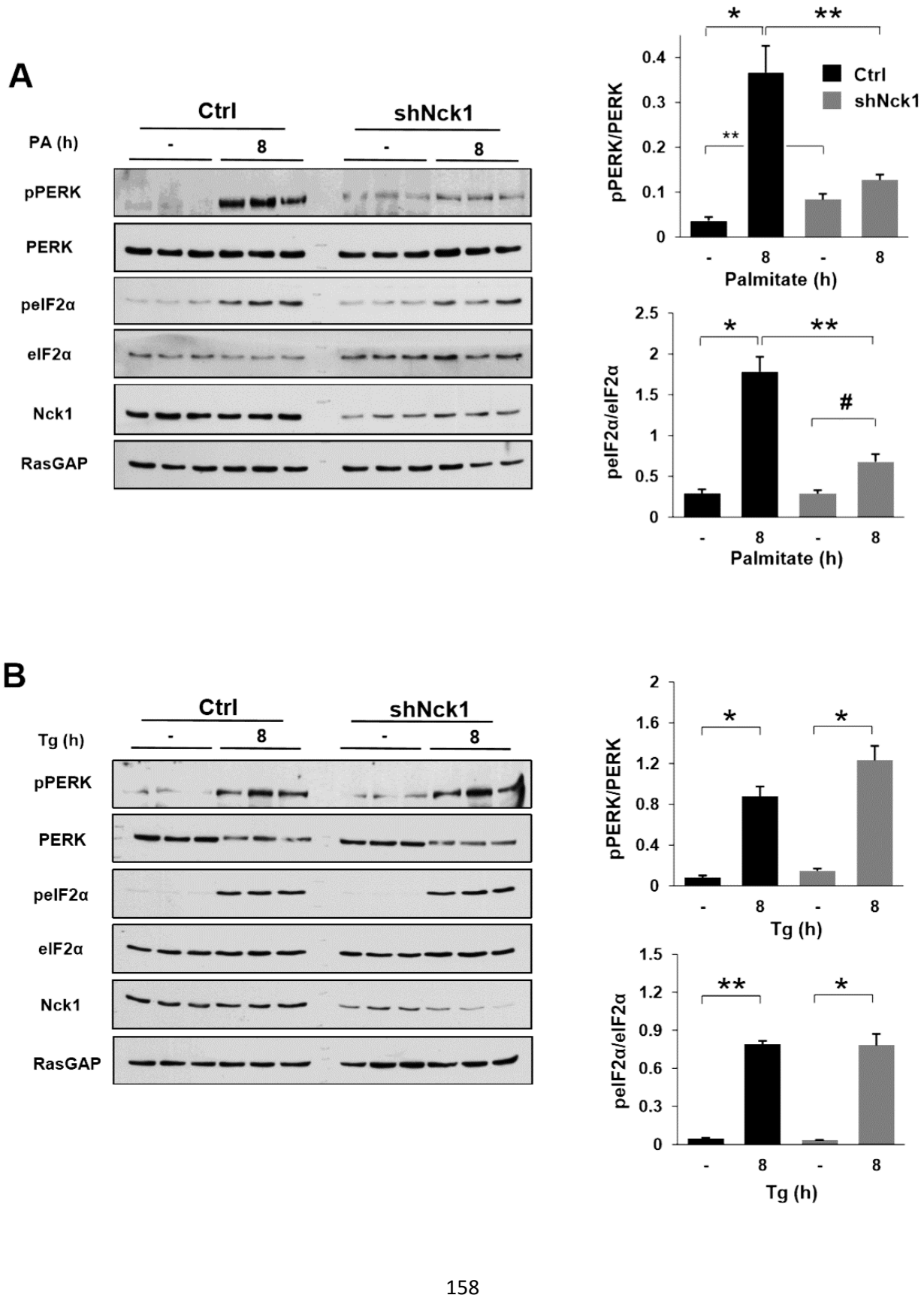

A
Ctrl
shNck1
PA (h)
-
8
-
8
pPERK
PERK
peIF2α
eIF2α
Nck1
RasGAP
pPERK/PERK
Ctrl
shNck1
Palmitate (h)
peIF2α/eIF2α
Palmitate (h)
B
Ctrl
shNck1
Tg (h)
pPERK
PERK
peIF2α
eIF2α
Nck1
RasGAP
pPERK/PERK
Tg (h)
peIF2α/eIF2α
Tg (h)

Figure 3. 3 Stress-induced PERK activation in control and Nck1-silenced MIN6 cells.

Total cell lysates from indicated MIN6 cells untreated or treated for 8 h with (A) 1 mM PA or (B) 1 μM Tg were subjected to Western blot with indicated antibodies. Western blot images and bar charts quantitation is representative of 3 independent experiments performed in triplicate. Black bars represent control (Ctrl) cells while gray bars represent shNck1 cells. Significant difference is indicated in (A) as $*p<0.01$, $**p<0.05$ for pPERK/PERK, and $*p<0.005$, $**p<0.01$, $^{\#}p<0.05$ for peIF2α/eIF2α; and in (B) $*p<0.005$, $**p<0.0001$ for both pPERK/PERK and peIF2α/eIF2α.

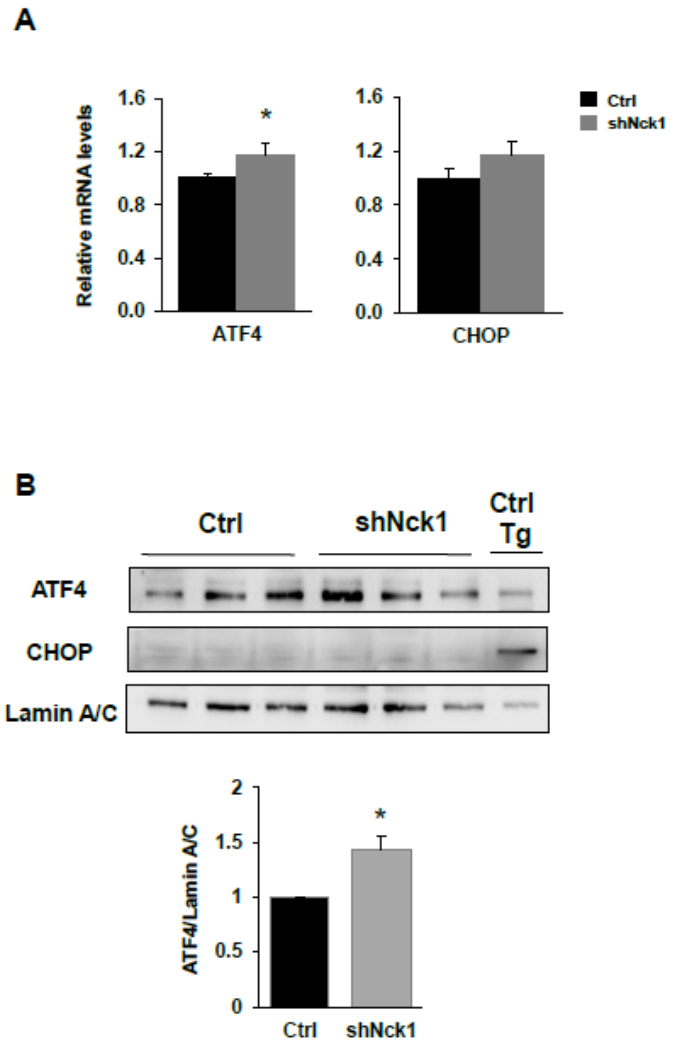
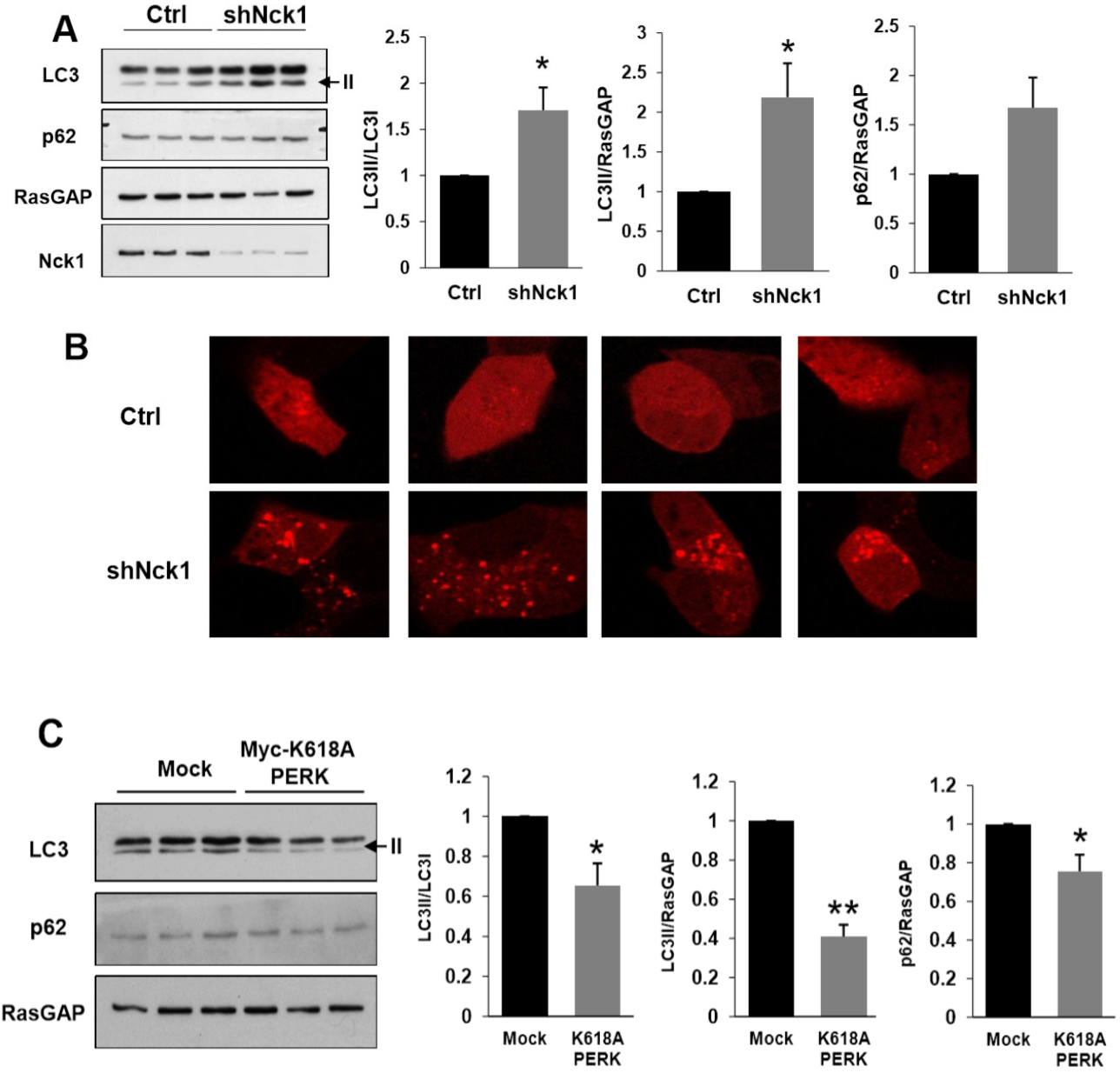


Figure 3. 4 ATF4 and CHOP expression in control and Nck1-silenced MIN6 cells.

(A) mRNA levels of ATF4 and CHOP in control (Ctrl) and shNck1 MIN6 cells as determined by qRT-PCR. Values were normalized to the housekeeping gene Cyclophilin b (Cycb). Data are mean ± SEM (n=9, *$p<0.05$). (B) Crude nuclear extracts from Ctrl and shNck1 MIN6 cells were subjected to Western blot using indicated antibodies. Last lane represents Ctrl cells treated with Tg (8 h). Bar chart is quantitation of ATF4 normalized to nuclear marker Lamin A/C from 3 independent experiments performed in triplicates. Data are mean ± SEM (n=3, *$p<0.05$).


A
Ctrl
shNck1
LC3
II
p62
RasGAP
Nck1
LC3II/LC3I
LC3II/RasGAP
p62/RasGAP
Ctrl
shNck1
B
Ctrl
shNck1
C
Mock
Myc-K618A PERK
LC3
II
p62
RasGAP
LC3II/LC3I
LC3II/RasGAP
p62/RasGAP
Mock
K618A PERK

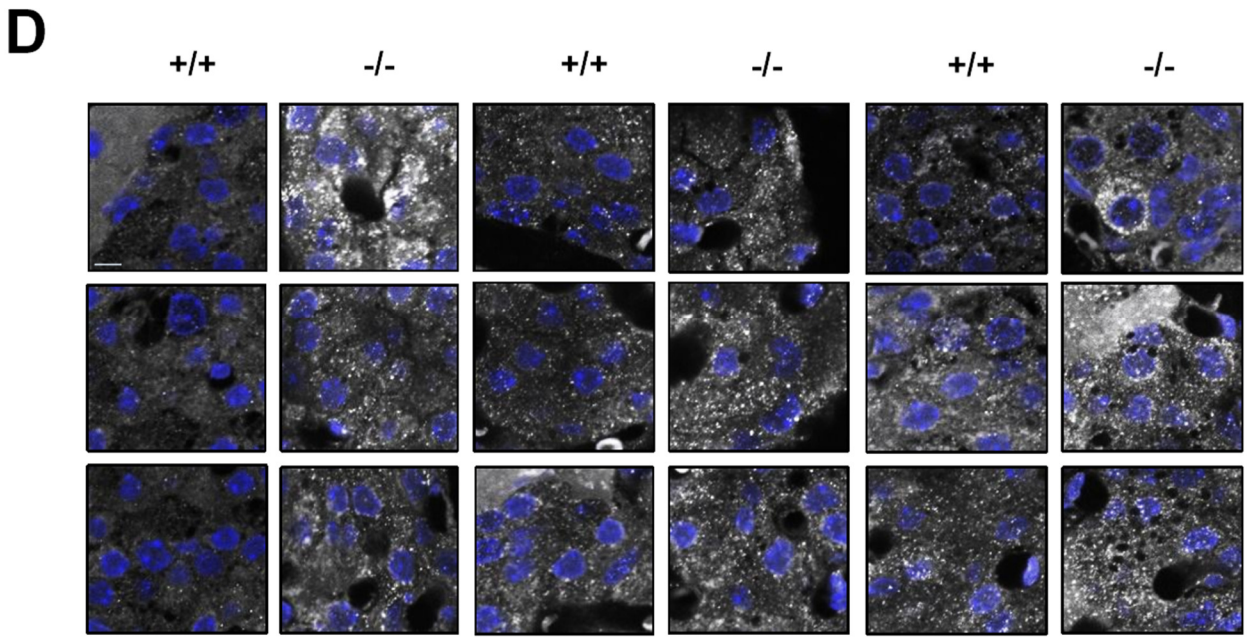


Figure 3. 5 Silencing Nck1 enhances autophagy in pancreatic β cells.

(A) Total cell lysates from control (Ctrl) and shNck1 were subjected to Western blot using indicated antibodies. For LC3, upper band is LC3I and lower band is LC3II, the lipidated form of LC3. Bar charts are quantitation of 4 independent experiments performed in triplicate, *$p<0.05$. (B) Confocal microscopy images (63X) from control (Ctrl) and shNck1 MIN6 cells transiently transfected with mCherry-GFP-LC3 plasmid (0.5 µg, n=3). (C) Total cell lysates from shNck1 MIN6 cells transiently transfected with pcDNA3.1 (Mock) or pcDNA3.1-PERK K618A (K618A) were subjected to Western blot using indicated antibodies. Bar charts represent ratio of quantified immunoreactive signals from 3 independent experiments performed in triplicate (*$p<0.05$, **$p<0.001$). (D) LC3 immunofluorescence staining confocal microscopy images of pancreatic tissue sections from 22 weeks old *Nck1*$^{+/+}$ and *Nck1*$^{-/-}$ mice (n=3 per group). Punctate LC3 staining is revealed by white dots and DAPI-stained nuclei in blue. Scale bar: 5 µm.

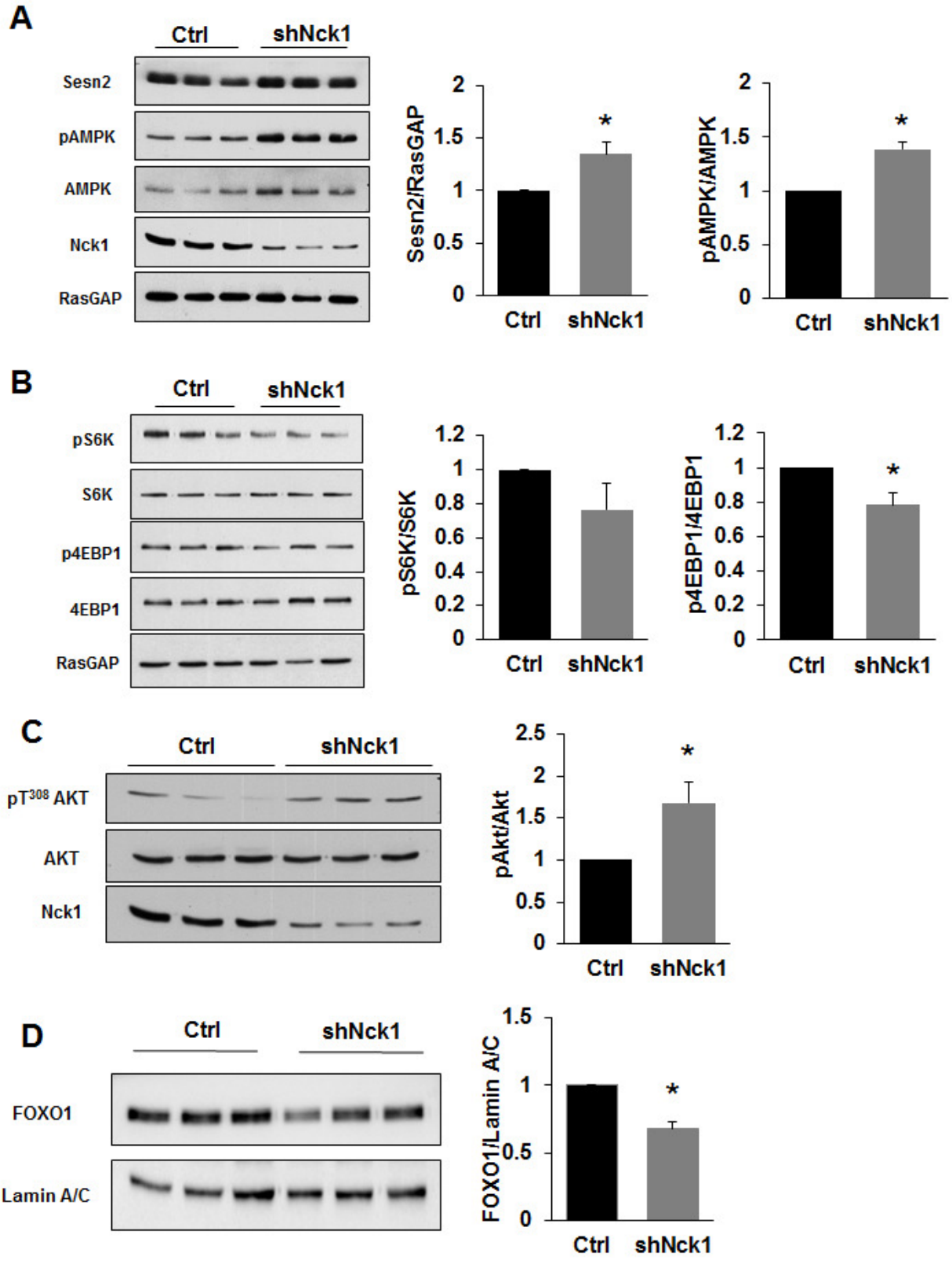

A
Ctrl
shNck1
Sesn2
pAMPK
AMPK
Nck1
RasGAP
Sesn2/RasGAP
pAMPK/AMPK
B
pS6K
S6K
p4EBP1
4EBP1
pS6K/S6K
p4EBP1/4EBP1
C
pT308 AKT
AKT
pAkt/Akt
D
FOXO1
Lamin A/C
FOXO1/Lamin A/C

Figure 3. 6 Silencing Nck1 in MIN6 cells enhances Sesn2-pAMPK-mTORC1 signaling and Akt activation.

Total cell lysates from control (Ctrl) and shNck1 MIN6 cells were subjected to Western blot using indicated antibodies. Western blots images are representative of at least 3 independent experiments performed in triplicate. Bar charts are densitometry quantitation of the ratio of (A) Sesn2/RasGAP (n=4, *$p<0.05$) and pAMPK/AMPK (n=5, *$p<0.001$), (B) pp70S6K/p70S6K and p4EBP1/4EBP1 (n=3, *<0.05), (C) pAkt/Akt (n=5, *$p<0.05$), and (D) nuclear FOXO1 protein levels and bar chart represents FOXO1/LaminA/C ratio (n=3, *$p<0.005$).

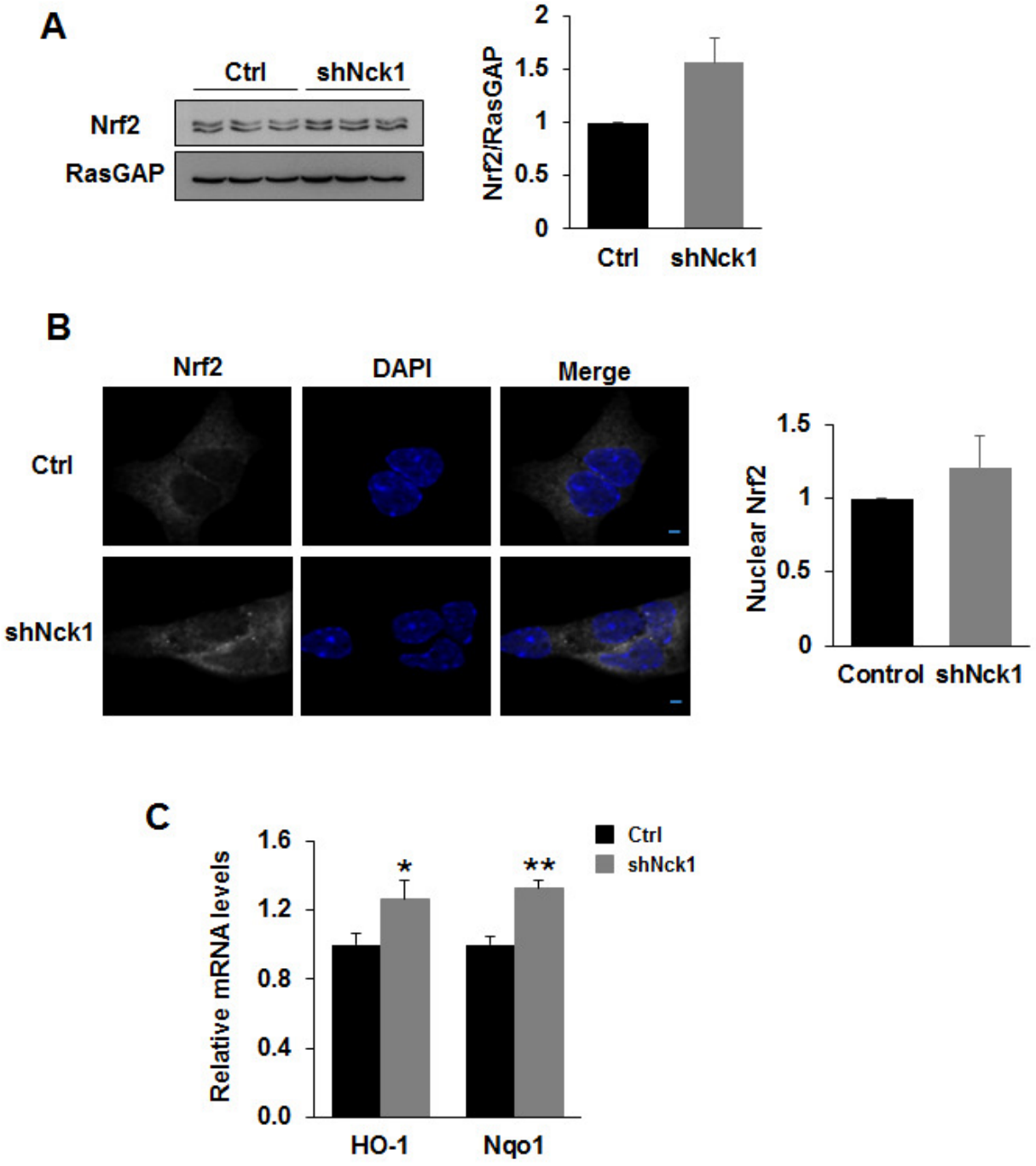

A
Ctrl
shNck1
Nrf2
RasGAP
Nrf2/RasGAP
2
1.5
1
0.5
0
Ctrl
shNck1
B
Nrf2
DAPI
Merge
Ctrl
shNck1
Nuclear Nrf2
1.5
1
0.5
0
Control
shNck1
C
Relative mRNA levels
1.6
1.2
0.8
0.4
0.0
*
**
Ctrl
shNck1
HO-1
Nqo1

Figure 3. 7 Silencing Nck1 in MIN6 cells promotes Nrf2 nuclear localization and expression of Nrf2-target antioxidant genes.

(A) Total cell lysates (RIPA buffer) from control (Ctrl) and shNck1 MIN6 cells were subjected to Western blot using indicated antibodies. Western blot images are typical of 3 independent experiments performed in triplicate. Bar charts show the ratio of Nrf2/RasGAP as determined by densitometry quantitation of individual bands (n=3). (B) Control (Ctrl) and shNck1 MIN6 cells were fixed and subjected to immunofluorescence staining with Nrf2 antibody. Confocal microscopy images are representative of 3 independent experiments. Nuclear DAPI staining is shown in blue, and Nrf2 shown in gray. Scale bar: 2 µm. Bar Chart shows quantitation of Nrf2 signal intensity in nuclear regions (n=3, ImageJ). (C) HO-1 and NqO-1 mRNA levels in control (Ctrl) and shNck1 MIN6 cells as determined by qRT-PCR. Values were normalized to the housekeeping gene Cyclophilin b (Cycb). Data are mean ± SEM (n=9, $*p<0.05$, $**p<0.0005$).

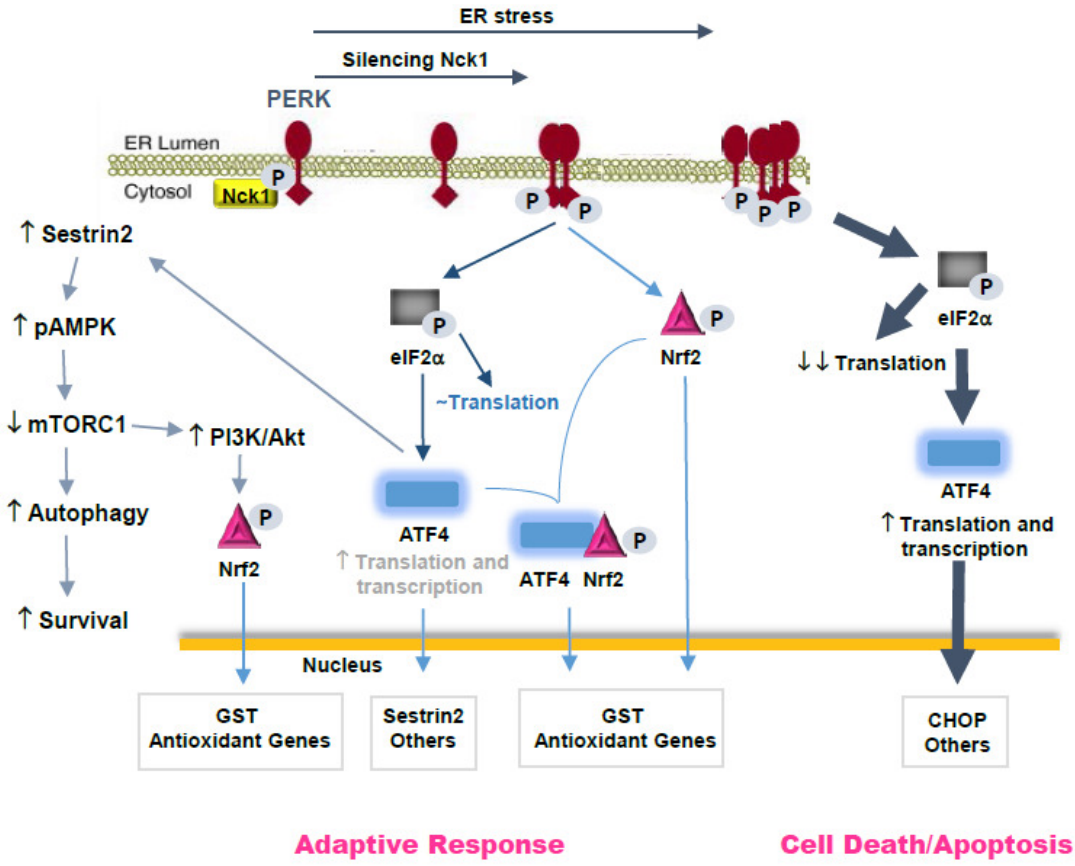
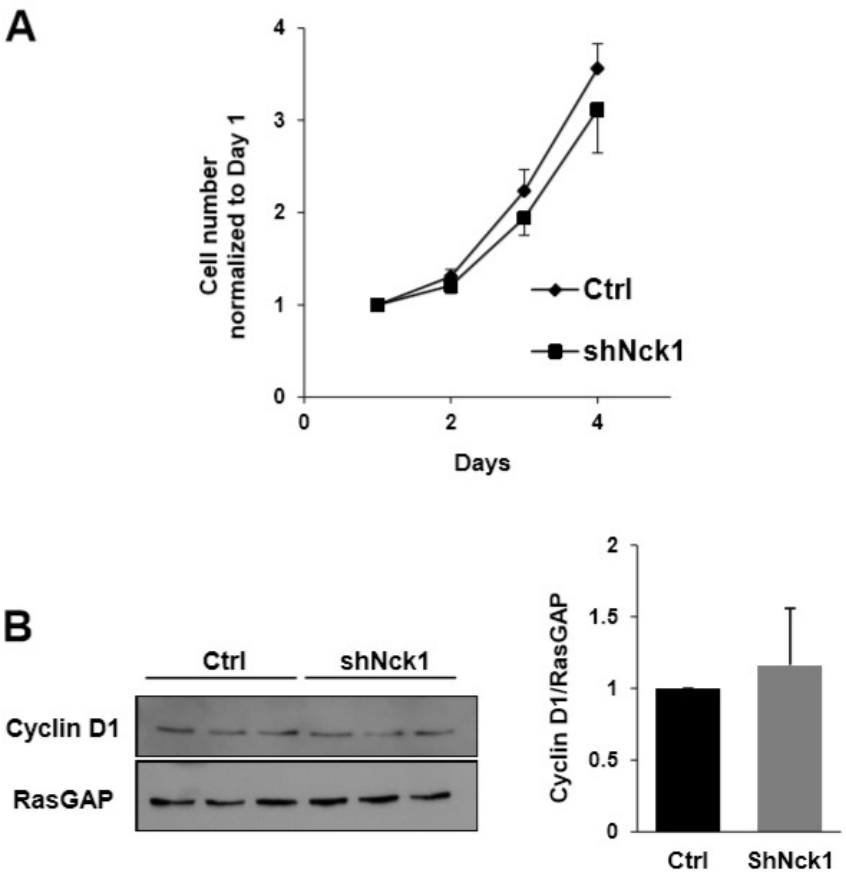


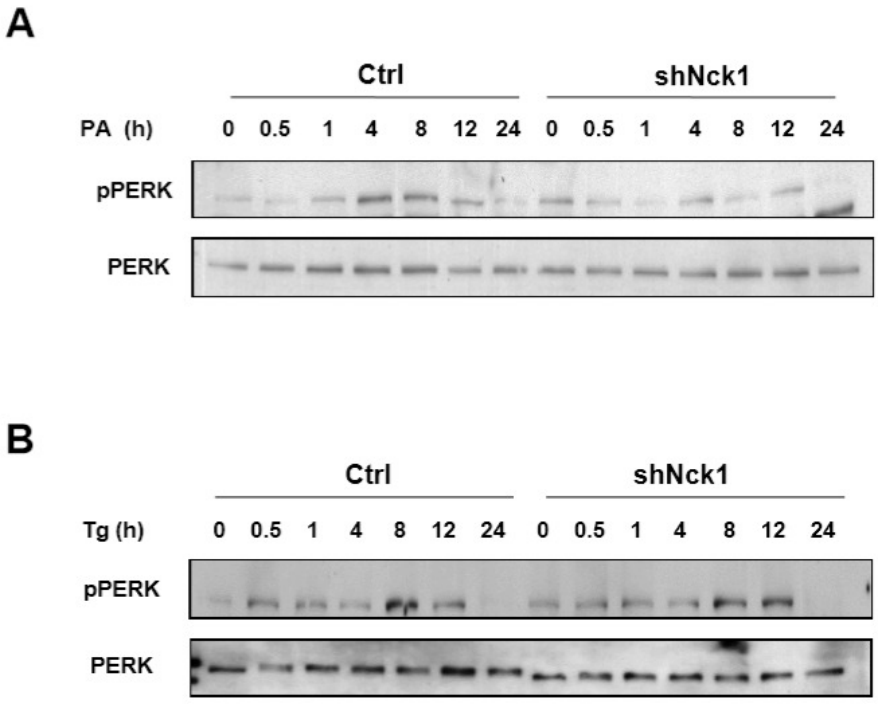
Figure 3. 8 PERK regulation of adaptive or apoptotic response.

Previous work from our group has demonstrated that by interacting with PERK, Nck1 limits PERK activation (Yamani et al, 2014). Therefore, in absence of Nck1, PERK basal activity, potentially increased through facilitated PERK dimerization, initiates an adaptive response involving ATF4 and Nrf2 regulation of genes that contribute to improve β cell function and survival. Basal autophagy, increased through ATF4/Sestrin2-mediated attenuation of mTORC1 activity, further contributes to the adaptive response. In contrast, under ER stress condition, PERK activation/signaling is strongly induced and leads to increased ATF4 and CHOP expression associated with β cell death potentially through apoptosis.



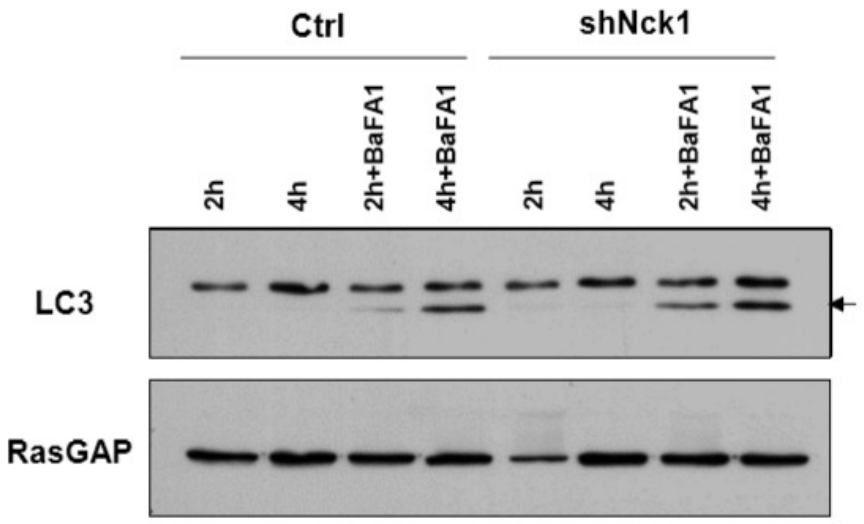
Supplementary Figure S3. 1 Proliferation in control and shNck1 MIN6 cells.

(A) Control (Ctrl) and shNck1 MIN6 cells plated at low density on day 0 were grown over 4 days in regular medium. Cell number was assessed every day from day 1 to 4 using MTT assays. Results, expressed as mean ± SEM, were normalized according to day 1. (B) Total cell lysates from control (Ctrl) and shNck1 MIN6 cells were subjected to Western blot using cyclin D1 and RasGAP antibodies. Left, representative Western blots with the indicated antibodies from 3 independent experiments performed in triplicate. Right, ratio of densitometry quantitation of cyclin D1/RasGAP expressed as mean ± SEM from 3 independent experiments.



Supplementary Figure S3. 2 Time course of PERK activation in palmitate (PA)- and thapsigargin (Tg)-treated control and shNck1 MIN6 cells.

Control (Ctrl) and shNck1 MIN6 cells were exposed to (A) PA 1 mM or (B) Tg 1 μM for 0 to 24 h. At indicated time points, total cell lysates were prepared and further subjected to Western blot with pPERK and PERK antibodies (n=1).



Supplementary Figure S3. 3 Enhanced autophagosomes formation in shNck1 MIN6 cells. Control (Ctrl) and shNck1 MIN6 cells were serum starved for 2 or 4 h in presence or absence of Bafilomycin (BafA1) (100 nM). Total cell lysates were subjected to Western blot with LC3 and RasGAP antibodies. Results are representative of 3 independent experiments. LC3II is indicated by the arrow and RasGAP represents loading control.

mouse gene	qPCR primer sequences (5' – 3')
ATF4-F	GCCTAAGCCATGGCGCTCTT
ATF4-R	GGTCATGTTGTGGGGCTTTGC
Chop-F	TAACAGCCGGAACCTGAGGAGA
Chop-R	GGTGCCCCCAATTTCATCTGAG
Cycb-F	TTCCTTTTGCTGCCCGGACC
Cycb-R	CGACTCGTCCTACAGATTCATCTCC
HO-1-F	CAAGCACAGGGTGACAGAAGAG
HO-1-R	GCTAGTGCTGATCTGGGGTTTC
Nqo1-F	TCAGCCAATCAGCGTTCGGT
Nqo1-R	AGGTCAGATTCGAGTACCTCCC

Supplementary Table S3. 1 Primer sequences used for qRT-PCR analysis.

CHAPTER 4. General discussion and future perspectives

In the past decade, several studies focused on understanding ER stress-induced UPR related molecular mechanisms leading to cell survival or cell death. Although recent progress has provided new insights adding to the complexity of the UPR signaling network, the exact mechanisms that carry out the transition from survival to apoptotic signals are not yet clearly understood. Identifying UPR-associated molecular mechanisms that favor cell survival as opposed to cell death is important as these can be powerful therapeutical targets to control cell dysfunction leading to the development of diseases. Altogether, the studies presented in this thesis uncover a PERK-mediated adaptive response initiated following silencing of the Src homology domain-containing adaptor protein Nck1 in pancreatic β cells.

4.1 Nck binds to PERK and regulates PERK activity

Our lab was the first to implicate the adaptor proteins Nck in the regulation of the UPR, where Nck1 overexpression was shown to increase mRNA translation by attenuating ER stress-induced eIF2αSer^{51} phosphorylation (Kebache et al, 2002; Kebache et al, 2004). Mechanistically, we provided strong evidence that Nck modulates translation by directly interacting with the β subunit of eIF2 (eIF2β) (Kebache et al, 2002) and the Ser/Thr protein phosphatase 1 (PP1c) that promotes dephosphorylation of phospho-eIF2αSer^{51} (Kebache et al, 2004; Latreille & Larose, 2006). Interestingly, we also uncovered that rescued expression of Nck1 in *Nck*$^{-/-}$ MEFs decreased phosphorylation of PERK on its activation site pThr^{980} under ER stress (Kebache et al, 2004), suggesting that in addition to regulating the levels of phosphorylated eIF2αSer^{51}, Nck1 negatively regulates PERK activity. Pursuing this hypothesis, I showed that Nck1 modulates PERK activity by directly interacting with PERK (Chapter 2). In fact, my work revealed that both Nck1 and Nck2 directly interact with PERK. We demonstrated that Nck1/PERK interaction involves the SH2

domain of Nck1 and PERK phosphorylated on Y^{561}, a novel autophosphorylation site in the PERK juxtamembrane domain (Figures 2.2-2.3). Furthermore, I demonstrated that PERK activity and downstream signaling in Nck1/2-deficient MEFs were increased compared to wild-type MEFs, in both basal and ER stress conditions (Figure 2.1), confirming that Nck not only regulates UPR signaling at the level of eIF2αSer^{51} phosphorylation, but also at the level of PERK.

Knowing that PERK is highly expressed and plays a crucial role in pancreatic β cell proteostasis and homeostasis (Delepine et al, 2000; Harding et al, 2001; Zhang et al, 2002a), I further addressed the significance of Nck1/PERK interaction in pancreatic β cells. By co-immunoprecipitation experiments involving endogenous proteins, I first confirmed Nck binding to PERK phosphorylated on pY^{561} in mouse insulinoma pancreatic β cell line MIN6 (Figure 2.6). Then, I hypothesized that Nck, as an adaptor protein interacting with PERK via its SH2 domain, may recruit through its SH3 domains a tyrosine protein phosphatase, such as PTP1B, in close proximity of PERK to mediate PERK pY^{561} dephosphorylation, to regulate PERK activation. Such PTP1B-dependent PERK modulation has been previously described, as PTP1B dephosphorylates PERK on pY^{619}, thereby regulating PERK activity (Bettaieb et al, 2011; Krishnan et al, 2011). However, Nck1 depletion in MIN6 cells, rather than decrease, enhanced PERK activation and signaling while decreased PERK phosphorylation on Y^{561}, making Nck1 protective of PERK phosphorylation on Y^{561} (Figure 2.7). Accordingly with phosphorylation of PERK on Y^{561} negatively modulating PERK activation, I demonstrated that it inversely correlates with PERK activation in MIN6 cells and that the activity of a PERK Y561F mutant, which escapes phosphorylation and Nck binding, was increased compared to PERK wild-type (Figure 2.4). Therefore, I proposed that Nck1 interaction with PERK phosphorylated on Y^{561} limits PERK

activation (Figure 2.5). Alternatively, it could also illustrate that by itself PERK phosphorylation on Y^{561} maintains PERK in a closed conformation, which favors monomeric PERK state and prevents unwanted PERK activation. Addressing basal and ER stress-induced activity of phosphomimetic PERK mutants (Y561E, Y561D) could further improve our understanding of the biological relevance of PERK phosphorylation on Y^{561}. Although additional investigation is required to further discriminate between these potential models, my first study identified a novel PERK autophosphorylation regulatory site (Y^{561}) and Nck1 as a novel interacting partner and regulator of PERK activation.

4.2 Other PERK binding proteins

PERK has been shown to be regulated by different effectors through direct interaction in a number of studies. One of the earliest binding proteins identified was GRP78/Bip, which binds PERK luminal domain, and prevents PERK dimerization and subsequent activation (Bertolotti et al, 2000; Hendershot, 2004). The co-chaperone of Bip in the ER, $p58^{IPK}$, also binds PERK luminal domain as part of a negative feedback mechanism to turn off PERK kinase activity during late stages of the UPR (Yan et al, 2002a; van Huizen et al, 2003). In addition, the ER protein disulfide isomerase A6 (PDIA6) binds PERK and negatively regulates PERK signaling, as it does for IRE1α upon binding to the IRE1α luminal domain (Eletto et al, 2014). Therefore, Bip, $p58^{IPK}$, and PDIA6 are all negative regulators of PERK. On the other hand, the Ca^{2+}-dependent phosphatase Calcineurin interacts with pre-activated PERK on its cytoplasmic domain to further enhance its activation upon ER stress (Bollo et al, 2010). In addition, PARP16, an ER-anchored ADP-ribosyltransferase protein, was found to induce PERK activity upon ER stress by interacting with PERK cytoplasmic domain and ADP-ribosylating PERK (Jwa & Chang, 2012). Moreover,

transducing β-like 2 (TBL2) was identified as a new PERK binding protein, which interacts with the cytoplasmic region of PERK upon ER stress. TBL2 was found to preferentially bind the phosphorylated active form of PERK, and interestingly, positively regulate ATF4 protein expression, affecting neither PERK activity nor peIF2αS^{51} levels (Tsukumo et al, 2014). Finally, the small GTPase Rheb, known to positively regulate mTORC1 activity, was also shown to interact with PERK, eliciting PERK signaling pathway and negatively regulating general protein synthesis (Tyagi et al, 2015).

Based on the above findings, it can be reasonable to propose that luminal-based interactions with PERK negatively regulate PERK activity and signaling, while most cytoplasmic-based interactions involving an area surrounding the kinase domain of PERK elicit PERK activity and signaling. However, little is known of potential PERK regulation by interacting with its juxtamembrane domain. We have shown that through its SH2 domain, Nck1 binds PERK juxtamembrane domain on pY^{561}. We also showed that this interaction limits PERK activity, since downregulation of Nck1 or Y561F point mutation primed PERK activity and signaling. But how does Nck1 regulate PERK activity? In order to elucidate the mechanism by which Nck1 regulates PERK activity, the role of phosphorylation of Y^{561} in the juxtamembrane domain should also be further understood. It is well known that phosphorylation in the juxtamembrane domain in several RTKs, such as the PDGFR and the ephrin receptors, is involved in the regulation of RTKs activation process. Specifically, it was shown that the juxtamembrane domain adopts a closed conformation and creates a barrier on the activation loop of the kinase domain when its tyrosine residues are not phosphorylated. However, upon stimulation, phosphorylation of tyrosine residues in the juxtamembrane domain adds a bulky charged group that induces conformational change

removing this barrier and allows activation of the kinase domain (Holland et al, 1997). Here, we demonstrate that loss of Y^{561} phosphorylation in PERK juxtamembrane domain leads to increased PERK kinase activity. This could be due to the fact that the phosphorylated juxtamembrane domain at Y^{561} prevents interaction between different monomers, thereby regulating PERK activity. In that case, loss of Y^{561} phosphorylation in the juxtamembrane domain leads to enhanced PERK activity by promoting PERK dimerization. By protecting Y^{561} phosphorylation, Nck1 may be hindering the spontaneous dimerization of PERK. On the other hand, Nck1 binding to PERK may allosterically affect the interaction of PERK luminal domain with Bip or any of the inhibitory PERK-binding partners. In addition, Nck1 interaction at pY^{561} PERK could recruit a phosphatase (such as PTP1B) through its SH3 domains, and allow PTP1B to dephosphorylate PERK on other activating tyrosine residues in the PERK kinase domain such as pY^{615} previously identified as a positive regulatory site for PERK activation (Su et al, 2008) and a target of PTP1B (Krishnan et al, 2011). Finally, Nck1 may inhibit or prevent PERK interaction with other PERK-binding regulators that positively enhance its activity.

4.3 Role of protein tyrosine phosphatases

The fact that phosphorylation of PERK on Y^{561} increased in pervanadate-treated cells, a general tyrosine phosphatase inhibitor, suggests the involvement of a tyrosine phosphatase in dephosphorylating PERK at pY^{561}, or activation of a protein kinase mediating this phosphorylation event, which we believe is PERK according to our data showing that recombinant active PERK is phosphorylated on Y^{561} *in vitro* (Figure 2.2). Protein tyrosine phosphatase 1B (PTP1B) and T cell protein tyrosine phosphatase (TCPTP), two non-receptor tyrosine phosphatases have been shown previously to regulate PERK's activity in a different manner (Bettaieb et al, 2011). PTP1B and

TCPTP share 72% sequence identity within their catalytic domains, as well as highly similar tertiary structure (Andersen et al, 2001; Tiganis & Bennett, 2007). PTP1B is attached to the cytoplasmic side of the ER while TCPTP is ubiquitously distributed in cells. Both PTP1B and TCPTP were shown to regulate IR activity and signaling. Indeed, PTP1B and TCPTP negatively modulate IR signaling through dephosphorylation of the IRβ at Y^{1162}/Y^{1163} (Salmeen et al, 2000; Galic et al, 2003; Galic et al, 2005). In MIN6 cells, knocking down of PTP1B was shown to enhance PERK activity and signaling while in contrast, knocking down of TCPTP has the opposite effect on PERK activation and signaling (Bettaieb et al, 2011). To identify a potential protein phosphatase involved in PERK pY^{561} dephosphorylation, I measured the levels of pY^{561}PERK in PTP1B-deficient and TCPTP heterozygous MEFs (Appendix 1). I showed that pY^{561}PERK increased in both PTP1B-deficient and TCPTP heterozygote MEFs compared to their wild-type counterparts, suggesting that both tyrosine phosphatases might be involved in regulating PERK phosphorylation on pY^{561}.

Interestingly, both PTP1B and TCPTP have been recently implicated in pancreatic β cell function. PTP1B deficiency delayed onset of diabetes in *IRS2*$^{-/-}$ mice (Kushner et al, 2004). However, silencing PTP1B in MIN6 cells was shown to sensitize β cells toward apoptosis in response to lipotoxicity and glucolipotoxicity (Bettaieb et al, 2011). In agreement, HFD feeding in pancreatic-specific *PTP1B*$^{-/-}$ mice lead to exacerbation of glucose intolerance due to loss of GSIS (Liu et al, 2014b). On the other hand, isolated islets from *PTP1B*$^{-/-}$ mice fed a normal chow diet (NCD) led to increased β cell mass and increased β cell proliferation, and this phenotype was recapitulated in MIN6 cells following PTP1B downregulation. In those mice, pancreatic β cells displayed an increase in Akt activation and GSIS, but no increase in β cell insulin content. Finally,

streptozotocin-treated *PTP1B*$^{-/-}$ mice displayed β cell mass and function recovery compared to wild-type mice (Fernandez-Ruiz et al, 2014). On the other hand, pancreatic-specific deletion of TCPTP generated glucose intolerance caused by lower GSIS and lower insulin content in mice fed a HFD. These findings were recapitulated in *ex-vivo* pancreatic islets, as well as in MIN6 cells treated with TCPTP inhibitor, or transfected with an shRNA silencing TCPTP expression (Xi et al, 2015). Although both PTP1B and TCPTP are potential candidates for dephosphorylation of pY^{561}PERK, I believe that the best candidate is TCPTP. Indeed, TCPTP deficiency in MEFs increased pY^{561}PERK (Appendix 1), correlating with the finding that TCPTP downregulation in MIN6 cells reduce PERK activity and signaling (Bettaieb et al, 2011). In addition, as already mentioned above, TCPTP deficiency in mice pancreatic β cells or MIN6 cells resulted in dysfunctionalities (Xi et al, 2015), which could be due to insufficient PERK activity, that leads to decreased β cell resilience facing stress conditions. It would be interesting to determine PERK activity in TCPTP and PTP1B-deficient pancreatic β cells in mice. Further investigation is required to better understand the exact contribution of these phosphatases to the process of pY^{561} PERK dephosphorylation and to mechanistically elucidate how they regulate PERK activity.

4.4 Nck1 silencing and pancreatic β cell insulin levels

One of the most striking findings in my first study is that pancreatic islets from *Nck1*$^{-/-}$ mice display an increase in insulin content. Similarly, Nck1 silencing in MIN6 cells increased insulin levels in a PERK-dependent manner, due to enhanced proinsulin synthesis rather than abnormal accumulation of proinsulin in these cells. In agreement, electron microscopy analysis demonstrated that Nck1-silenced MIN6 cells displayed bigger and increased number of secretory granules, while general morphology was preserved. In short, silencing Nck1 in MIN6 cells promotes proinsulin

synthesis and content (Figure 2.8) without altering ER morphology (Figure 3.1), and these findings might represent robust β cells, with a higher capacity to synthesize, process and store proinsulin.

4.5 Nck1 silencing and pancreatic β cell survival

Increased proinsulin synthesis implies that the ER workload must be greater in Nck1-silenced MIN6 cells and potentially generate more ROS production that could make these cells more vulnerable to stress. Surprisingly, Nck1-silenced MIN6 cells are more resistant to glucotoxic, lipotoxic or oxidative stress, all stresses relevant to β cell dysfunction associated with type 2 diabetes (Figure 3.2). However, Nck1-silenced MIN6 cells are not protected against pharmacological induction of ER stress using thapsigargin or dithiothreitol. Therefore, it appears that silencing Nck1 in MIN6 cells elicits a protective cellular response to mild stress, but not to very strong stress targeting the ER. To identify the mechanisms by which Nck1 silencing protects MIN6 cells against certain stresses, we further analyzed PERK activation in these conditions. Interestingly, we discovered that palmitate-induced PERK activation in Nck1-silenced MIN6 cells was attenuated compared to control cells while Tg-induced PERK activation was similar in both shNck1 and control MIN6 cells (Figure 3.3). This clearly illustrates that according to intensity and duration of PERK's activation, cell fate might adopt diametrically different directions. For example, while a short term treatment with palmitate for 1 h led to increased general mRNA translation, after 72 h, it was reported to inhibit protein translation to the same extent as a 4 h thapsigargin treatment (Hatanaka et al, 2014). Further supporting this concept, a recent study demonstrated that different types of stresses, such as low glucose and thapsigargin, can activate different PERK-mediated mechanisms leading to drastically different cell fates (Shin et al, 2015).

Mechanistically, I provided strong evidence revealing that the antioxidant Nrf2 pathway, autophagy and Akt activation are all upregulated in Nck1-silenced MIN6 cells and could contribute to create an adaptive response, which generates cells that are more resistant to mild stress. As Nrf2 is a direct target of PERK and synergizes with ATF4 activity (Cullinan et al, 2003), I predicted that it could be activated in Nck1-silenced MIN6 cells. Indeed, I found that both Nrf2 nuclear localization and dependent gene expression of antioxidant genes were increased in these cells. Although a statistically significant level of Nrf2 nuclear localization was not reached upon depletion of Nck1, its increase was sufficient to significantly upregulate expression of Nrf2-target genes (Chapter 3). Therefore, I believe that together all these changes contribute to a better control of ROS levels and related resilience to mild stress in Nck1-silenced MIN6 cells.

Besides increased PERK activation and signaling involving Nrf2, I also discovered that autophagy was enhanced in a PERK-dependent manner in Nck1-silenced MIN6 cells. Autophagy is a cellular degradation-recycling system for aggregated proteins and damaged organelles to regenerate new pools of materials for cellular needs (Klionsky & Emr, 2000). Several studies investigating the effect of metabolic stress on autophagy showed that even though metabolic stress such as high glucose and saturated fatty acids increase autophagosome formation, autophagy turnover was impaired and ultimately leads to cell death (Mir et al, 2015). Mechanistically, physiological stresses such as palmitate initially activate mTORC1, which blocks autophagy and simultaneously contribute to increase mRNA translation and ER protein load (Hatanaka et al, 2014). Eventually, longer exposure triggers sustained activation of the UPR due to increased protein load, which blocks mRNA translation and ultimately leads to β cell death (Hatanaka et al, 2014). In this thesis, I report that upregulation of Sesn2, by inducing activation of AMPK, inhibits

mTORC1 activity in Nck1-silenced MIN6 cells. Therefore, I believe that activation of AMPK leading to attenuation of mTORC1 activity contributes to increased autophagy steering towards an increased adaptive capacity in these cells (Figure 3.5).

In addition to increasing the capacity of the ER and autophagy, Akt activation in Nck1-silenced pancreatic β cells can play a role in protecting cells against lipotoxic, glucotoxic and oxidative stress-induced apoptosis (Figure 3.6C). Akt is known to promote nuclear exclusion and degradation of FOXO1 via phosphorylation (Biggs et al, 1999), a process that we also observe in Nck1-silenced MIN6 cells (Figure 3.6D). Early induction of FOXO1 activity has been shown to promote palmitate-induced apoptosis (Martinez et al, 2008). On the other hand, expression of a dominant negative FOXO1 protected cells against palmitate-induced cell death (Martinez et al, 2008). Interestingly, our group has recently demonstrated that silencing Nck1, which spontaneously enhances PI3K/Akt signaling in HepG2 cells correlates with reduced FOXO1 nuclear localization (Li et al, 2014a). Therefore, increased phosphorylation/activation of Akt in Nck1-silenced MIN6 cells potentially leads to nuclear exclusion and degradation of FOXO1, thereby preventing FOXO1-dependent apoptotic signaling during stress. In addition, nuclear expression of FOXO1 and ATF4 in osteoblasts was shown to have a synergistic effect in inhibiting pancreatic β cell proliferation, further highlighting a negative role for FOXO1 expression in cells (Kode et al, 2012). Moreover, a recent study showed that upon mild metabolic stress, such as low glucose, Akt phosphorylation is increased in a PERK-dependent manner, activating an adaptive response that enables cell survival (Shin et al, 2015). Eventually, once Akt activation is dampened, cells undergo apoptosis (Shin et al, 2015). It has also been shown that in response to oxidative stress, peIF2αS^{51} can determine cell fate through activation of Akt (Rajesh et al 2015). Specifically,

it was shown that under oxidative stress, proficiency in eIF2αS^{51} phosphorylation facilitates Akt activation through downregulation of mTORC1 activity and enhances cell survival (Rajesh et al, 2015).

The main role of pancreatic β cells is to synthesize and release insulin in response to high glucose. Proinsulin represents 20% of total β cell mRNAs (Van Lommel et al, 2006) and glucose regulated insulin translation can account for up to 50% of total protein synthesis in β cells (Schuit et al, 1988). One intriguing finding is that Nck1 silencing in MIN6 cells enhances eIF2αSer^{51} phosphorylation while at the same time leads to increased proinsulin synthesis. Although this might seem contradictory since eIF2αSer^{51} phosphorylation is known to attenuate general protein synthesis, it also promotes preferential translation of specific mRNAs. The best example of that is ATF4, whose translational expression increases upon eIF2αSer^{51} phosphorylation. Also, basal activation of PERK could promote upregulation of specific RNA binding proteins that bind and stabilize insulin mRNA and allow its recruitment to ribosomal fractions for preferential increased synthesis. Although PERK and eIF2α deficiency phenotypically lead to pancreatic β cell dysfunction, differences in protein synthesis were observed. Mice with β cell-specific mutation in eIF2α preventing its phosphorylation (eIF2αS51A) display β cell dysfunction due to their inability to attenuate protein translation, thus overloading the ER and creating ER stress, which leads to apoptosis (Scheuner et al, 2005). On the other hand, pancreatic islets from *PERK*$^{-/-}$ mice showed no difference in protein translation compared to islets from wild-type mice (Gupta et al, 2010). Similarly, INS832/13 β cells transfected with dominant negative PERK showed no difference in general protein synthesis compared to control cells (Feng et al, 2009). Interestingly, downregulating the expression of PERK in INS832/13 cells reduced proinsulin levels by about

half. Moreover, PERK deficiency led to defects in protein processing associated with apoptosis (Gupta et al, 2010). Finally, there was no obvious pancreatic β cell defect in mice lacking other eIF2α kinases: HRI, PKR, or GCN2, further highlighting the exclusive importance of PERK among eIF2α kinases in pancreatic β cell function and homeostasis (Yang et al, 1995; Han et al, 2001; Zhang et al, 2002b). All combined, these findings indicate that PERK, in addition to its role in eIF2αSer^{51} phosphorylation, plays a distinct and essential role in pancreatic β cell proteostasis and resilience.

Upon ER stress, the UPR is activated in order to expand the folding capacity of the ER. Uniquely, PERK activation attenuates general protein synthesis by phosphorylation of eIF2α on Ser^{51} to prevent overloading of the ER with newly synthesized proteins to process. Depending on the type and intensity of the stress, as well as the UPR signaling, cells survive or undergo apoptosis. Palmitate has been shown to induce PERK activation and downstream translational and transcriptional signaling in primary rodent and human β cells (Cunha et al, 2008). In cases of transient or periodic stresses, the UPR is activated, allowing cells to respond, and then return to basal physiological state when the stress subsides (Figure 4.1A). In contrast, if the stresses are chronic, PERK remains activated and signals towards prominent induction of CHOP, which contributes to ER stress-induced cell death (Oyadomari et al, 2001). In agreement, CHOP depletion partially protects INS832 cells against palmitate-induced apoptosis (Cunha et al, 2008). However, in the case of silencing Nck1, basal PERK activity and signaling are enhanced, thereby promoting Nrf2-antioxidant signaling, increased autophagy, and Akt activation, which all together increase the folding capacity and function of the ER, and fine tunes an adaptive response that renders cells more resilient to stresses (Figure 4.1B). It is important to note that enhanced basal

PERK activity and signaling in Nck1-silenced MIN6 cells, although significant, is weak compared to ER stress-induced PERK activation and signaling. In agreement, spontaneous increase in PERK activity and signaling does initiate proapoptotic signaling, but on the long run is sufficient to generate an adaptive state that is protective against mild stresses relevant to type 2 diabetes.

4.6 Possible role for Nck1 in other tissues

The findings in my thesis studies pertain to the role of Nck1 in pancreatic β cells, but this could be expanded in other tissue. We previously reported a beneficial role for Nck1 deficiency in mice fed a HFD (Latreille et al, 2011). Indeed, our group previously demonstrated that obesity-induced activation of ER stress in liver and adipose tissue was attenuated in Nck1 null mice fed a HFD. Specifically, HFD Nck1 null mice showed decreased hepatic PERK activity and signaling compared to HFD wild-type mice. In addition, liver from Nck1 null mice on HFD was more insulin sensitive as we observed increased *in vivo* hepatic insulin-induced Akt phosphorylation (Latreille et al, 2011). Furthermore, we also demonstrated that Nck1 deficiency in HepG2 cells also leads to increased insulin signaling, as insulin-dependent Akt activation was enhanced (Latreille et al, 2011; Li et al, 2014a). In conclusion, the concept that by sensitizing PERK signaling, silencing Nck1 has a protective role might apply to other cell types or tissues. However, given that PERK activity is crucial for pancreatic β cell function, I believe that the protective effect of Nck1 regulation of PERK activity may be more biologically relevant in pancreatic β cells.

4.7 Future perspectives

Based on our findings, spontaneously enhancing PERK basal activity and signaling in pancreatic β cells improve β cell function and response to mild stress by promoting an adaptive state that expands the ER capacity to synthesize and process insulin, and enhances a signaling

network steering towards cell survival. To further validate the significance of my findings, I hypothesize that reducing Nck1 expression in heterozygous Akita mice should delay loss of pancreatic β cell function and apoptosis. Akita mice harbor a mutation in *Ins2* gene, which results in accumulation of misfolded insulin in the ER, creating ER stress that eventually induces β cell death (Wang et al, 1999). Silencing Nck1 specifically in Akita pancreatic β cells is expected to enhance ER folding capacity and function, as well as antioxidant response, which enable β cells to survive better than in control Akita mice β cells. Therefore, I predict that silencing Nck1 in Akita pancreatic β cells could prevent progression of hyperglycemia and β cell loss, classical hallmarks of T2D development in heterozygous Akita mice.

On the other hand, the novel emerging concept that in β cells, sensitizing PERK by preventing its interaction with Nck1 creates robust β cells equipped with an efficient ER in its capacity to produce and secrete bioactive insulin, and protect β cells from stress, strongly suggest that preventing Nck1 and PERK interaction could be a potential significant target for the development of therapies promoting β cell functionality and resilience. To block the interaction between PERK and Nck1 in β cells, our laboratory has designed a phosphopeptide representing the minimal sequence for PERK binding to Nck1 (PERKpY^{561}: ENKpYDSVSGE). As controls, the same peptide unphosphorylated, harbouring the Y/F substitution or scrambled are also considered. As phosphopeptides *per se* show low cell membrane penetration, these peptides have been synthesized conjugated to the cell penetrating peptide TAT (GRKKRRQRRRPQ) from HIV, a powerful system to deliver phospho/unphosphopeptides into cells (Kim et al, 2014b). Preliminary experiments using MIN6 cells revealed that these peptides efficiently enter the cells. Furthermore, we observed that the peptide representing the minimal PERK sequence to bind Nck1

appears to spontaneously increase basal levels of PERK activity and signaling while the negative control peptides failed to do so. Additional experiments are in progress to further validate the effects of the peptide that prevents Nck1/PERK interaction, but we are confident that this approach as a proof of principle, will confirm that efficient sensitization of PERK physiological basal activity contributes to promote β cell function and survival to stresses relevant to diabetes. For people with high risk of developing diabetes, targeting Nck1 and PERK interaction in pancreatic β cells can be efficient in preventing cell dysfunction and development of diabetes.

In conclusion, this thesis reports novel findings demonstrating that Nck1 through its SH2 domain binds PERK phosphorylated at Y^{561} and by protecting this site against dephosphorylation, Nck1 limits PERK activation and signaling. Therefore, my findings revealed Y^{561} in the PERK juxtamembrane as a novel PERK autophosphorylation site that plays a significant role in preventing unwanted PERK activation. In addition, I provided strong data demonstrating that priming PERK basal activity and signaling by silencing Nck1 in pancreatic β cells leads to fine tuning of adaptive mechanisms involved in protecting β cells against mild stresses that otherwise induce β cell apoptosis. Collectively, data reported in this thesis shed more light on mechanisms regulating pancreatic β cell proteostasis and homeostasis, and contribute to open new avenues for developing efficient therapeutical approaches to prevent β cell dysfunction and diabetes onset, but also treat and/or cure type 2 diabetes.

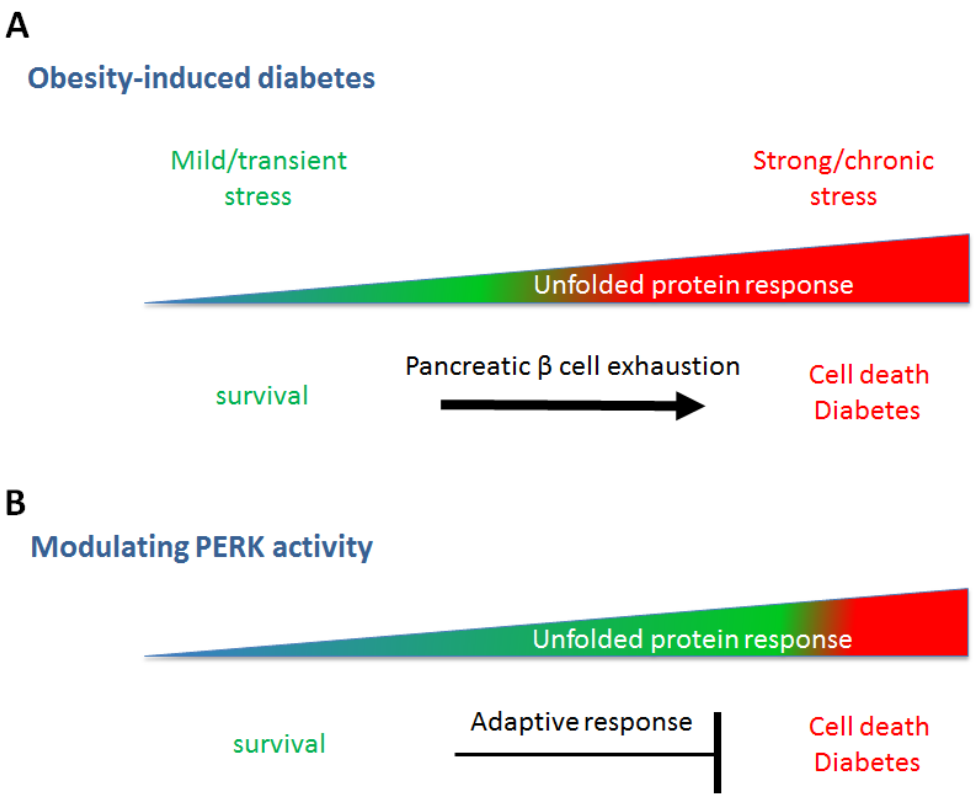


Figure 4. 1 Nck1 modulation of the UPR through PERK signaling increases the robustness of pancreatic β cells in response to stresses.

(A) In pancreatic β cells, activation of the UPR under transient or periodic stresses allows protection and survival of cells. With continuous exposure to strong or chronic stresses, pancreatic β cells become exhausted, and strong activation of the UPR leads to initiation of proapoptotic signaling pathways. (B) Nck1 silencing in pancreatic β cells enhances basal activation of PERK and its signaling pathway, which includes activation of autophagy, antioxidant Nrf2, and Akt. These pathways fine tune the adaptive response by expanding the folding capacity of the ER and function, while increasing insulin synthesis. Consequently, pancreatic β cells become robust in the face of metabolic stresses that can result in β cell dysfunction.

CHAPTER 5. Contribution to original research

In chapter 2, I demonstrated that genetic deletion of Nck in mouse embryonic fibroblast cells (MEFs) increases PERK activity under ER stress conditions as well as basal conditions. In addition, I showed that Nck binds through its SH2 domain to PERK at pY^{561} on the juxtamembrane domain, making Nck one of the few PERK-binding proteins identified thus far. Moreover, I revealed that Nck1 binding to PERK negatively regulates PERK kinase activity. Furthermore, I showed that stable downregulation of Nck1 expression in MIN6 cells enhances PERK activity and its signaling pathway. More importantly, Nck1 depletion in MIN6 cells increases insulin levels in a PERK activity-dependent manner, and that the increase is due to enhanced proinsulin synthesis. In parallel, isolated islets from *Nck1*$^{-/-}$ mice were found to have more insulin content compared to islets from wild-type mice. Together, my study describes novel molecular mechanisms regulating the activity of PERK and pancreatic β cell function.

In chapter 3, I investigate the effect Nck1 silencing would have on pancreatic β cell survival against stresses. First, I visually displayed using electron microscopy that Nck1-silenced MIN6 cells contain more mature insulin granules. I then demonstrated that Nck1 silencing in MIN6 cells enhances survival to diabetes-related stresses such as glucotoxicity, lipotoxicity, and oxidative stress, and that survival correlated with lower level of PERK activation after exposure to stress. I determined that PERK subtle activation in Nck1-silenced MIN6 cells upregulates 3 different prosurvival mechanisms: autophagy, antioxidant Nrf2, and Akt activation. All these pathways equip cells with a stronger adaptive response that can better handle stress. This is the first study that links PERK activity with autophagy in pancreatic β cells. It also introduces a remarkable aspect on enhancing pancreatic β cell function through modulation of PERK activity. In

conclusion, I showed that a subtle increase in PERK activity by silencing Nck1 generates robust pancreatic β cells capable of surviving stresses related to diabetes.

Summary and Conclusion

This thesis reports a study that supports a role for Nck1 in PERK activation and pancreatic β cell function. First, I demonstrated that Nck1 interacts with PERK in an SH2-pY^{561}-dependent manner *in vitro* and in mouse insulinoma pancreatic β MIN6 cells. Mutated PERK Y561F, which escapes phosphorylation at the mutated residue, abolishes Nck1/PERK binding, and enhances PERK kinase activity compared to wild-type PERK. Inversely, increased phosphorylation at Y^{561} in MIN6 cells delayed PERK activation upon ER stress. I then generated MIN6 cells depleted of Nck1 expression using shRNA against Nck1. Nck1 depletion in MIN6 cells increased PERK basal activity and signaling. Interestingly, these cells displayed increased insulin content in a PERK-dependent manner, resulting from enhanced insulin biosynthesis. I then demonstrated that Nck1-silenced MIN6 cells display increased survival to diabetes-related stresses, and this increase correlated with lower PERK activation in response to the stress. Furthermore, I showed that Nck1-silenced MIN6 cells displayed a PERK-dependent increase in autophagy, increased Nrf2-antioxidant gene expression, and finally increased Akt activation. Altogether, these pathways enhance the adaptive response of Nck1-silenced MIN6 cells, which increases the survival of pancreatic β cells against diabetes-related stresses.

In conclusion, the work presented in this thesis strongly demonstrate that a subtle increase of PERK activity and signaling fine tunes downstream molecular mechanisms, which enhances the adaptive response of pancreatic β cells against stresses. These findings provide insights on the mechanisms that can be potential therapeutical targets to generate resilient pancreatic β cells that better survive diabetes-related stresses.

REFERENCES

Abel JJ (1926) Crystalline Insulin. *Proc Natl Acad Sci U S A* **12:** 132-136

Abella JV, Vaillancourt R, Frigault MM, Ponzo MG, Zuo D, Sangwan V, Larose L, Park M (2010) The Gab1 scaffold regulates RTK-dependent dorsal ruffle formation through the adaptor Nck. *J Cell Sci* **123:** 1306-1319

Aguilar-Bryan L, Bryan J (1999) Molecular biology of adenosine triphosphate-sensitive potassium channels. *Endocr Rev* **20:** 101-135

Aguirre V, Werner ED, Giraud J, Lee YH, Shoelson SE, White MF (2002) Phosphorylation of Ser307 in insulin receptor substrate-1 blocks interactions with the insulin receptor and inhibits insulin action. *J Biol Chem* **277:** 1531-1537

Aitio O, Hellman M, Kesti T, Kleino I, Samuilova O, Paakkonen K, Tossavainen H, Saksela K, Permi P (2008) Structural basis of PxxDY motif recognition in SH3 binding. *J Mol Biol* **382:** 167-178

Alquier T, Peyot ML, Latour MG, Kebede M, Sorensen CM, Gesta S, Ronald Kahn C, Smith RD, Jetton TL, Metz TO, Prentki M, Poitout V (2009) Deletion of GPR40 impairs glucose-induced insulin secretion in vivo in mice without affecting intracellular fuel metabolism in islets. *Diabetes* **58:** 2607-2615

Ameri K, Lewis CE, Raida M, Sowter H, Hai T, Harris AL (2004) Anoxic induction of ATF-4 through HIF-1-independent pathways of protein stabilization in human cancer cells. *Blood* **103:** 1876-1882

Andersen JN, Mortensen OH, Peters GH, Drake PG, Iversen LF, Olsen OH, Jansen PG, Andersen HS, Tonks NK, Moller NP (2001) Structural and evolutionary relationships among protein tyrosine phosphatase domains. *Mol Cell Biol* **21:** 7117-7136

Andrews NC, Erdjument-Bromage H, Davidson MB, Tempst P, Orkin SH (1993) Erythroid transcription factor NF-E2 is a haematopoietic-specific basic-leucine zipper protein. *Nature* **362:** 722-728

Anton IM, Lu W, Mayer BJ, Ramesh N, Geha RS (1998) The Wiskott-Aldrich syndrome protein-interacting protein (WIP) binds to the adaptor protein Nck. *J Biol Chem* **273:** 20992-20995

Araki E, Lipes MA, Patti ME, Bruning JC, Haag B, 3rd, Johnson RS, Kahn CR (1994) Alternative pathway of insulin signalling in mice with targeted disruption of the IRS-1 gene. *Nature* **372:** 186-190

Araki E, Oyadomari S, Mori M (2003) Endoplasmic reticulum stress and diabetes mellitus. *Intern Med* **42:** 7-14

Ashcroft FM, Harrison DE, Ashcroft SJ (1984) Glucose induces closure of single potassium channels in isolated rat pancreatic beta-cells. *Nature* **312:** 446-448

Atkinson MA, Maclaren NK (1994) The pathogenesis of insulin-dependent diabetes mellitus. *N Engl J Med* **331:** 1428-1436

Atkinson MA, Eisenbarth GS (2001) Type 1 diabetes: new perspectives on disease pathogenesis and treatment. *Lancet* **358:** 221-229

Atkinson MA, Rhodes CJ (2005) Pancreatic regeneration in type 1 diabetes: dreams on a deserted islet? *Diabetologia* **48:** 2200-2202

Back SH, Scheuner D, Han J, Song B, Ribick M, Wang J, Gildersleeve RD, Pennathur S, Kaufman RJ (2009) Translation attenuation through eIF2alpha phosphorylation prevents oxidative stress and maintains the differentiated state in beta cells. *Cell Metab* **10:** 13-26

Bae SH, Sung SH, Oh SY, Lim JM, Lee SK, Park YN, Lee HE, Kang D, Rhee SG (2013) Sestrins activate Nrf2 by promoting p62-dependent autophagic degradation of Keap1 and prevent oxidative liver damage. *Cell Metab* **17:** 73-84

Bae SS, Cho H, Mu J, Birnbaum MJ (2003) Isoform-specific regulation of insulin-dependent glucose uptake by Akt/protein kinase B. *J Biol Chem* **278:** 49530-49536

Barda-Saad M, Shirasu N, Pauker MH, Hassan N, Perl O, Balbo A, Yamaguchi H, Houtman JC, Appella E, Schuck P, Samelson LE (2010) Cooperative interactions at the SLP-76 complex are critical for actin polymerization. *EMBO J* **29:** 2315-2328

Barletta GM, Kovari IA, Verma RK, Kerjaschki D, Holzman LB (2003) Nephrin and Neph1 co-localize at the podocyte foot process intercellular junction and form cis hetero-oligomers. *J Biol Chem* **278:** 19266-19271

Baynes JW (1991) Role of oxidative stress in development of complications in diabetes. *Diabetes* **40:** 405-412

Bernal-Mizrachi E, Fatrai S, Johnson JD, Ohsugi M, Otani K, Han Z, Polonsky KS, Permutt MA (2004) Defective insulin secretion and increased susceptibility to experimental diabetes are induced by reduced Akt activity in pancreatic islet beta cells. *J Clin Invest* **114:** 928-936

Bernales S, McDonald KL, Walter P (2006) Autophagy counterbalances endoplasmic reticulum expansion during the unfolded protein response. *PLoS Biol* **4:** e423

Bertolotti A, Zhang Y, Hendershot LM, Harding HP, Ron D (2000) Dynamic interaction of BiP and ER stress transducers in the unfolded-protein response. *Nat Cell Biol* **2:** 326-332

Bertolotti A, Wang X, Novoa I, Jungreis R, Schlessinger K, Cho JH, West AB, Ron D (2001) Increased sensitivity to dextran sodium sulfate colitis in IRE1beta-deficient mice. *J Clin Invest* **107:** 585-593

Best CH, Haist RE (1941) The effect of insulin administration on the insulin content of the pancreas. *J Physiol* **100:** 142-146

Bettaieb A, Liu S, Xi Y, Nagata N, Matsuo K, Matsuo I, Chahed S, Bakke J, Keilhack H, Tiganis T, Haj FG (2011) Differential regulation of endoplasmic reticulum stress by protein tyrosine phosphatase 1B and T cell protein tyrosine phosphatase. *J Biol Chem* **286:** 9225-9235

Bettaieb A, Matsuo K, Matsuo I, Wang S, Melhem R, Koromilas AE, Haj FG (2012) Protein tyrosine phosphatase 1B deficiency potentiates PERK/eIF2alpha signaling in brown adipocytes. *PLoS One* **7:** e34412

Biddinger SB, Hernandez-Ono A, Rask-Madsen C, Haas JT, Aleman JO, Suzuki R, Scapa EF, Agarwal C, Carey MC, Stephanopoulos G, Cohen DE, King GL, Ginsberg HN, Kahn CR (2008) Hepatic insulin resistance is sufficient to produce dyslipidemia and susceptibility to atherosclerosis. *Cell Metab* **7:** 125-134

Biggs WH, 3rd, Meisenhelder J, Hunter T, Cavenee WK, Arden KC (1999) Protein kinase B/Akt-mediated phosphorylation promotes nuclear exclusion of the winged helix transcription factor FKHR1. *Proc Natl Acad Sci U S A* **96:** 7421-7426

Bladt F, Aippersbach E, Gelkop S, Strasser GA, Nash P, Tafuri A, Gertler FB, Pawson T (2003) The murine Nck SH2/SH3 adaptors are important for the development of mesoderm-derived embryonic structures and for regulating the cellular actin network. *Mol Cell Biol* **23:** 4586-4597

Blais JD, Filipenko V, Bi M, Harding HP, Ron D, Koumenis C, Wouters BG, Bell JC (2004) Activating transcription factor 4 is translationally regulated by hypoxic stress. *Mol Cell Biol* **24:** 7469-7482

Blasutig IM, New LA, Thanabalasuriar A, Dayarathna TK, Goudreault M, Quaggin SE, Li SS, Gruenheid S, Jones N, Pawson T (2008) Phosphorylated YDXV motifs and Nck SH2/SH3 adaptors act cooperatively to induce actin reorganization. *Mol Cell Biol* **28:** 2035-2046

Bluestone JA, Herold K, Eisenbarth G (2010) Genetics, pathogenesis and clinical interventions in type 1 diabetes. *Nature* **464:** 1293-1300

Bokoch GM, Wang Y, Bohl BP, Sells MA, Quilliam LA, Knaus UG (1996) Interaction of the Nck adapter protein with p21-activated kinase (PAK1). *J Biol Chem* **271:** 25746-25749

Bollo M, Paredes RM, Holstein D, Zheleznova N, Camacho P, Lechleiter JD (2010) Calcineurin interacts with PERK and dephosphorylates calnexin to relieve ER stress in mammals and frogs. *PLoS One* **5:** e11925

Bong YS, Park YH, Lee HS, Mood K, Ishimura A, Daar IO (2004) Tyr-298 in ephrinB1 is critical for an interaction with the Grb4 adaptor protein. *Biochem J* **377:** 499-507

Bonini JA, Colca J, Hofmann C (1995a) Altered expression of insulin signaling components in streptozotocin-treated rats. *Biochem Biophys Res Commun* **212:** 933-938

Bonini JA, Colca JR, Dailey C, White M, Hofmann C (1995b) Compensatory alterations for insulin signal transduction and glucose transport in insulin-resistant diabetes. *Am J Physiol* **269:** E759-765

Bonner-Weir S, Orci L (1982) New perspectives on the microvasculature of the islets of Langerhans in the rat. *Diabetes* **31:** 883-889

Borroto A, Arellano I, Dopfer EP, Prouza M, Suchanek M, Fuentes M, Orfao A, Schamel WW, Alarcon B (2013) Nck recruitment to the TCR required for ZAP70 activation during thymic development. *J Immunol* **190:** 1103-1112

Bottazzo GF, Florin-Christensen A, Doniach D (1974) Islet-cell antibodies in diabetes mellitus with autoimmune polyendocrine deficiencies. *Lancet* **2:** 1279-1283

Braverman LE, Quilliam LA (1999) Identification of Grb4/Nckbeta, a src homology 2 and 3 domain-containing adapter protein having similar binding and biological properties to Nck. *J Biol Chem* **274:** 5542-5549

Briscoe CP, Tadayyon M, Andrews JL, Benson WG, Chambers JK, Eilert MM, Ellis C, Elshourbagy NA, Goetz AS, Minnick DT, Murdock PR, Sauls HR, Jr., Shabon U, Spinage LD, Strum JC, Szekeres PG, Tan KB, Way JM, Ignar DM, Wilson S, Muir AI (2003) The orphan G protein-coupled receptor GPR40 is activated by medium and long chain fatty acids. *J Biol Chem* **278:** 11303-11311

Brissova M, Fowler MJ, Nicholson WE, Chu A, Hirshberg B, Harlan DM, Powers AC (2005) Assessment of human pancreatic islet architecture and composition by laser scanning confocal microscopy. *J Histochem Cytochem* **53:** 1087-1097

Brunet A, Bonni A, Zigmond MJ, Lin MZ, Juo P, Hu LS, Anderson MJ, Arden KC, Blenis J, Greenberg ME (1999) Akt promotes cell survival by phosphorylating and inhibiting a Forkhead transcription factor. *Cell* **96:** 857-868

Bruning A, Rahmeh M, Friese K (2013) Nelfinavir and bortezomib inhibit mTOR activity via ATF4-mediated sestrin-2 regulation. *Mol Oncol* **7:** 1012-1018

Brush MH, Weiser DC, Shenolikar S (2003) Growth arrest and DNA damage-inducible protein GADD34 targets protein phosphatase 1 alpha to the endoplasmic reticulum and promotes dephosphorylation of the alpha subunit of eukaryotic translation initiation factor 2. *Mol Cell Biol* **23:** 1292-1303

Budanov AV, Shoshani T, Faerman A, Zelin E, Kamer I, Kalinski H, Gorodin S, Fishman A, Chajut A, Einat P, Skaliter R, Gudkov AV, Chumakov PM, Feinstein E (2002) Identification of a novel stress-responsive gene Hi95 involved in regulation of cell viability. *Oncogene* **21:** 6017-6031

Budanov AV, Karin M (2008) p53 target genes sestrin1 and sestrin2 connect genotoxic stress and mTOR signaling. *Cell* **134:** 451-460

Buday L, Wunderlich L, Tamas P (2002) The Nck family of adapter proteins: regulators of actin cytoskeleton. *Cell Signal* **14:** 723-731

Burkhardt JK, Carrizosa E, Shaffer MH (2008) The actin cytoskeleton in T cell activation. *Annu Rev Immunol* **26:** 233-259

Butler AE, Janson J, Bonner-Weir S, Ritzel R, Rizza RA, Butler PC (2003) Beta-cell deficit and increased beta-cell apoptosis in humans with type 2 diabetes. *Diabetes* **52:** 102-110

Cabrera O, Berman DM, Kenyon NS, Ricordi C, Berggren PO, Caicedo A (2006) The unique cytoarchitecture of human pancreatic islets has implications for islet cell function. *Proc Natl Acad Sci U S A* **103:** 2334-2339

Cai D, Dhe-Paganon S, Melendez PA, Lee J, Shoelson SE (2003) Two new substrates in insulin signaling, IRS5/DOK4 and IRS6/DOK5. *J Biol Chem* **278:** 25323-25330

Calfon M, Zeng H, Urano F, Till JH, Hubbard SR, Harding HP, Clark SG, Ron D (2002) IRE1 couples endoplasmic reticulum load to secretory capacity by processing the XBP-1 mRNA. *Nature* **415:** 92-96

Campbell IL, Hellquist LN, Taylor KW (1982) Insulin biosynthesis and its regulation. *Clin Sci (Lond)* **62:** 449-455

Cao SS, Kaufman RJ (2012) Unfolded protein response. *Curr Biol* **22:** R622-626

Cardin E, Latreille M, Khoury C, Greenwood MT, Larose L (2007) Nck-1 selectively modulates eIF2alphaSer51 phosphorylation by a subset of eIF2alpha-kinases. *FEBS J* **274:** 5865-5875

Cardin E, Larose L (2008) Nck-1 interacts with PKR and modulates its activation by dsRNA. *Biochem Biophys Res Commun* **377:** 231-235

Cardozo AK, Ortis F, Storling J, Feng YM, Rasschaert J, Tonnesen M, Van Eylen F, Mandrup-Poulsen T, Herchuelz A, Eizirik DL (2005) Cytokines downregulate the sarcoendoplasmic reticulum pump Ca2+ ATPase 2b and deplete endoplasmic reticulum Ca2+, leading to induction of endoplasmic reticulum stress in pancreatic beta-cells. *Diabetes* **54:** 452-461

Carrara M, Prischi F, Nowak PR, Ali MM (2015) Crystal structures reveal transient PERK luminal domain tetramerization in endoplasmic reticulum stress signaling. *EMBO J* **34:** 1589-1600

Castello A, Gaya M, Tucholski J, Oellerich T, Lu KH, Tafuri A, Pawson T, Wienands J, Engelke M, Batista FD (2013) Nck-mediated recruitment of BCAP to the BCR regulates the PI(3)K-Akt pathway in B cells. *Nat Immunol* **14:** 966-975

Chaki SP, Barhoumi R, Berginski ME, Sreenivasappa H, Trache A, Gomez SM, Rivera GM (2013) Nck enables directional cell migration through the coordination of polarized membrane protrusion with adhesion dynamics. *J Cell Sci* **126:** 1637-1649

Chan K, Kan YW (1999) Nrf2 is essential for protection against acute pulmonary injury in mice. *Proc Natl Acad Sci U S A* **96:** 12731-12736

Chan TO, Rittenhouse SE, Tsichlis PN (1999) AKT/PKB and other D3 phosphoinositide-regulated kinases: kinase activation by phosphoinositide-dependent phosphorylation. *Annu Rev Biochem* **68:** 965-1014

Chang-Chen KJ, Mullur R, Bernal-Mizrachi E (2008) Beta-cell failure as a complication of diabetes. *Reviews in endocrine & metabolic disorders* **9:** 329-343

Chen HD, Frankel G (2005) Enteropathogenic Escherichia coli: unravelling pathogenesis. *FEMS Microbiol Rev* **29:** 83-98

Chen M, She H, Davis EM, Spicer CM, Kim L, Ren R, Le Beau MM, Li W (1998) Identification of Nck family genes, chromosomal localization, expression, and signaling specificity. *J Biol Chem* **273:** 25171-25178

Chen M, She H, Kim A, Woodley DT, Li W (2000) Nckbeta adapter regulates actin polymerization in NIH 3T3 fibroblasts in response to platelet-derived growth factor bb. *Mol Cell Biol* **20:** 7867-7880

Chen WS, Xu PZ, Gottlob K, Chen ML, Sokol K, Shiyanova T, Roninson I, Weng W, Suzuki R, Tobe K, Kadowaki T, Hay N (2001) Growth retardation and increased apoptosis in mice with homozygous disruption of the Akt1 gene. *Genes Dev* **15:** 2203-2208

Chen X, Shen J, Prywes R (2002) The luminal domain of ATF6 senses endoplasmic reticulum (ER) stress and causes translocation of ATF6 from the ER to the Golgi. *J Biol Chem* **277:** 13045-13052

Chiasson JL, Liljenquist JE, Sinclair-Smith BC, Lacy WW (1975) Gluconeogenesis from alanine in normal postabsorptive man. Intrahepatic stimulatory effect of glucagon. *Diabetes* **24:** 574-584

Cho H, Mu J, Kim JK, Thorvaldsen JL, Chu Q, Crenshaw EB, 3rd, Kaestner KH, Bartolomei MS, Shulman GI, Birnbaum MJ (2001a) Insulin resistance and a diabetes mellitus-like syndrome in mice lacking the protein kinase Akt2 (PKB beta). *Science* **292:** 1728-1731

Cho H, Thorvaldsen JL, Chu Q, Feng F, Birnbaum MJ (2001b) Akt1/PKBalpha is required for normal growth but dispensable for maintenance of glucose homeostasis in mice. *J Biol Chem* **276:** 38349-38352

Choi SE, Lee SM, Lee YJ, Li LJ, Lee SJ, Lee JH, Kim Y, Jun HS, Lee KW, Kang Y (2009) Protective role of autophagy in palmitate-induced INS-1 beta-cell death. *Endocrinology* **150:** 126-134

Chou MM, Fajardo JE, Hanafusa H (1992) The SH2- and SH3-containing Nck protein transforms mammalian fibroblasts in the absence of elevated phosphotyrosine levels. *Mol Cell Biol* **12:** 5834-5842

Clemens JC, Ursuliak Z, Clemens KK, Price JV, Dixon JE (1996) A Drosophila protein-tyrosine phosphatase associates with an adapter protein required for axonal guidance. *J Biol Chem* **271:** 17002-17005

Cnop M, Welsh N, Jonas JC, Jorns A, Lenzen S, Eizirik DL (2005) Mechanisms of pancreatic beta-cell death in type 1 and type 2 diabetes: many differences, few similarities. *Diabetes* **54 Suppl 2:** S97-107

Cohen GB, Ren R, Baltimore D (1995) Modular binding domains in signal transduction proteins. *Cell* **80:** 237-248

Cohen P (2006) The twentieth century struggle to decipher insulin signalling. *Nat Rev Mol Cell Biol* **7:** 867-873

Connor JH, Weiser DC, Li S, Hallenbeck JM, Shenolikar S (2001) Growth arrest and DNA damage-inducible protein GADD34 assembles a novel signaling complex containing protein phosphatase 1 and inhibitor 1. *Mol Cell Biol* **21:** 6841-6850

Cook DL, Hales CN (1984) Intracellular ATP directly blocks K+ channels in pancreatic B-cells. *Nature* **311:** 271-273

Copps KD, Hancer NJ, Opare-Ado L, Qiu W, Walsh C, White MF (2010) Irs1 serine 307 promotes insulin sensitivity in mice. *Cell Metab* **11:** 84-92

Cowan CA, Henkemeyer M (2001) The SH2/SH3 adaptor Grb4 transduces B-ephrin reverse signals. *Nature* **413:** 174-179

Cox RD, Meier-Ewert S, Ross M, Larin Z, Monaco AP, Lehrach H (1993) Genome mapping and cloning of mutations using yeast artificial chromosomes. *Methods Enzymol* **225:** 637-653

Credle JJ, Finer-Moore JS, Papa FR, Stroud RM, Walter P (2005) On the mechanism of sensing unfolded protein in the endoplasmic reticulum. *Proc Natl Acad Sci U S A* **102:** 18773-18784

Crespin SR, Greenough WB, 3rd, Steinberg D (1969) Stimulation of insulin secretion by infusion of free fatty acids. *J Clin Invest* **48:** 1934-1943

Cui W, Li J, Ron D, Sha B (2011) The structure of the PERK kinase domain suggests the mechanism for its activation. *Acta Crystallogr D Biol Crystallogr* **67:** 423-428

Cullinan SB, Zhang D, Hannink M, Arvisais E, Kaufman RJ, Diehl JA (2003) Nrf2 is a direct PERK substrate and effector of PERK-dependent cell survival. *Mol Cell Biol* **23:** 7198-7209

Cullinan SB, Diehl JA (2004) PERK-dependent activation of Nrf2 contributes to redox homeostasis and cell survival following endoplasmic reticulum stress. *J Biol Chem* **279:** 20108-20117

Cullinan SB, Gordan JD, Jin J, Harper JW, Diehl JA (2004) The Keap1-BTB protein is an adaptor that bridges Nrf2 to a Cul3-based E3 ligase: oxidative stress sensing by a Cul3-Keap1 ligase. *Mol Cell Biol* **24:** 8477-8486

Cullinan SB, Diehl JA (2006) Coordination of ER and oxidative stress signaling: the PERK/Nrf2 signaling pathway. *Int J Biochem Cell Biol* **38:** 317-332

Cunha DA, Hekerman P, Ladriere L, Bazarra-Castro A, Ortis F, Wakeham MC, Moore F, Rasschaert J, Cardozo AK, Bellomo E, Overbergh L, Mathieu C, Lupi R, Hai T, Herchuelz A,

Marchetti P, Rutter GA, Eizirik DL, Cnop M (2008) Initiation and execution of lipotoxic ER stress in pancreatic beta-cells. *J Cell Sci* **121:** 2308-2318

Curry DL, Bennett LL, Grodsky GM (1968) Dynamics of insulin secretion by the perfused rat pancreas. *Endocrinology* **83:** 572-584

Daneman D (2006) Type 1 diabetes. *Lancet* **367:** 847-858

Daniel S, Noda M, Straub SG, Sharp GW (1999) Identification of the docked granule pool responsible for the first phase of glucose-stimulated insulin secretion. *Diabetes* **48:** 1686-1690

Davidson HW, Rhodes CJ, Hutton JC (1988) Intraorganellar calcium and pH control proinsulin cleavage in the pancreatic beta cell via two distinct site-specific endopeptidases. *Nature* **333:** 93-96

de Haro C, Mendez R, Santoyo J (1996) The eIF-2alpha kinases and the control of protein synthesis. *FASEB J* **10:** 1378-1387

De Meyts P (2004) Insulin and its receptor: structure, function and evolution. *Bioessays* **26:** 1351-1362

De Meyts P (2008) The insulin receptor: a prototype for dimeric, allosteric membrane receptors? *Trends Biochem Sci* **33:** 376-384

de Wit NJ, Rijntjes J, Diepstra JH, van Kuppevelt TH, Weidle UH, Ruiter DJ, van Muijen GN (2005) Analysis of differential gene expression in human melanocytic tumour lesions by custom made oligonucleotide arrays. *Br J Cancer* **92:** 2249-2261

Delepine M, Nicolino M, Barrett T, Golamaully M, Lathrop GM, Julier C (2000) EIF2AK3, encoding translation initiation factor 2-alpha kinase 3, is mutated in patients with Wolcott-Rallison syndrome. *Nat Genet* **25:** 406-409

Desai CJ, Garrity PA, Keshishian H, Zipursky SL, Zinn K (1999) The Drosophila SH2-SH3 adapter protein Dock is expressed in embryonic axons and facilitates synapse formation by the RP3 motoneuron. *Development* **126:** 1527-1535

Dickson SL, Egecioglu E, Landgren S, Skibicka KP, Engel JA, Jerlhag E (2011) The role of the central ghrelin system in reward from food and chemical drugs. *Mol Cell Endocrinol* **340:** 80-87

Dobbins RL, Chester MW, Stevenson BE, Daniels MB, Stein DT, McGarry JD (1998) A fatty acid- dependent step is critically important for both glucose- and non-glucose-stimulated insulin secretion. *J Clin Invest* **101:** 2370-2376

Dong XC, Copps KD, Guo S, Li Y, Kollipara R, DePinho RA, White MF (2008) Inactivation of hepatic Foxo1 by insulin signaling is required for adaptive nutrient homeostasis and endocrine growth regulation. *Cell Metab* **8:** 65-76

Dor Y, Brown J, Martinez OI, Melton DA (2004) Adult pancreatic beta-cells are formed by self-duplication rather than stem-cell differentiation. *Nature* **429:** 41-46

Ebato C, Uchida T, Arakawa M, Komatsu M, Ueno T, Komiya K, Azuma K, Hirose T, Tanaka K, Kominami E, Kawamori R, Fujitani Y, Watada H (2008) Autophagy is important in islet homeostasis and compensatory increase of beta cell mass in response to high-fat diet. *Cell Metab* **8:** 325-332

Efrat S, Russ HA (2012) Making beta cells from adult tissues. *Trends Endocrinol Metab* **23:** 278-285

Egea PF, Stroud RM, Walter P (2005) Targeting proteins to membranes: structure of the signal recognition particle. *Curr Opin Struct Biol* **15:** 213-220

Egile C, Loisel TP, Laurent V, Li R, Pantaloni D, Sansonetti PJ, Carlier MF (1999) Activation of the CDC42 effector N-WASP by the Shigella flexneri IcsA protein promotes actin nucleation by Arp2/3 complex and bacterial actin-based motility. *J Cell Biol* **146:** 1319-1332

Eizirik DL, Cardozo AK, Cnop M (2008) The role for endoplasmic reticulum stress in diabetes mellitus. *Endocr Rev* **29:** 42-61

El-Assaad W, Buteau J, Peyot ML, Nolan C, Roduit R, Hardy S, Joly E, Dbaibo G, Rosenberg L, Prentki M (2003) Saturated fatty acids synergize with elevated glucose to cause pancreatic beta-cell death. *Endocrinology* **144:** 4154-4163

Eletto D, Eletto D, Dersh D, Gidalevitz T, Argon Y (2014) Protein disulfide isomerase A6 controls the decay of IRE1alpha signaling via disulfide-dependent association. *Mol Cell* **53:** 562-576

Ellgaard L, Frickel EM (2003) Calnexin, calreticulin, and ERp57: teammates in glycoprotein folding. *Cell Biochem Biophys* **39:** 223-247

Ellgaard L, Helenius A (2003) Quality control in the endoplasmic reticulum. *Nat Rev Mol Cell Biol* **4:** 181-191

Engin F, Yermalovich A, Nguyen T, Hummasti S, Fu W, Eizirik DL, Mathis D, Hotamisligil GS (2013) Restoration of the unfolded protein response in pancreatic beta cells protects mice against type 1 diabetes. *Sci Transl Med* **5:** 211ra156

Fairweather D, Rose NR (2002) Type 1 diabetes: virus infection or autoimmune disease? *Nat Immunol* **3:** 338-340

Fantin VR, Sparling JD, Slot JW, Keller SR, Lienhard GE, Lavan BE (1998) Characterization of insulin receptor substrate 4 in human embryonic kidney 293 cells. *J Biol Chem* **273:** 10726-10732

Fantin VR, Wang Q, Lienhard GE, Keller SR (2000) Mice lacking insulin receptor substrate 4 exhibit mild defects in growth, reproduction, and glucose homeostasis. *Am J Physiol Endocrinol Metab* **278:** E127-133

Fava E, Dehghany J, Ouwendijk J, Muller A, Niederlein A, Verkade P, Meyer-Hermann M, Solimena M (2012) Novel standards in the measurement of rat insulin granules combining electron microscopy, high-content image analysis and in silico modelling. *Diabetologia* **55:** 1013-1023

Fawcett JP, Georgiou J, Ruston J, Bladt F, Sherman A, Warner N, Saab BJ, Scott R, Roder JC, Pawson T (2007) Nck adaptor proteins control the organization of neuronal circuits important for walking. *Proc Natl Acad Sci U S A* **104:** 20973-20978

Federici M, Hribal ML, Ranalli M, Marselli L, Porzio O, Lauro D, Borboni P, Lauro R, Marchetti P, Melino G, Sesti G (2001) The common Arg972 polymorphism in insulin receptor substrate-1 causes apoptosis of human pancreatic islets. *FASEB J* **15:** 22-24

Feng D, Wei J, Gupta S, McGrath BC, Cavener DR (2009) Acute ablation of PERK results in ER dysfunctions followed by reduced insulin secretion and cell proliferation. *BMC Cell Biol* **10:** 61

Ferdaoussi M, Bergeron V, Zarrouki B, Kolic J, Cantley J, Fielitz J, Olson EN, Prentki M, Biden T, MacDonald PE, Poitout V (2012) G protein-coupled receptor (GPR)40-dependent potentiation of insulin secretion in mouse islets is mediated by protein kinase D1. *Diabetologia* **55:** 2682-2692

Fernandez-Ruiz R, Vieira E, Garcia-Roves PM, Gomis R (2014) Protein tyrosine phosphatase-1B modulates pancreatic beta-cell mass. *PLoS One* **9:** e90344

Frame S, Cohen P (2001) GSK3 takes centre stage more than 20 years after its discovery. *Biochem J* **359:** 1-16

Frese S, Schubert WD, Findeis AC, Marquardt T, Roske YS, Stradal TE, Heinz DW (2006) The phosphotyrosine peptide binding specificity of Nck1 and Nck2 Src homology 2 domains. *J Biol Chem* **281:** 18236-18245

Friedl P, Wolf K (2010) Plasticity of cell migration: a multiscale tuning model. *J Cell Biol* **188:** 11-19

Frischknecht F, Cudmore S, Moreau V, Reckmann I, Rottger S, Way M (1999) Tyrosine phosphorylation is required for actin-based motility of vaccinia but not Listeria or Shigella. *Curr Biol* **9:** 89-92

Froguel P, Zouali H, Vionnet N, Velho G, Vaxillaire M, Sun F, Lesage S, Stoffel M, Takeda J, Passa P, et al. (1993) Familial hyperglycemia due to mutations in glucokinase. Definition of a subtype of diabetes mellitus. *N Engl J Med* **328:** 697-702

Fu J, Zheng H, Wang H, Yang B, Zhao R, Lu C, Liu Z, Hou Y, Xu Y, Zhang Q, Qu W, Pi J (2015) Protective Role of Nuclear Factor E2-Related Factor 2 against Acute Oxidative Stress-Induced Pancreatic beta -Cell Damage. *Oxid Med Cell Longev* **2015:** 639191

Fujimoto WY, Ensinck JW, Williams RH (1974) Somatostatin inhibits insulin and glucagon release by monolayer cell cultures of rat endocrine pancreas. *Life Sci* **15:** 1999-2004

Furukawa M, Xiong Y (2005) BTB protein Keap1 targets antioxidant transcription factor Nrf2 for ubiquitination by the Cullin 3-Roc1 ligase. *Mol Cell Biol* **25:** 162-171

Gaisano HY (2014) Here come the newcomer granules, better late than never. *Trends Endocrinol Metab* **25:** 381-388

Galic S, Klingler-Hoffmann M, Fodero-Tavoletti MT, Puryer MA, Meng TC, Tonks NK, Tiganis T (2003) Regulation of insulin receptor signaling by the protein tyrosine phosphatase TCPTP. *Mol Cell Biol* **23:** 2096-2108

Galic S, Hauser C, Kahn BB, Haj FG, Neel BG, Tonks NK, Tiganis T (2005) Coordinated regulation of insulin signaling by the protein tyrosine phosphatases PTP1B and TCPTP. *Mol Cell Biol* **25:** 819-829

Galisteo ML, Chernoff J, Su YC, Skolnik EY, Schlessinger J (1996) The adaptor protein Nck links receptor tyrosine kinases with the serine-threonine kinase Pak1. *J Biol Chem* **271:** 20997-21000

Gao Y, Sartori DJ, Li C, Yu QC, Kushner JA, Simon MC, Diehl JA (2012) PERK is required in the adult pancreas and is essential for maintenance of glucose homeostasis. *Mol Cell Biol* **32:** 5129-5139

Garrity PA, Rao Y, Salecker I, McGlade J, Pawson T, Zipursky SL (1996) Drosophila photoreceptor axon guidance and targeting requires the dreadlocks SH2/SH3 adapter protein. *Cell* **85:** 639-650

Gebauer F, Hentze MW (2004) Molecular mechanisms of translational control. *Nat Rev Mol Cell Biol* **5:** 827-835

George S, Rochford JJ, Wolfrum C, Gray SL, Schinner S, Wilson JC, Soos MA, Murgatroyd PR, Williams RM, Acerini CL, Dunger DB, Barford D, Umpleby AM, Wareham NJ, Davies HA, Schafer AJ, Stoffel M, O'Rahilly S, Barroso I (2004) A family with severe insulin resistance and diabetes due to a mutation in AKT2. *Science* **304:** 1325-1328

Gil D, Schamel WW, Montoya M, Sanchez-Madrid F, Alarcon B (2002) Recruitment of Nck by CD3 epsilon reveals a ligand-induced conformational change essential for T cell receptor signaling and synapse formation. *Cell* **109:** 901-912

Grote E, Carr CM, Novick PJ (2000) Ordering the final events in yeast exocytosis. *J Cell Biol* **151:** 439-452

Gruenheid S, DeVinney R, Bladt F, Goosney D, Gelkop S, Gish GD, Pawson T, Finlay BB (2001) Enteropathogenic E. coli Tir binds Nck to initiate actin pedestal formation in host cells. *Nat Cell Biol* **3:** 856-859

Gu Y, Lindner J, Kumar A, Yuan W, Magnuson MA (2011) Rictor/mTORC2 is essential for maintaining a balance between beta-cell proliferation and cell size. *Diabetes* **60:** 827-837

Gupta S, McGrath B, Cavener DR (2010) PERK (EIF2AK3) regulates proinsulin trafficking and quality control in the secretory pathway. *Diabetes* **59:** 1937-1947

Haataja L, Gurlo T, Huang CJ, Butler PC (2008) Islet amyloid in type 2 diabetes, and the toxic oligomer hypothesis. *Endocr Rev* **29:** 303-316

Hai T, Curran T (1991) Cross-family dimerization of transcription factors Fos/Jun and ATF/CREB alters DNA binding specificity. *Proc Natl Acad Sci U S A* **88:** 3720-3724

Hake MJ, Choowongkomon K, Kostenko O, Carlin CR, Sonnichsen FD (2008) Specificity determinants of a novel Nck interaction with the juxtamembrane domain of the epidermal growth factor receptor. *Biochemistry* **47:** 3096-3108

Hamada S, Hara K, Hamada T, Yasuda H, Moriyama H, Nakayama R, Nagata M, Yokono K (2009) Upregulation of the mammalian target of rapamycin complex 1 pathway by Ras homolog enriched in brain in pancreatic beta-cells leads to increased beta-cell mass and prevention of hyperglycemia. *Diabetes* **58:** 1321-1332

Hamanaka RB, Bennett BS, Cullinan SB, Diehl JA (2005) PERK and GCN2 contribute to eIF2alpha phosphorylation and cell cycle arrest after activation of the unfolded protein response pathway. *Mol Biol Cell* **16:** 5493-5501

Hamilton JA, Kamp F (1999) How are free fatty acids transported in membranes? Is it by proteins or by free diffusion through the lipids? *Diabetes* **48:** 2255-2269

Han AP, Yu C, Lu L, Fujiwara Y, Browne C, Chin G, Fleming M, Leboulch P, Orkin SH, Chen JJ (2001) Heme-regulated eIF2alpha kinase (HRI) is required for translational regulation and survival of erythroid precursors in iron deficiency. *EMBO J* **20:** 6909-6918

Han D, Lerner AG, Vande Walle L, Upton JP, Xu W, Hagen A, Backes BJ, Oakes SA, Papa FR (2009) IRE1alpha kinase activation modes control alternate endoribonuclease outputs to determine divergent cell fates. *Cell* **138:** 562-575

Han J, Back SH, Hur J, Lin YH, Gildersleeve R, Shan J, Yuan CL, Krokowski D, Wang S, Hatzoglou M, Kilberg MS, Sartor MA, Kaufman RJ (2013) ER-stress-induced transcriptional regulation increases protein synthesis leading to cell death. *Nat Cell Biol* **15:** 481-490

Hancock JF, Parton RG (2005) Ras plasma membrane signalling platforms. *Biochem J* **389:** 1-11

Harden N, Lee J, Loh HY, Ong YM, Tan I, Leung T, Manser E, Lim L (1996) A Drosophila homolog of the Rac- and Cdc42-activated serine/threonine kinase PAK is a potential focal adhesion and focal complex protein that colocalizes with dynamic actin structures. *Mol Cell Biol* **16:** 1896-1908

Harding HP, Zhang Y, Ron D (1999) Protein translation and folding are coupled by an endoplasmic-reticulum-resident kinase. *Nature* **397:** 271-274

Harding HP, Novoa I, Zhang Y, Zeng H, Wek R, Schapira M, Ron D (2000a) Regulated translation initiation controls stress-induced gene expression in mammalian cells. *Mol Cell* **6:** 1099-1108

Harding HP, Zhang Y, Bertolotti A, Zeng H, Ron D (2000b) Perk is essential for translational regulation and cell survival during the unfolded protein response. *Mol Cell* **5:** 897-904

Harding HP, Zeng H, Zhang Y, Jungries R, Chung P, Plesken H, Sabatini DD, Ron D (2001) Diabetes mellitus and exocrine pancreatic dysfunction in perk-/- mice reveals a role for translational control in secretory cell survival. *Mol Cell* **7:** 1153-1163

Harding HP, Zhang Y, Zeng H, Novoa I, Lu PD, Calfon M, Sadri N, Yun C, Popko B, Paules R, Stojdl DF, Bell JC, Hettmann T, Leiden JM, Ron D (2003) An integrated stress response regulates amino acid metabolism and resistance to oxidative stress. *Mol Cell* **11:** 619-633

Harding HP, Zyryanova AF, Ron D (2012) Uncoupling proteostasis and development in vitro with a small molecule inhibitor of the pancreatic endoplasmic reticulum kinase, PERK. *J Biol Chem* **287:** 44338-44344

Hashimoto N, Kido Y, Uchida T, Asahara S, Shigeyama Y, Matsuda T, Takeda A, Tsuchihashi D, Nishizawa A, Ogawa W, Fujimoto Y, Okamura H, Arden KC, Herrera PL, Noda T, Kasuga M (2006) Ablation of PDK1 in pancreatic beta cells induces diabetes as a result of loss of beta cell mass. *Nat Genet* **38:** 589-593

Hatanaka M, Maier B, Sims EK, Templin AT, Kulkarni RN, Evans-Molina C, Mirmira RG (2014) Palmitate induces mRNA translation and increases ER protein load in islet beta-cells via activation of the mammalian target of rapamycin pathway. *Diabetes* **63:** 3404-3415

Hayes JD, McMahon M (2001) Molecular basis for the contribution of the antioxidant responsive element to cancer chemoprevention. *Cancer Lett* **174:** 103-113

Haze K, Yoshida H, Yanagi H, Yura T, Mori K (1999) Mammalian transcription factor ATF6 is synthesized as a transmembrane protein and activated by proteolysis in response to endoplasmic reticulum stress. *Mol Biol Cell* **10:** 3787-3799

Haze K, Okada T, Yoshida H, Yanagi H, Yura T, Negishi M, Mori K (2001) Identification of the G13 (cAMP-response-element-binding protein-related protein) gene product related to activating transcription factor 6 as a transcriptional activator of the mammalian unfolded protein response. *Biochem J* **355:** 19-28

He CH, Gong P, Hu B, Stewart D, Choi ME, Choi AM, Alam J (2001) Identification of activating transcription factor 4 (ATF4) as an Nrf2-interacting protein. Implication for heme oxygenase-1 gene regulation. *J Biol Chem* **276:** 20858-20865

Hem CD, Sundvold-Gjerstad V, Granum S, Koll L, Abrahamsen G, Buday L, Spurkland A (2015) T cell specific adaptor protein (TSAd) promotes interaction of Nck with Lck and SLP-76 in T cells. *Cell Commun Signal* **13:** 31

Hendershot LM (2004) The ER function BiP is a master regulator of ER function. *Mt Sinai J Med* **71:** 289-297

Henquin JC (2000) Triggering and amplifying pathways of regulation of insulin secretion by glucose. *Diabetes* **49:** 1751-1760

Herold KC, Gitelman SE, Ehlers MR, Gottlieb PA, Greenbaum CJ, Hagopian W, Boyle KD, Keyes-Elstein L, Aggarwal S, Phippard D, Sayre PH, McNamara J, Bluestone JA, Ab ATEST (2013) Teplizumab (anti-CD3 mAb) treatment preserves C-peptide responses in patients with new-onset type 1 diabetes in a randomized controlled trial: metabolic and immunologic features at baseline identify a subgroup of responders. *Diabetes* **62:** 3766-3774

Hess DA, Humphrey SE, Ishibashi J, Damsz B, Lee AH, Glimcher LH, Konieczny SF (2011) Extensive pancreas regeneration following acinar-specific disruption of Xbp1 in mice. *Gastroenterology* **141:** 1463-1472

Hetz C, Martinon F, Rodriguez D, Glimcher LH (2011) The unfolded protein response: integrating stress signals through the stress sensor IRE1alpha. *Physiol Rev* **91:** 1219-1243

Hetz C, Chevet E, Harding HP (2013) Targeting the unfolded protein response in disease. *Nat Rev Drug Discov* **12:** 703-719

Higgs HN, Pollard TD (1999) Regulation of actin polymerization by Arp2/3 complex and WASp/Scar proteins. *J Biol Chem* **274:** 32531-32534

Hing H, Xiao J, Harden N, Lim L, Zipursky SL (1999) Pak functions downstream of Dock to regulate photoreceptor axon guidance in Drosophila. *Cell* **97:** 853-863

Hinnebusch AG (2005) Translational regulation of GCN4 and the general amino acid control of yeast. *Annu Rev Microbiol* **59:** 407-450

Hodish I, Liu M, Rajpal G, Larkin D, Holz RW, Adams A, Liu L, Arvan P (2010) Misfolded proinsulin affects bystander proinsulin in neonatal diabetes. *J Biol Chem* **285:** 685-694

Holcik M, Sonenberg N (2005) Translational control in stress and apoptosis. *Nat Rev Mol Cell Biol* **6:** 318-327

Holland SJ, Gale NW, Gish GD, Roth RA, Songyang Z, Cantley LC, Henkemeyer M, Yancopoulos GD, Pawson T (1997) Juxtamembrane tyrosine residues couple the Eph family receptor EphB2/Nuk to specific SH2 domain proteins in neuronal cells. *EMBO J* **16:** 3877-3888

Hollien J, Weissman JS (2006) Decay of endoplasmic reticulum-localized mRNAs during the unfolded protein response. *Science* **313:** 104-107

Hollien J, Lin JH, Li H, Stevens N, Walter P, Weissman JS (2009) Regulated Ire1-dependent decay of messenger RNAs in mammalian cells. *J Cell Biol* **186:** 323-331

Holman RR (1998) Assessing the potential for alpha-glucosidase inhibitors in prediabetic states. *Diabetes research and clinical practice* **40 Suppl:** S21-25

Hong EG, Jung DY, Ko HJ, Zhang Z, Ma Z, Jun JY, Kim JH, Sumner AD, Vary TC, Gardner TW, Bronson SK, Kim JK (2007) Nonobese, insulin-deficient Ins2Akita mice develop type 2 diabetes phenotypes including insulin resistance and cardiac remodeling. *Am J Physiol Endocrinol Metab* **293:** E1687-1696

Howell SL, Tyhurst M (1986) The cytoskeleton and insulin secretion. *Diabetes Metab Rev* **2:** 107-123

Hu Q, Milfay D, Williams LT (1995) Binding of NCK to SOS and activation of ras-dependent gene expression. *Mol Cell Biol* **15:** 1169-1174

Hu Y, Xu XH, He K, Zhang LL, Wang SK, Pan YQ, He BS, Feng TT, Mao XM (2014) Genome-wide analysis of DNA methylation variations caused by chronic glucolipotoxicity in beta-cells. *Exp Clin Endocrinol Diabetes* **122:** 71-78

Huang HC, Nguyen T, Pickett CB (2000) Regulation of the antioxidant response element by protein kinase C-mediated phosphorylation of NF-E2-related factor 2. *Proc Natl Acad Sci U S A* **97:** 12475-12480

Huebner K, Kastury K, Druck T, Salcini AE, Lanfrancone L, Pelicci G, Lowenstein E, Li W, Park SH, Cannizzaro L, et al. (1994) Chromosome locations of genes encoding human signal transduction adapter proteins, Nck (NCK), Shc (SHC1), and Grb2 (GRB2). *Genomics* **22:** 281-287

Huh WJ, Esen E, Geahlen JH, Bredemeyer AJ, Lee AH, Shi G, Konieczny SF, Glimcher LH, Mills JC (2010) XBP1 controls maturation of gastric zymogenic cells by induction of MIST1 and expansion of the rough endoplasmic reticulum. *Gastroenterology* **139:** 2038-2049

Hur KY, So JS, Ruda V, Frank-Kamenetsky M, Fitzgerald K, Koteliansky V, Iwawaki T, Glimcher LH, Lee AH (2012) IRE1alpha activation protects mice against acetaminophen-induced hepatotoxicity. *J Exp Med* **209:** 307-318

Ido Y, Vindigni A, Chang K, Stramm L, Chance R, Heath WF, DiMarchi RD, Di Cera E, Williamson JR (1997) Prevention of vascular and neural dysfunction in diabetic rats by C-peptide. *Science* **277:** 563-566

Inoki K, Li Y, Zhu T, Wu J, Guan KL (2002) TSC2 is phosphorylated and inhibited by Akt and suppresses mTOR signalling. *Nat Cell Biol* **4:** 648-657

Itoh K, Chiba T, Takahashi S, Ishii T, Igarashi K, Katoh Y, Oyake T, Hayashi N, Satoh K, Hatayama I, Yamamoto M, Nabeshima Y (1997) An Nrf2/small Maf heterodimer mediates the induction of phase II detoxifying enzyme genes through antioxidant response elements. *Biochem Biophys Res Commun* **236:** 313-322

Itoh K, Wakabayashi N, Katoh Y, Ishii T, Igarashi K, Engel JD, Yamamoto M (1999) Keap1 represses nuclear activation of antioxidant responsive elements by Nrf2 through binding to the amino-terminal Neh2 domain. *Genes Dev* **13:** 76-86

Itoh Y, Kawamata Y, Harada M, Kobayashi M, Fujii R, Fukusumi S, Ogi K, Hosoya M, Tanaka Y, Uejima H, Tanaka H, Maruyama M, Satoh R, Okubo S, Kizawa H, Komatsu H, Matsumura F, Noguchi Y, Shinohara T, Hinuma S, Fujisawa Y, Fujino M (2003) Free fatty acids regulate insulin secretion from pancreatic beta cells through GPR40. *Nature* **422:** 173-176

Iwakoshi NN, Lee AH, Glimcher LH (2003) The X-box binding protein-1 transcription factor is required for plasma cell differentiation and the unfolded protein response. *Immunol Rev* **194:** 29-38

Iwasaki N, Sugiyama Y, Miyazaki S, Nakagawa H, Nishimura K, Matsuo S (2015) An ATF4-Signal-Modulating Machine Other Than GADD34 Acts in ATF4-to-CHOP Signaling to Block CHOP Expression in ER-Stress-Related Autophagy. *J Cell Biochem* **116:** 1300-1309

Iwawaki T, Akai R, Yamanaka S, Kohno K (2009) Function of IRE1 alpha in the placenta is essential for placental development and embryonic viability. *Proc Natl Acad Sci U S A* **106:** 16657-16662

Iwawaki T, Akai R, Kohno K (2010) IRE1alpha disruption causes histological abnormality of exocrine tissues, increase of blood glucose level, and decrease of serum immunoglobulin level. *PLoS One* **5:** e13052

Jarosch E, Lenk U, Sommer T (2003) Endoplasmic reticulum-associated protein degradation. *Int Rev Cytol* **223:** 39-81

Jiang ZY, Zhou QL, Coleman KA, Chouinard M, Boese Q, Czech MP (2003) Insulin signaling through Akt/protein kinase B analyzed by small interfering RNA-mediated gene silencing. *Proc Natl Acad Sci U S A* **100:** 7569-7574

Jones N, Blasutig IM, Eremina V, Ruston JM, Bladt F, Li H, Huang H, Larose L, Li SS, Takano T, Quaggin SE, Pawson T (2006) Nck adaptor proteins link nephrin to the actin cytoskeleton of kidney podocytes. *Nature* **440:** 818-823

Jones N, New LA, Fortino MA, Eremina V, Ruston J, Blasutig IM, Aoudjit L, Zou Y, Liu X, Yu GL, Takano T, Quaggin SE, Pawson T (2009) Nck proteins maintain the adult glomerular filtration barrier. *J Am Soc Nephrol* **20:** 1533-1543

Joseph N, Reicher B, David A, Matalon O, Barda-Saad M (2014) Ubiquitylation-dependent downregulation of Nck regulates its functional activity. *FEBS Lett* **588:** 3808-3815

Julier C, Nicolino M (2010) Wolcott-Rallison syndrome. *Orphanet J Rare Dis* **5:** 29

Jung HJ, Suh Y (2014) Regulation of IGF -1 signaling by microRNAs. *Front Genet* **5:** 472

Jung HS, Chung KW, Won Kim J, Kim J, Komatsu M, Tanaka K, Nguyen YH, Kang TM, Yoon KH, Kim JW, Jeong YT, Han MS, Lee MK, Kim KW, Shin J, Lee MS (2008) Loss of autophagy diminishes pancreatic beta cell mass and function with resultant hyperglycemia. *Cell Metab* **8:** 318-324

Jurkin J, Henkel T, Nielsen AF, Minnich M, Popow J, Kaufmann T, Heindl K, Hoffmann T, Busslinger M, Martinez J (2014) The mammalian tRNA ligase complex mediates splicing of XBP1 mRNA and controls antibody secretion in plasma cells. *EMBO J* **33:** 2922-2936

Jwa M, Chang P (2012) PARP16 is a tail-anchored endoplasmic reticulum protein required for the PERK- and IRE1alpha-mediated unfolded protein response. *Nat Cell Biol* **14:** 1223-1230

Kalwat MA, Yoder SM, Wang Z, Thurmond DC (2013) A p21-activated kinase (PAK1) signaling cascade coordinately regulates F-actin remodeling and insulin granule exocytosis in pancreatic beta cells. *Biochem Pharmacol* **85:** 808-816

Kane S, Sano H, Liu SC, Asara JM, Lane WS, Garner CC, Lienhard GE (2002) A method to identify serine kinase substrates. Akt phosphorylates a novel adipocyte protein with a Rab GTPase-activating protein (GAP) domain. *J Biol Chem* **277:** 22115-22118

Kansanen E, Jyrkkanen HK, Levonen AL (2012) Activation of stress signaling pathways by electrophilic oxidized and nitrated lipids. *Free Radic Biol Med* **52:** 973-982

Karpinski BA, Morle GD, Huggenvik J, Uhler MD, Leiden JM (1992) Molecular cloning of human CREB-2: an ATF/CREB transcription factor that can negatively regulate transcription from the cAMP response element. *Proc Natl Acad Sci U S A* **89:** 4820-4824

Kaufman RJ (1999) Stress signaling from the lumen of the endoplasmic reticulum: coordination of gene transcriptional and translational controls. *Genes Dev* **13:** 1211-1233

Kaufman RJ (2002) Orchestrating the unfolded protein response in health and disease. *J Clin Invest* **110:** 1389-1398

Kaung HL (1994) Growth dynamics of pancreatic islet cell populations during fetal and neonatal development of the rat. *Dev Dyn* **200:** 163-175

Kebache S, Zuo D, Chevet E, Larose L (2002) Modulation of protein translation by Nck-1. *Proc Natl Acad Sci U S A* **99:** 5406-5411

Kebache S, Cardin E, Nguyen DT, Chevet E, Larose L (2004) Nck-1 antagonizes the endoplasmic reticulum stress-induced inhibition of translation. *J Biol Chem* **279:** 9662-9671

Kee Y, Yoo JS, Hazuka CD, Peterson KE, Hsu SC, Scheller RH (1997) Subunit structure of the mammalian exocyst complex. *Proc Natl Acad Sci U S A* **94:** 14438-14443

Kelly RP, Sutton R, Ashcroft FM (1991) Voltage-activated calcium and potassium currents in human pancreatic beta-cells. *J Physiol* **443:** 175-192

Kemmler W, Peterson JD, Rubenstein AH, Steiner DF (1972) On the biosynthesis, intracellular transport and mechanism of conversion of proinsulin to insulin and C-peptide. *Diabetes* **21:** 572-581

Kenny B, DeVinney R, Stein M, Reinscheid DJ, Frey EA, Finlay BB (1997) Enteropathogenic E. coli (EPEC) transfers its receptor for intimate adherence into mammalian cells. *Cell* **91:** 511-520

Kestila M, Lenkkeri U, Mannikko M, Lamerdin J, McCready P, Putaala H, Ruotsalainen V, Morita T, Nissinen M, Herva R, Kashtan CE, Peltonen L, Holmberg C, Olsen A, Tryggvason K (1998) Positionally cloned gene for a novel glomerular protein--nephrin--is mutated in congenital nephrotic syndrome. *Mol Cell* **1:** 575-582

Khoshnoodi J, Sigmundsson K, Ofverstedt LG, Skoglund U, Obrink B, Wartiovaara J, Tryggvason K (2003) Nephrin promotes cell-cell adhesion through homophilic interactions. *Am J Pathol* **163:** 2337-2346

Kim J, Kundu M, Viollet B, Guan KL (2011) AMPK and mTOR regulate autophagy through direct phosphorylation of Ulk1. *Nat Cell Biol* **13:** 132-141

Kim J, Cheon H, Jeong YT, Quan W, Kim KH, Cho JM, Lim YM, Oh SH, Jin SM, Kim JH, Lee MK, Kim S, Komatsu M, Kang SW, Lee MS (2014a) Amyloidogenic peptide oligomer accumulation in autophagy-deficient beta cells induces diabetes. *J Clin Invest* **124:** 3311-3324

Kim SM, Chae MK, Lee C, Yim MS, Bang JK, Ryu EK (2014b) Enhanced cellular uptake of a TAT-conjugated peptide inhibitor targeting the polo-box domain of polo-like kinase 1. *Amino Acids* **46:** 2595-2603

Kim YB, Inoue T, Nakajima R, Shirai-Morishita Y, Tokuyama K, Suzuki M (1999) Effect of long-term exercise on gene expression of insulin signaling pathway intermediates in skeletal muscle. *Biochem Biophys Res Commun* **254:** 720-727

Klionsky DJ, Emr SD (2000) Autophagy as a regulated pathway of cellular degradation. *Science* **290:** 1717-1721

Kode A, Mosialou I, Silva BC, Joshi S, Ferron M, Rached MT, Kousteni S (2012) FoxO1 protein cooperates with ATF4 protein in osteoblasts to control glucose homeostasis. *J Biol Chem* **287:** 8757-8768

Kosmaczewski SG, Edwards TJ, Han SM, Eckwahl MJ, Meyer BI, Peach S, Hesselberth JR, Wolin SL, Hammarlund M (2014) The RtcB RNA ligase is an essential component of the metazoan unfolded protein response. *EMBO Rep* **15:** 1278-1285

Koumenis C, Naczki C, Koritzinsky M, Rastani S, Diehl A, Sonenberg N, Koromilas A, Wouters BG (2002) Regulation of protein synthesis by hypoxia via activation of the endoplasmic reticulum kinase PERK and phosphorylation of the translation initiation factor eIF2alpha. *Mol Cell Biol* **22:** 7405-7416

Kouroku Y, Fujita E, Tanida I, Ueno T, Isoai A, Kumagai H, Ogawa S, Kaufman RJ, Kominami E, Momoi T (2007) ER stress (PERK/eIF2alpha phosphorylation) mediates the polyglutamine-induced LC3 conversion, an essential step for autophagy formation. *Cell death and differentiation* **14:** 230-239

Krishnakumar SS, Radoff DT, Kummel D, Giraudo CG, Li F, Khandan L, Baguley SW, Coleman J, Reinisch KM, Pincet F, Rothman JE (2011) A conformational switch in complexin is required for synaptotagmin to trigger synaptic fusion. *Nat Struct Mol Biol* **18:** 934-940

Krishnan N, Fu C, Pappin DJ, Tonks NK (2011) H2S-Induced sulfhydration of the phosphatase PTP1B and its role in the endoplasmic reticulum stress response. *Sci Signal* **4:** ra86

Kriz W (2007) Ontogenetic development of the filtration barrier. *Nephron Exp Nephrol* **106:** e44-50

Kubota N, Tobe K, Terauchi Y, Eto K, Yamauchi T, Suzuki R, Tsubamoto Y, Komeda K, Nakano R, Miki H, Satoh S, Sekihara H, Sciacchitano S, Lesniak M, Aizawa S, Nagai R, Kimura S, Akanuma Y, Taylor SI, Kadowaki T (2000) Disruption of insulin receptor substrate 2 causes type 2 diabetes because of liver insulin resistance and lack of compensatory beta-cell hyperplasia. *Diabetes* **49:** 1880-1889

Kuhne MR, Pawson T, Lienhard GE, Feng GS (1993) The insulin receptor substrate 1 associates with the SH2-containing phosphotyrosine phosphatase Syp. *J Biol Chem* **268:** 11479-11481

Kulkarni RN, Zisman A (2003) Lessons for human diabetes from experimental mouse models. *Curr Diab Rep* **3:** 168-175

Kushner JA, Haj FG, Klaman LD, Dow MA, Kahn BB, Neel BG, White MF (2004) Islet-sparing effects of protein tyrosine phosphatase-1b deficiency delays onset of diabetes in IRS2 knockout mice. *Diabetes* **53:** 61-66

Kwak MK, Wakabayashi N, Greenlaw JL, Yamamoto M, Kensler TW (2003) Antioxidants enhance mammalian proteasome expression through the Keap1-Nrf2 signaling pathway. *Mol Cell Biol* **23:** 8786-8794

Labelle-Cote M, Dusseault J, Ismail S, Picard-Cloutier A, Siegel PM, Larose L (2011) Nck2 promotes human melanoma cell proliferation, migration and invasion in vitro and primary melanoma-derived tumor growth in vivo. *BMC Cancer* **11:** 443

Labelle-Cote M, Larose L (2011) [A uNick protein]. *Med Sci (Paris)* **27:** 746-752

Latreille M, Larose L (2006) Nck in a complex containing the catalytic subunit of protein phosphatase 1 regulates eukaryotic initiation factor 2alpha signaling and cell survival to endoplasmic reticulum stress. *J Biol Chem* **281:** 26633-26644

Latreille M, Laberge MK, Bourret G, Yamani L, Larose L (2011) Deletion of Nck1 attenuates hepatic ER stress signaling and improves glucose tolerance and insulin signaling in liver of obese mice. *Am J Physiol Endocrinol Metab* **300:** E423-434

Lauffenburger DA, Horwitz AF (1996) Cell migration: a physically integrated molecular process. *Cell* **84:** 359-369

Lavan BE, Lane WS, Lienhard GE (1997) The 60-kDa phosphotyrosine protein in insulin-treated adipocytes is a new member of the insulin receptor substrate family. *J Biol Chem* **272:** 11439-11443

Lawe DC, Hahn C, Wong AJ (1997) The Nck SH2/SH3 adaptor protein is present in the nucleus and associates with the nuclear protein SAM68. *Oncogene* **14:** 223-231

Lawrence MC, McKern NM, Ward CW (2007) Insulin receptor structure and its implications for the IGF-1 receptor. *Curr Opin Struct Biol* **17:** 699-705

Lebastchi J, Deng S, Lebastchi AH, Beshar I, Gitelman S, Willi S, Gottlieb P, Akirav EM, Bluestone JA, Herold KC (2013) Immune therapy and beta-cell death in type 1 diabetes. *Diabetes* **62:** 1676-1680

Lee AH, Iwakoshi NN, Glimcher LH (2003) XBP-1 regulates a subset of endoplasmic reticulum resident chaperone genes in the unfolded protein response. *Mol Cell Biol* **23:** 7448-7459

Lee AH, Chu GC, Iwakoshi NN, Glimcher LH (2005) XBP-1 is required for biogenesis of cellular secretory machinery of exocrine glands. *EMBO J* **24:** 4368-4380

Lee AH, Scapa EF, Cohen DE, Glimcher LH (2008) Regulation of hepatic lipogenesis by the transcription factor XBP1. *Science* **320:** 1492-1496

Lee AH, Heidtman K, Hotamisligil GS, Glimcher LH (2011) Dual and opposing roles of the unfolded protein response regulated by IRE1alpha and XBP1 in proinsulin processing and insulin secretion. *Proc Natl Acad Sci U S A* **108:** 8885-8890

Lee AS (1992) Mammalian stress response: induction of the glucose-regulated protein family. *Curr Opin Cell Biol* **4:** 267-273

Lee CH, Li W, Nishimura R, Zhou M, Batzer AG, Myers MG, Jr., White MF, Schlessinger J, Skolnik EY (1993) Nck associates with the SH2 domain-docking protein IRS-1 in insulin-stimulated cells. *Proc Natl Acad Sci U S A* **90:** 11713-11717

Lee JH, Budanov AV, Park EJ, Birse R, Kim TE, Perkins GA, Ocorr K, Ellisman MH, Bodmer R, Bier E, Karin M (2010) Sestrin as a feedback inhibitor of TOR that prevents age-related pathologies. *Science* **327:** 1223-1228

Lee JH, Budanov AV, Talukdar S, Park EJ, Park HL, Park HW, Bandyopadhyay G, Li N, Aghajan M, Jang I, Wolfe AM, Perkins GA, Ellisman MH, Bier E, Scadeng M, Foretz M, Viollet B, Olefsky J, Karin M (2012a) Maintenance of metabolic homeostasis by Sestrin2 and Sestrin3. *Cell Metab* **16:** 311-321

Lee K, Tirasophon W, Shen X, Michalak M, Prywes R, Okada T, Yoshida H, Mori K, Kaufman RJ (2002) IRE1-mediated unconventional mRNA splicing and S2P-mediated ATF6 cleavage merge to regulate XBP1 in signaling the unfolded protein response. *Genes Dev* **16:** 452-466

Lee S, Hur EG, Ryoo IG, Jung KA, Kwak J, Kwak MK (2012b) Involvement of the Nrf2-proteasome pathway in the endoplasmic reticulum stress response in pancreatic beta-cells. *Toxicol Appl Pharmacol* **264:** 431-438

Leeuw T, Wu C, Schrag JD, Whiteway M, Thomas DY, Leberer E (1998) Interaction of a G-protein beta-subunit with a conserved sequence in Ste20/PAK family protein kinases. *Nature* **391:** 191-195

Lefebvre PJ (1983) *Glucagon*, Berlin ; New York: Springer-Verlag.

Lefèbvre PJ, Unger RH (1972) *Glucagon; molecular physiology, clinical and therapeutic implications*, 1st edn. Oxford, New York,: Pergamon Press.

Legg JA, Bompard G, Dawson J, Morris HL, Andrew N, Cooper L, Johnston SA, Tramountanis G, Machesky LM (2007) N-WASP involvement in dorsal ruffle formation in mouse embryonic fibroblasts. *Mol Biol Cell* **18:** 678-687

Lehmann JM, Riethmuller G, Johnson JP (1990) Nck, a melanoma cDNA encoding a cytoplasmic protein consisting of the src homology units SH2 and SH3. *Nucleic Acids Res* **18:** 1048

Letourneau PC, Cypher C (1991) Regulation of growth cone motility. *Cell Motil Cytoskeleton* **20:** 267-271

Letourneau PC, Condic ML, Snow DM (1992) Extracellular matrix and neurite outgrowth. *Curr Opin Genet Dev* **2:** 625-634

Lettau M, Qian J, Linkermann A, Latreille M, Larose L, Kabelitz D, Janssen O (2006) The adaptor protein Nck interacts with Fas ligand: Guiding the death factor to the cytotoxic immunological synapse. *Proc Natl Acad Sci U S A* **103:** 5911-5916

Lettau M, Schmidt H, Kabelitz D, Janssen O (2007) Secretory lysosomes and their cargo in T and NK cells. *Immunol Lett* **108:** 10-19

Lettau M, Paulsen M, Kabelitz D, Janssen O (2008) Storage, expression and function of Fas ligand, the key death factor of immune cells. *Curr Med Chem* **15:** 1684-1696

Lettau M, Pieper J, Janssen O (2009) Nck adapter proteins: functional versatility in T cells. *Cell Commun Signal* **7:** 1

Levine B, Kroemer G (2008) Autophagy in the pathogenesis of disease. *Cell* **132:** 27-42

Li B, Pi Z, Liu L, Zhang B, Huang X, Hu P, Chevet E, Yi P, Liu J (2013) FGF-2 prevents cancer cells from ER stress-mediated apoptosis via enhancing proteasome-mediated Nck degradation. *Biochem J* **452:** 139-145

Li F, Pincet F, Perez E, Giraudo CG, Tareste D, Rothman JE (2011) Complexin activates and clamps SNAREpins by a common mechanism involving an intermediate energetic state. *Nat Struct Mol Biol* **18:** 941-946

Li H, Zhu J, Aoudjit L, Latreille M, Kawachi H, Larose L, Takano T (2006) Rat nephrin modulates cell morphology via the adaptor protein Nck. *Biochem Biophys Res Commun* **349:** 310-316

Li H, Dusseault J, Larose L (2014a) Nck1 depletion induces activation of the PI3K/Akt pathway by attenuating PTP1B protein expression. *Cell Commun Signal* **12:** 71

Li J, Luo R, Kowluru A, Li G (2004) Novel regulation by Rac1 of glucose- and forskolin-induced insulin secretion in INS-1 beta-cells. *Am J Physiol Endocrinol Metab* **286:** E818-827

Li W, Hu P, Skolnik EY, Ullrich A, Schlessinger J (1992) The SH2 and SH3 domain-containing Nck protein is oncogenic and a common target for phosphorylation by different surface receptors. *Mol Cell Biol* **12:** 5824-5833

Li W, Wu W, Song H, Wang F, Li H, Chen L, Lai Y, Janicki JS, Ward KW, Meyer CJ, Wang XL, Tang D, Cui T (2014b) Targeting Nrf2 by dihydro-CDDO-trifluoroethyl amide enhances autophagic clearance and viability of beta-cells in a setting of oxidative stress. *FEBS Lett* **588:** 2115-2124

Li X, Meriane M, Triki I, Shekarabi M, Kennedy TE, Larose L, Lamarche-Vane N (2002) The adaptor protein Nck-1 couples the netrin-1 receptor DCC (deleted in colorectal cancer) to the activation of the small GTPase Rac1 through an atypical mechanism. *J Biol Chem* **277:** 37788-37797

Lin JH, Li H, Yasumura D, Cohen HR, Zhang C, Panning B, Shokat KM, Lavail MM, Walter P (2007) IRE1 signaling affects cell fate during the unfolded protein response. *Science* **318:** 944-949

Lin JH, Li H, Zhang Y, Ron D, Walter P (2009) Divergent effects of PERK and IRE1 signaling on cell viability. *PLoS One* **4:** e4170

Lipson KL, Fonseca SG, Ishigaki S, Nguyen LX, Foss E, Bortell R, Rossini AA, Urano F (2006) Regulation of insulin biosynthesis in pancreatic beta cells by an endoplasmic reticulum-resident protein kinase IRE1. *Cell Metab* **4:** 245-254

Lipson KL, Ghosh R, Urano F (2008) The role of IRE1alpha in the degradation of insulin mRNA in pancreatic beta-cells. *PLoS One* **3:** e1648

Liu M, Wright J, Guo H, Xiong Y, Arvan P (2014a) Proinsulin entry and transit through the endoplasmic reticulum in pancreatic beta cells. *Vitam Horm* **95:** 35-62

Liu S, Xi Y, Bettaieb A, Matsuo K, Matsuo I, Kulkarni RN, Haj FG (2014b) Disruption of protein-tyrosine phosphatase 1B expression in the pancreas affects beta-cell function. *Endocrinology* **155:** 3329-3338

Liu SC, Wang Q, Lienhard GE, Keller SR (1999) Insulin receptor substrate 3 is not essential for growth or glucose homeostasis. *J Biol Chem* **274:** 18093-18099

Long YC, Cheng Z, Copps KD, White MF (2011) Insulin receptor substrates Irs1 and Irs2 coordinate skeletal muscle growth and metabolism via the Akt and AMPK pathways. *Mol Cell Biol* **31:** 430-441

Lu KH, Keppler S, Leithauser F, Mattfeldt T, Castello A, Kostezka U, Kublbeck G, Schmitt S, Klevenz A, Prokosch S, Pougialis G, Pawson T, Batista F, Tafuri A, Arnold B (2015) Nck adaptor proteins modulate differentiation and effector function of T cells. *J Leukoc Biol*

Lu PD, Harding HP, Ron D (2004) Translation reinitiation at alternative open reading frames regulates gene expression in an integrated stress response. *J Cell Biol* **167:** 27-33

Lu W, Katz S, Gupta R, Mayer BJ (1997) Activation of Pak by membrane localization mediated by an SH3 domain from the adaptor protein Nck. *Curr Biol* **7:** 85-94

Luo Y, Frey EA, Pfuetzner RA, Creagh AL, Knoechel DG, Haynes CA, Finlay BB, Strynadka NC (2000) Crystal structure of enteropathogenic Escherichia coli intimin-receptor complex. *Nature* **405:** 1073-1077

Luschnig S, Krauss J, Bohmann K, Desjeux I, Nusslein-Volhard C (2000) The Drosophila SHC adaptor protein is required for signaling by a subset of receptor tyrosine kinases. *Mol Cell* **5:** 231-241

Lussier G, Larose L (1997) A casein kinase I activity is constitutively associated with Nck. *J Biol Chem* **272:** 2688-2694

Ma K, Vattem KM, Wek RC (2002) Dimerization and release of molecular chaperone inhibition facilitate activation of eukaryotic initiation factor-2 kinase in response to endoplasmic reticulum stress. *J Biol Chem* **277:** 18728-18735

Ma Y, Lu Y, Zeng H, Ron D, Mo W, Neubert TA (2001) Characterization of phosphopeptides from protein digests using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry and nanoelectrospray quadrupole time-of-flight mass spectrometry. *Rapid Commun Mass Spectrom* **15:** 1693-1700

Ma Y, Hendershot LM (2003) Delineation of a negative feedback regulatory loop that controls protein translation during endoplasmic reticulum stress. *J Biol Chem* **278:** 34864-34873

Magnusson I, Rothman DL, Gerard DP, Katz LD, Shulman GI (1995) Contribution of hepatic glycogenolysis to glucose production in humans in response to a physiological increase in plasma glucagon concentration. *Diabetes* **44:** 185-189

Maiuri MC, Malik SA, Morselli E, Kepp O, Criollo A, Mouchel PL, Carnuccio R, Kroemer G (2009) Stimulation of autophagy by the p53 target gene Sestrin2. *Cell Cycle* **8:** 1571-1576

Makino S, Kunimoto K, Muraoka Y, Mizushima Y, Katagiri K, Tochino Y (1980) Breeding of a non-obese, diabetic strain of mice. *Jikken Dobutsu* **29:** 1-13

Mancini AD, Poitout V (2013) The fatty acid receptor FFA1/GPR40 a decade later: how much do we know? *Trends Endocrinol Metab* **24:** 398-407

Manser E, Leung T, Salihuddin H, Zhao ZS, Lim L (1994) A brain serine/threonine protein kinase activated by Cdc42 and Rac1. *Nature* **367:** 40-46

Manser E, Huang HY, Loo TH, Chen XQ, Dong JM, Leung T, Lim L (1997) Expression of constitutively active alpha-PAK reveals effects of the kinase on actin and focal complexes. *Mol Cell Biol* **17:** 1129-1143

Manser E, Loo TH, Koh CG, Zhao ZS, Chen XQ, Tan L, Tan I, Leung T, Lim L (1998) PAK kinases are directly coupled to the PIX family of nucleotide exchange factors. *Mol Cell* **1:** 183-192

Marchetti P, Dotta F, Lauro D, Purrello F (2008) An overview of pancreatic beta-cell defects in human type 2 diabetes: implications for treatment. *Regul Pept* **146:** 4-11

Marciniak SJ, Garcia-Bonilla L, Hu J, Harding HP, Ron D (2006) Activation-dependent substrate recruitment by the eukaryotic translation initiation factor 2 kinase PERK. *J Cell Biol* **172:** 201-209

Margolis B, Silvennoinen O, Comoglio F, Roonprapunt C, Skolnik E, Ullrich A, Schlessinger J (1992) High-efficiency expression/cloning of epidermal growth factor-receptor-binding proteins with Src homology 2 domains. *Proceedings of the National Academy of Sciences of the United States of America* **89:** 8894-8898

Markussen J, Schiff HE (1973) Molecular parameters of C-peptide from bovine proinsulin. *Int J Pept Protein Res* **5:** 69-72

Martin BC, Warram JH, Krolewski AS, Bergman RN, Soeldner JS, Kahn CR (1992) Role of glucose and insulin resistance in development of type 2 diabetes mellitus: results of a 25-year follow-up study. *Lancet* **340:** 925-929

Martinez SC, Tanabe K, Cras-Meneur C, Abumrad NA, Bernal-Mizrachi E, Permutt MA (2008) Inhibition of Foxo1 protects pancreatic islet beta-cells against fatty acid and endoplasmic reticulum stress-induced apoptosis. *Diabetes* **57:** 846-859

Martinon F, Chen X, Lee AH, Glimcher LH (2010) TLR activation of the transcription factor XBP1 regulates innate immune responses in macrophages. *Nat Immunol* **11:** 411-418

Matsumoto H, Miyazaki S, Matsuyama S, Takeda M, Kawano M, Nakagawa H, Nishimura K, Matsuo S (2013) Selection of autophagy or apoptosis in cells exposed to ER-stress depends on ATF4 expression pattern with or without CHOP expression. *Biol Open* **2:** 1084-1090

Mayer BJ (2001) SH3 domains: complexity in moderation. *J Cell Sci* **114:** 1253-1263

McCarty JH (1998) The Nck SH2/SH3 adaptor protein: a regulator of multiple intracellular signal transduction events. *Bioessays* **20:** 913-921

McGarry JD, Dobbins RL (1999) Fatty acids, lipotoxicity and insulin secretion. *Diabetologia* **42:** 128-138

Meier JJ (2008) Beta cell mass in diabetes: a realistic therapeutic target? *Diabetologia* **51:** 703-713

Meisenhelder J, Hunter T (1992) The SH2/SH3 domain-containing protein Nck is recognized by certain anti-phospholipase C-gamma 1 monoclonal antibodies, and its phosphorylation on tyrosine is stimulated by platelet-derived growth factor and epidermal growth factor treatment. *Mol Cell Biol* **12:** 5843-5856

Melanitou E, Fain P, Eisenbarth GS (2003) Genetics of type 1A (immune mediated) diabetes. *J Autoimmun* **21:** 93-98

Meury T, Akhouayri O, Jafarov T, Mandic V, St-Arnaud R (2010) Nuclear alpha NAC influences bone matrix mineralization and osteoblast maturation in vivo. *Mol Cell Biol* **30:** 43-53

Michael MD, Kulkarni RN, Postic C, Previs SF, Shulman GI, Magnuson MA, Kahn CR (2000) Loss of insulin signaling in hepatocytes leads to severe insulin resistance and progressive hepatic dysfunction. *Mol Cell* **6:** 87-97

Milani M, Rzymski T, Mellor HR, Pike L, Bottini A, Generali D, Harris AL (2009) The role of ATF4 stabilization and autophagy in resistance of breast cancer cells treated with Bortezomib. *Cancer Res* **69:** 4415-4423

Mir SU, George NM, Zahoor L, Harms R, Guinn Z, Sarvetnick NE (2015) Inhibition of autophagic turnover in beta-cells by fatty acids and glucose leads to apoptotic cell death. *J Biol Chem* **290:** 6071-6085

Molinete M, Irminger JC, Tooze SA, Halban PA (2000) Trafficking/sorting and granule biogenesis in the beta-cell. *Semin Cell Dev Biol* **11:** 243-251

Moon HW, Whipp SC, Argenzio RA, Levine MM, Giannella RA (1983) Attaching and effacing activities of rabbit and human enteropathogenic Escherichia coli in pig and rabbit intestines. *Infect Immun* **41:** 1340-1351

Mora A, Komander D, van Aalten DM, Alessi DR (2004) PDK1, the master regulator of AGC kinase signal transduction. *Semin Cell Dev Biol* **15:** 161-170

Mori H, Guan KL (2012) Tissue-specific ablation of Tsc1 in pancreatic beta-cells. *Methods Mol Biol* **821:** 407-419

Mori K, Kawahara T, Yoshida H, Yanagi H, Yura T (1996) Signalling from endoplasmic reticulum to nucleus: transcription factor with a basic-leucine zipper motif is required for the unfolded protein-response pathway. *Genes Cells* **1:** 803-817

Moskalenko S, Henry DO, Rosse C, Mirey G, Camonis JH, White MA (2002) The exocyst is a Ral effector complex. *Nat Cell Biol* **4:** 66-72

Mothe I, Van Obberghen E (1996) Phosphorylation of insulin receptor substrate-1 on multiple serine residues, 612, 632, 662, and 731, modulates insulin action. *J Biol Chem* **271:** 11222-11227

Mukherjee C, Bakthavachalu B, Schoenberg DR (2014) The cytoplasmic capping complex assembles on adapter protein nck1 bound to the proline-rich C-terminus of Mammalian capping enzyme. *PLoS Biol* **12:** e1001933

Mundel P, Kriz W (1995) Structure and function of podocytes: an update. *Anat Embryol (Berl)* **192:** 385-397

Murphy R, Ellard S, Hattersley AT (2008) Clinical implications of a molecular genetic classification of monogenic beta-cell diabetes. *Nat Clin Pract Endocrinol Metab* **4:** 200-213

Myers MG, Jr., Grammer TC, Wang LM, Sun XJ, Pierce JH, Blenis J, White MF (1994) Insulin receptor substrate-1 mediates phosphatidylinositol 3'-kinase and p70S6k signaling during insulin, insulin-like growth factor-1, and interleukin-4 stimulation. *J Biol Chem* **269:** 28783-28789

Nagamatsu S, Steiner DF (1992) Altered glucose regulation of insulin biosynthesis in insulinoma cells: mouse beta TC3 cells secrete insulin-related peptides predominantly via a constitutive pathway. *Endocrinology* **130:** 748-754

Natarajan K, Meyer MR, Jackson BM, Slade D, Roberts C, Hinnebusch AG, Marton MJ (2001) Transcriptional profiling shows that Gcn4p is a master regulator of gene expression during amino acid starvation in yeast. *Mol Cell Biol* **21:** 4347-4368

Negi S, Park SH, Jetha A, Aikin R, Tremblay M, Paraskevas S (2012) Evidence of endoplasmic reticulum stress mediating cell death in transplanted human islets. *Cell Transplant* **21:** 889-900

Nevins AK, Thurmond DC (2003) Glucose regulates the cortical actin network through modulation of Cdc42 cycling to stimulate insulin secretion. *Am J Physiol Cell Physiol* **285:** C698-710

New LA, Keyvani Chahi A, Jones N (2013) Direct regulation of nephrin tyrosine phosphorylation by Nck adaptor proteins. *J Biol Chem* **288:** 1500-1510

Newgard CB, McGarry JD (1995) Metabolic coupling factors in pancreatic beta-cell signal transduction. *Annu Rev Biochem* **64:** 689-719

Nguyen DT, Kebache S, Fazel A, Wong HN, Jenna S, Emadali A, Lee EH, Bergeron JJ, Kaufman RJ, Larose L, Chevet E (2004) Nck-dependent activation of extracellular signal-regulated kinase-1 and regulation of cell survival during endoplasmic reticulum stress. *Mol Biol Cell* **15:** 4248-4260

Nguyen T, Nioi P, Pickett CB (2009) The Nrf2-antioxidant response element signaling pathway and its activation by oxidative stress. *J Biol Chem* **284:** 13291-13295

Nishimura R, Li W, Kashishian A, Mondino A, Zhou M, Cooper J, Schlessinger J (1993) Two signaling molecules share a phosphotyrosine-containing binding site in the platelet-derived growth factor receptor. *Mol Cell Biol* **13:** 6889-6896

Nishitoh H, Matsuzawa A, Tobiume K, Saegusa K, Takeda K, Inoue K, Hori S, Kakizuka A, Ichijo H (2002) ASK1 is essential for endoplasmic reticulum stress-induced neuronal cell death triggered by expanded polyglutamine repeats. *Genes Dev* **16:** 1345-1355

Nolan CJ, Madiraju MS, Delghingaro-Augusto V, Peyot ML, Prentki M (2006) Fatty acid signaling in the beta-cell and insulin secretion. *Diabetes* **55 Suppl 2:** S16-23

Novoa I, Zhang Y, Zeng H, Jungreis R, Harding HP, Ron D (2003) Stress-induced gene expression requires programmed recovery from translational repression. *EMBO J* **22:** 1180-1187

Odisho T, Zhang L, Volchuk A (2015) ATF6beta regulates the Wfs1 gene and has a cell survival role in the ER stress response in pancreatic beta-cells. *Exp Cell Res* **330:** 111-122

Onken B, Driscoll M (2010) Metformin induces a dietary restriction-like state and the oxidative stress response to extend C. elegans Healthspan via AMPK, LKB1, and SKN-1. *PLoS One* **5:** e8758

Orci L, Ravazzola M, Amherdt M, Madsen O, Perrelet A, Vassalli JD, Anderson RG (1986) Conversion of proinsulin to insulin occurs coordinately with acidification of maturing secretory vesicles. *J Cell Biol* **103:** 2273-2281

Orci L, Ravazzola M, Storch MJ, Anderson RG, Vassalli JD, Perrelet A (1987) Proteolytic maturation of insulin is a post-Golgi event which occurs in acidifying clathrin-coated secretory vesicles. *Cell* **49:** 865-868

Otani K, Kulkarni RN, Baldwin AC, Krutzfeldt J, Ueki K, Stoffel M, Kahn CR, Polonsky KS (2004) Reduced beta-cell mass and altered glucose sensing impair insulin-secretory function in betaIRKO mice. *Am J Physiol Endocrinol Metab* **286:** E41-49

Oyadomari S, Takeda K, Takiguchi M, Gotoh T, Matsumoto M, Wada I, Akira S, Araki E, Mori M (2001) Nitric oxide-induced apoptosis in pancreatic beta cells is mediated by the endoplasmic reticulum stress pathway. *Proc Natl Acad Sci U S A* **98:** 10845-10850

Oyadomari S, Araki E, Mori M (2002) Endoplasmic reticulum stress-mediated apoptosis in pancreatic beta-cells. *Apoptosis* **7:** 335-345

Ozbek MN, Senee V, Aydemir S, Kotan LD, Mungan NO, Yuksel B, Julier C, Topaloglu AK (2010) Wolcott-Rallison syndrome due to the same mutation (W522X) in EIF2AK3 in two unrelated families and review of the literature. *Pediatr Diabetes* **11:** 279-285

Ozcan U, Cao Q, Yilmaz E, Lee AH, Iwakoshi NN, Ozdelen E, Tuncman G, Gorgun C, Glimcher LH, Hotamisligil GS (2004) Endoplasmic reticulum stress links obesity, insulin action, and type 2 diabetes. *Science* **306:** 457-461

Ozcan U, Yilmaz E, Ozcan L, Furuhashi M, Vaillancourt E, Smith RO, Gorgun CZ, Hotamisligil GS (2006) Chemical chaperones reduce ER stress and restore glucose homeostasis in a mouse model of type 2 diabetes. *Science* **313:** 1137-1140

Panzhinskiy E, Ren J, Nair S (2013) Protein tyrosine phosphatase 1B and insulin resistance: role of endoplasmic reticulum stress/reactive oxygen species/nuclear factor kappa B axis. *PLoS One* **8:** e77228

Park D, Rhee SG (1992) Phosphorylation of Nck in response to a variety of receptors, phorbol myristate acetate, and cyclic AMP. *Mol Cell Biol* **12:** 5816-5823

Park HW, Park H, Ro SH, Jang I, Semple IA, Kim DN, Kim M, Nam M, Zhang D, Yin L, Lee JH (2014) Hepatoprotective role of Sestrin2 against chronic ER stress. *Nature communications* **5:** 4233

Park SW, Zhou Y, Lee J, Lee J, Ozcan U (2010) Sarco(endo)plasmic reticulum Ca2+-ATPase 2b is a major regulator of endoplasmic reticulum stress and glucose homeostasis in obesity. *Proc Natl Acad Sci U S A* **107:** 19320-19325

Parmigiani A, Nourbakhsh A, Ding B, Wang W, Kim YC, Akopiants K, Guan KL, Karin M, Budanov AV (2014) Sestrins inhibit mTORC1 kinase activation through the GATOR complex. *Cell Rep* **9:** 1281-1291

Patzelt C, Labrecque AD, Duguid JR, Carroll RJ, Keim PS, Heinrikson RL, Steiner DF (1978) Detection and kinetic behavior of preproinsulin in pancreatic islets. *Proc Natl Acad Sci U S A* **75:** 1260-1264

Pauker MH, Barda-Saad M (2011) Studies of novel interactions between Nck and VAV SH3 domains. *Commun Integr Biol* **4:** 175-177

Pavitt GD (2005) eIF2B, a mediator of general and gene-specific translational control. *Biochem Soc Trans* **33:** 1487-1492

Pawson T (1993) Signal transduction--a conserved pathway from the membrane to the nucleus. *Dev Genet* **14:** 333-338

Pawson T (1995) Protein modules and signalling networks. *Nature* **373:** 573-580

Pawson T, Scott JD (1997) Signaling through scaffold, anchoring, and adaptor proteins. *Science* **278:** 2075-2080

Paz K, Hemi R, LeRoith D, Karasik A, Elhanany E, Kanety H, Zick Y (1997) A molecular basis for insulin resistance. Elevated serine/threonine phosphorylation of IRS-1 and IRS-2 inhibits their binding to the juxtamembrane region of the insulin receptor and impairs their ability to undergo insulin-induced tyrosine phosphorylation. *J Biol Chem* **272:** 29911-29918

Pederson TM, Kramer DL, Rondinone CM (2001) Serine/threonine phosphorylation of IRS-1 triggers its degradation: possible regulation by tyrosine phosphorylation. *Diabetes* **50:** 24-31

Pelkonen R, Miettinen TA, Taskinen MR, Nikkila EA (1968) Effect of acute elevation of plasma glycerol, triglyceride and FFA levels on glucose utilization and plasma insulin. *Diabetes* **17:** 76-82

Peng M, Yin N, Li MO (2014) Sestrins function as guanine nucleotide dissociation inhibitors for Rag GTPases to control mTORC1 signaling. *Cell* **159:** 122-133

Pessin JE, Thurmond DC, Elmendorf JS, Coker KJ, Okada S (1999) Molecular basis of insulin-stimulated GLUT4 vesicle trafficking. Location! Location! Location! *J Biol Chem* **274:** 2593-2596

Pilkis SJ, Granner DK (1992) Molecular physiology of the regulation of hepatic gluconeogenesis and glycolysis. *Annu Rev Physiol* **54:** 885-909

Poitout V (2003) The ins and outs of fatty acids on the pancreatic beta cell. *Trends Endocrinol Metab* **14:** 201-203

Poitout V, Hagman D, Stein R, Artner I, Robertson RP, Harmon JS (2006) Regulation of the insulin gene by glucose and fatty acids. *J Nutr* **136:** 873-876

Porte D, Jr. (2001) Clinical importance of insulin secretion and its interaction with insulin resistance in the treatment of type 2 diabetes mellitus and its complications. *Diabetes Metab Res Rev* **17:** 181-188

Posner BI, Faure R, Burgess JW, Bevan AP, Lachance D, Zhang-Sun G, Fantus IG, Ng JB, Hall DA, Lum BS, et al. (1994) Peroxovanadium compounds. A new class of potent phosphotyrosine phosphatase inhibitors which are insulin mimetics. *J Biol Chem* **269:** 4596-4604

Pouli AE, Emmanouilidou E, Zhao C, Wasmeier C, Hutton JC, Rutter GA (1998) Secretory-granule dynamics visualized in vivo with a phogrin-green fluorescent protein chimaera. *Biochem J* **333 (Pt 1):** 193-199

Pouyssegur J, Volmat V, Lenormand P (2002) Fidelity and spatio-temporal control in MAP kinase (ERKs) signalling. *Biochem Pharmacol* **64:** 755-763

Prado CL, Pugh-Bernard AE, Elghazi L, Sosa-Pineda B, Sussel L (2004) Ghrelin cells replace insulin-producing beta cells in two mouse models of pancreas development. *Proc Natl Acad Sci U S A* **101:** 2924-2929

Pramatarova A, Ochalski PG, Chen K, Gropman A, Myers S, Min KT, Howell BW (2003) Nck beta interacts with tyrosine-phosphorylated disabled 1 and redistributes in Reelin-stimulated neurons. *Mol Cell Biol* **23:** 7210-7221

Prehoda KE, Scott JA, Mullins RD, Lim WA (2000) Integration of multiple signals through cooperative regulation of the N-WASP-Arp2/3 complex. *Science* **290:** 801-806

Prentki M, Corkey BE (1996) Are the beta-cell signaling molecules malonyl-CoA and cystolic long-chain acyl-CoA implicated in multiple tissue defects of obesity and NIDDM? *Diabetes* **45:** 273-283

Prentki M, Joly E, El-Assaad W, Roduit R (2002) Malonyl-CoA signaling, lipid partitioning, and glucolipotoxicity: role in beta-cell adaptation and failure in the etiology of diabetes. *Diabetes* **51 Suppl 3:** S405-413

Prentki M, Nolan CJ (2006) Islet beta cell failure in type 2 diabetes. *J Clin Invest* **116:** 1802-1812

Puthalakath H, O'Reilly LA, Gunn P, Lee L, Kelly PN, Huntington ND, Hughes PD, Michalak EM, McKimm-Breschkin J, Motoyama N, Gotoh T, Akira S, Bouillet P, Strasser A (2007) ER stress triggers apoptosis by activating BH3-only protein Bim. *Cell* **129:** 1337-1349

Quan W, Hur KY, Lim Y, Oh SH, Lee JC, Kim KH, Kim GH, Kim SW, Kim HL, Lee MK, Kim KW, Kim J, Komatsu M, Lee MS (2012) Autophagy deficiency in beta cells leads to compromised unfolded protein response and progression from obesity to diabetes in mice. *Diabetologia* **55:** 392-403

Rachdi L, Balcazar N, Osorio-Duque F, Elghazi L, Weiss A, Gould A, Chang-Chen KJ, Gambello MJ, Bernal-Mizrachi E (2008) Disruption of Tsc2 in pancreatic beta cells induces beta cell mass expansion and improved glucose tolerance in a TORC1-dependent manner. *Proc Natl Acad Sci U S A* **105:** 9250-9255

Rajesh K, Krishnamoorthy J, Kazimierczak U, Tenkerian C, Papadakis AI, Wang S, Huang S, Koromilas AE (2015) Phosphorylation of the translation initiation factor eIF2alpha at serine 51 determines the cell fate decisions of Akt in response to oxidative stress. *Cell Death Dis* **6:** e1591

Rankin MM, Kushner JA (2009) Adaptive beta-cell proliferation is severely restricted with advanced age. *Diabetes* **58:** 1365-1372

Rao Y, Zipursky SL (1998) Domain requirements for the Dock adapter protein in growth- cone signaling. *Proc Natl Acad Sci U S A* **95:** 2077-2082

Reimold AM, Etkin A, Clauss I, Perkins A, Friend DS, Zhang J, Horton HF, Scott A, Orkin SH, Byrne MC, Grusby MJ, Glimcher LH (2000) An essential role in liver development for transcription factor XBP-1. *Genes Dev* **14:** 152-157

Reimold AM, Iwakoshi NN, Manis J, Vallabhajosyula P, Szomolanyi-Tsuda E, Gravallese EM, Friend D, Grusby MJ, Alt F, Glimcher LH (2001) Plasma cell differentiation requires the transcription factor XBP-1. *Nature* **412:** 300-307

Rhee JS, Betz A, Pyott S, Reim K, Varoqueaux F, Augustin I, Hesse D, Sudhof TC, Takahashi M, Rosenmund C, Brose N (2002) Beta phorbol ester- and diacylglycerol-induced augmentation of transmitter release is mediated by Munc13s and not by PKCs. *Cell* **108:** 121-133

Rhodes CJ, Halban PA (1987) Newly synthesized proinsulin/insulin and stored insulin are released from pancreatic B cells predominantly via a regulated, rather than a constitutive, pathway. *J Cell Biol* **105:** 145-153

Rhodes CJ, Lucas CA, Mutkoski RL, Orci L, Halban PA (1987) Stimulation by ATP of proinsulin to insulin conversion in isolated rat pancreatic islet secretory granules. Association with the ATP-dependent proton pump. *J Biol Chem* **262:** 10712-10717

Rietdorf J, Ploubidou A, Reckmann I, Holmstrom A, Frischknecht F, Zettl M, Zimmermann T, Way M (2001) Kinesin-dependent movement on microtubules precedes actin-based motility of vaccinia virus. *Nat Cell Biol* **3:** 992-1000

Rivera GM, Briceno CA, Takeshima F, Snapper SB, Mayer BJ (2004) Inducible clustering of membrane-targeted SH3 domains of the adaptor protein Nck triggers localized actin polymerization. *Curr Biol* **14:** 11-22

Rivera GM, Antoku S, Gelkop S, Shin NY, Hanks SK, Pawson T, Mayer BJ (2006) Requirement of Nck adaptors for actin dynamics and cell migration stimulated by platelet-derived growth factor B. *Proc Natl Acad Sci U S A* **103:** 9536-9541

Rivera GM, Vasilescu D, Papayannopoulos V, Lim WA, Mayer BJ (2009) A reciprocal interdependence between Nck and PI(4,5)P(2) promotes localized N-WASp-mediated actin polymerization in living cells. *Mol Cell* **36:** 525-535

Rivera JF, Costes S, Gurlo T, Glabe CG, Butler PC (2014) Autophagy defends pancreatic beta cells from human islet amyloid polypeptide-induced toxicity. *J Clin Invest* **124:** 3489-3500

Rivero-Lezcano OM, Marcilla A, Sameshima JH, Robbins KC (1995) Wiskott-Aldrich syndrome protein physically associates with Nck through Src homology 3 domains. *Mol Cell Biol* **15:** 5725-5731

Rohatgi R, Ho HY, Kirschner MW (2000) Mechanism of N-WASP activation by CDC42 and phosphatidylinositol 4, 5-bisphosphate. *J Cell Biol* **150:** 1299-1310

Rohatgi R, Nollau P, Ho HY, Kirschner MW, Mayer BJ (2001) Nck and phosphatidylinositol 4,5-bisphosphate synergistically activate actin polymerization through the N-WASP-Arp2/3 pathway. *J Biol Chem* **276:** 26448-26452

Rolland F, Winderickx J, Thevelein JM (2001) Glucose-sensing mechanisms in eukaryotic cells. *Trends Biochem Sci* **26:** 310-317

Ron D, Habener JF (1992) CHOP, a novel developmentally regulated nuclear protein that dimerizes with transcription factors C/EBP and LAP and functions as a dominant-negative inhibitor of gene transcription. *Genes Dev* **6:** 439-453

Ron D (2002) Translational control in the endoplasmic reticulum stress response. *J Clin Invest* **110:** 1383-1388

Ron D, Walter P (2007) Signal integration in the endoplasmic reticulum unfolded protein response. *Nat Rev Mol Cell Biol* **8:** 519-529

Rorsman P, Renstrom E (2003) Insulin granule dynamics in pancreatic beta cells. *Diabetologia* **46:** 1029-1045

Rouschop KM, Wouters BG (2009) Regulation of autophagy through multiple independent hypoxic signaling pathways. *Curr Mol Med* **9:** 417-424

Rowe AH (1923) Insulin Treatment of Diabetes Mellitus. *Cal State J Med* **21:** 204-208

Rowlands AG, Panniers R, Henshaw EC (1988) The catalytic mechanism of guanine nucleotide exchange factor action and competitive inhibition by phosphorylated eukaryotic initiation factor 2. *J Biol Chem* **263:** 5526-5533

Rozakis-Adcock M, McGlade J, Mbamalu G, Pelicci G, Daly R, Li W, Batzer A, Thomas S, Brugge J, Pelicci PG, Schlessinger J, Pawson T, et al. (1992) Association of the Shc and Grb2/Sem5 SH2-containing proteins is implicated in activation of the Ras pathway by tyrosine kinases. *Nature* **360:** 689-692

Rubio-Cabezas O, Patch AM, Minton JA, Flanagan SE, Edghill EL, Hussain K, Balafrej A, Deeb A, Buchanan CR, Jefferson IG, Mutair A, Neonatal Diabetes International Collaborative G, Hattersley AT, Ellard S (2009) Wolcott-Rallison syndrome is the most common genetic cause of permanent neonatal diabetes in consanguineous families. *J Clin Endocrinol Metab* **94:** 4162-4170

Ruotsalainen V, Ljungberg P, Wartiovaara J, Lenkkeri U, Kestila M, Jalanko H, Holmberg C, Tryggvason K (1999) Nephrin is specifically located at the slit diaphragm of glomerular podocytes. *Proc Natl Acad Sci U S A* **96:** 7962-7967

Ruotsalainen V, Patrakka J, Tissari P, Reponen P, Hess M, Kestila M, Holmberg C, Salonen R, Heikinheimo M, Wartiovaara J, Tryggvason K, Jalanko H (2000) Role of nephrin in cell junction formation in human nephrogenesis. *Am J Pathol* **157:** 1905-1916

Rutkowski DT, Arnold SM, Miller CN, Wu J, Li J, Gunnison KM, Mori K, Sadighi Akha AA, Raden D, Kaufman RJ (2006) Adaptation to ER stress is mediated by differential stabilities of pro-survival and pro-apoptotic mRNAs and proteins. *PLoS Biol* **4:** e374

Rutkowski DT, Kang SW, Goodman AG, Garrison JL, Taunton J, Katze MG, Kaufman RJ, Hegde RS (2007) The role of p58IPK in protecting the stressed endoplasmic reticulum. *Mol Biol Cell* **18:** 3681-3691

Rzymski T, Milani M, Singleton DC, Harris AL (2009) Role of ATF4 in regulation of autophagy and resistance to drugs and hypoxia. *Cell Cycle* **8:** 3838-3847

Salmeen A, Andersen JN, Myers MP, Tonks NK, Barford D (2000) Molecular basis for the dephosphorylation of the activation segment of the insulin receptor by protein tyrosine phosphatase 1B. *Mol Cell* **6:** 1401-1412

Saltiel AR, Kahn CR (2001) Insulin signalling and the regulation of glucose and lipid metabolism. *Nature* **414:** 799-806

Sano H, Kane S, Sano E, Miinea CP, Asara JM, Lane WS, Garner CW, Lienhard GE (2003) Insulin-stimulated phosphorylation of a Rab GTPase-activating protein regulates GLUT4 translocation. *J Biol Chem* **278:** 14599-14602

Sarbassov DD, Guertin DA, Ali SM, Sabatini DM (2005) Phosphorylation and regulation of Akt/PKB by the rictor-mTOR complex. *Science* **307:** 1098-1101

Scheuner D, Song B, McEwen E, Liu C, Laybutt R, Gillespie P, Saunders T, Bonner-Weir S, Kaufman RJ (2001) Translational control is required for the unfolded protein response and in vivo glucose homeostasis. *Mol Cell* **7:** 1165-1176

Scheuner D, Vander Mierde D, Song B, Flamez D, Creemers JW, Tsukamoto K, Ribick M, Schuit FC, Kaufman RJ (2005) Control of mRNA translation preserves endoplasmic reticulum function in beta cells and maintains glucose homeostasis. *Nat Med* **11:** 757-764

Scheuner D, Kaufman RJ (2008) The unfolded protein response: a pathway that links insulin demand with beta-cell failure and diabetes. *Endocr Rev* **29:** 317-333

Schlaepfer DD, Broome MA, Hunter T (1997) Fibronectin-stimulated signaling from a focal adhesion kinase-c-Src complex: involvement of the Grb2, p130cas, and Nck adaptor proteins. *Mol Cell Biol* **17:** 1702-1713

Schmucker D, Clemens JC, Shu H, Worby CA, Xiao J, Muda M, Dixon JE, Zipursky SL (2000) Drosophila Dscam is an axon guidance receptor exhibiting extraordinary molecular diversity. *Cell* **101:** 671-684

Schuit FC, In't Veld PA, Pipeleers DG (1988) Glucose stimulates proinsulin biosynthesis by a dose-dependent recruitment of pancreatic beta cells. *Proc Natl Acad Sci U S A* **85:** 3865-3869

Sells MA, Chernoff J (1997) Emerging from the Pak: the p21-activated protein kinase family. *Trends Cell Biol* **7:** 162-167

Sells MA, Knaus UG, Bagrodia S, Ambrose DM, Bokoch GM, Chernoff J (1997) Human p21-activated kinase (Pak1) regulates actin organization in mammalian cells. *Curr Biol* **7:** 202-210

Senee V, Vattem KM, Delepine M, Rainbow LA, Haton C, Lecoq A, Shaw NJ, Robert JJ, Rooman R, Diatloff-Zito C, Michaud JL, Bin-Abbas B, Taha D, Zabel B, Franceschini P, Topaloglu AK, Lathrop GM, Barrett TG, Nicolino M, Wek RC, Julier C (2004) Wolcott-Rallison Syndrome: clinical, genetic, and functional study of EIF2AK3 mutations and suggestion of genetic heterogeneity. *Diabetes* **53:** 1876-1883

Sevier CS, Kaiser CA (2008) Ero1 and redox homeostasis in the endoplasmic reticulum. *Biochim Biophys Acta* **1783:** 549-556

Shaffer AL, Shapiro-Shelef M, Iwakoshi NN, Lee AH, Qian SB, Zhao H, Yu X, Yang L, Tan BK, Rosenwald A, Hurt EM, Petroulakis E, Sonenberg N, Yewdell JW, Calame K, Glimcher LH, Staudt LM (2004) XBP1, downstream of Blimp-1, expands the secretory apparatus and other organelles, and increases protein synthesis in plasma cell differentiation. *Immunity* **21:** 81-93

Shamu CE, Walter P (1996) Oligomerization and phosphorylation of the Ire1p kinase during intracellular signaling from the endoplasmic reticulum to the nucleus. *EMBO J* **15:** 3028-3039

Shen J, Chen X, Hendershot L, Prywes R (2002) ER stress regulation of ATF6 localization by dissociation of BiP/GRP78 binding and unmasking of Golgi localization signals. *Dev Cell* **3:** 99-111

Shi Y, Vattem KM, Sood R, An J, Liang J, Stramm L, Wek RC (1998) Identification and characterization of pancreatic eukaryotic initiation factor 2 alpha-subunit kinase, PEK, involved in translational control. *Mol Cell Biol* **18:** 7499-7509

Shields BM, Hicks S, Shepherd MH, Colclough K, Hattersley AT, Ellard S (2010) Maturity-onset diabetes of the young (MODY): how many cases are we missing? *Diabetologia* **53:** 2504-2508

Shigihara N, Fukunaka A, Hara A, Komiya K, Honda A, Uchida T, Abe H, Toyofuku Y, Tamaki M, Ogihara T, Miyatsuka T, Hiddinga HJ, Sakagashira S, Koike M, Uchiyama Y, Yoshimori T, Eberhardt NL, Fujitani Y, Watada H (2014) Human IAPP-induced pancreatic beta cell toxicity and its regulation by autophagy. *J Clin Invest* **124:** 3634-3644

Shin S, Buel GR, Wolgamott L, Plas DR, Asara JM, Blenis J, Yoon SO (2015) ERK2 Mediates Metabolic Stress Response to Regulate Cell Fate. *Mol Cell*

Sidrauski C, Walter P (1997) The transmembrane kinase Ire1p is a site-specific endonuclease that initiates mRNA splicing in the unfolded protein response. *Cell* **90:** 1031-1039

Skolnik EY, Lee CH, Batzer A, Vicentini LM, Zhou M, Daly R, Myers MJ, Jr., Backer JM, Ullrich A, White MF, et al. (1993) The SH2/SH3 domain-containing protein GRB2 interacts with tyrosine-phosphorylated IRS1 and Shc: implications for insulin control of ras signalling. *EMBO J* **12:** 1929-1936

Sollner T, Whiteheart SW, Brunner M, Erdjument-Bromage H, Geromanos S, Tempst P, Rothman JE (1993) SNAP receptors implicated in vesicle targeting and fusion. *Nature* **362:** 318-324

Song J, Wu L, Chen Z, Kohanski RA, Pick L (2003) Axons guided by insulin receptor in Drosophila visual system. *Science* **300:** 502-505

Songyang Z, Shoelson SE, Chaudhuri M, Gish G, Pawson T, Haser WG, King F, Roberts T, Ratnofsky S, Lechleider RJ, et al. (1993) SH2 domains recognize specific phosphopeptide sequences. *Cell* **72:** 767-778

Sood R, Porter AC, Ma K, Quilliam LA, Wek RC (2000) Pancreatic eukaryotic initiation factor-2alpha kinase (PEK) homologues in humans, Drosophila melanogaster and Caenorhabditis elegans that mediate translational control in response to endoplasmic reticulum stress. *Biochem J* **346 Pt 2:** 281-293

Sou SN, Ilieva KM, Polizzi KM (2012) Binding of human BiP to the ER stress transducers IRE1 and PERK requires ATP. *Biochem Biophys Res Commun* **420:** 473-478

Stein DT, Esser V, Stevenson BE, Lane KE, Whiteside JH, Daniels MB, Chen S, McGarry JD (1996) Essentiality of circulating fatty acids for glucose-stimulated insulin secretion in the fasted rat. *J Clin Invest* **97:** 2728-2735

Stein E, Huynh-Do U, Lane AA, Cerretti DP, Daniel TO (1998) Nck recruitment to Eph receptor, EphB1/ELK, couples ligand activation to c-Jun kinase. *J Biol Chem* **273:** 1303-1308

Steiner DF, Oyer PE (1967) The biosynthesis of insulin and a probable precursor of insulin by a human islet cell adenoma. *Proc Natl Acad Sci U S A* **57:** 473-480

Steiner DF, Philipson LH (2000) Insulin Biosynthesis, Secretion, Structure, and Structure-Activity Relationships. In *Endotext*, De Groot LJ, Beck-Peccoz P, Chrousos G, Dungan K, Grossman A, Hershman JM, Koch C, McLachlan R, New M, Rebar R, Singer F, Vinik A, Weickert MO (eds). South Dartmouth (MA)

Steiner DF, Park SY, Stoy J, Philipson LH, Bell GI (2009) A brief perspective on insulin production. *Diabetes Obes Metab* **11 Suppl 4:** 189-196

Stoy J, Edghill EL, Flanagan SE, Ye H, Paz VP, Pluzhnikov A, Below JE, Hayes MG, Cox NJ, Lipkind GM, Lipton RB, Greeley SA, Patch AM, Ellard S, Steiner DF, Hattersley AT, Philipson LH, Bell GI, Neonatal Diabetes International Collaborative G (2007) Insulin gene mutations as a cause of permanent neonatal diabetes. *Proc Natl Acad Sci U S A* **104:** 15040-15044

Straub SG, Sharp GW (2002) Glucose-stimulated signaling pathways in biphasic insulin secretion. *Diabetes Metab Res Rev* **18:** 451-463

Su Q, Wang S, Baltzis D, Qu LK, Wong AH, Koromilas AE (2006) Tyrosine phosphorylation acts as a molecular switch to full-scale activation of the eIF2alpha RNA-dependent protein kinase. *Proc Natl Acad Sci U S A* **103:** 63-68

Su Q, Wang S, Gao HQ, Kazemi S, Harding HP, Ron D, Koromilas AE (2008) Modulation of the eukaryotic initiation factor 2 alpha-subunit kinase PERK by tyrosine phosphorylation. *J Biol Chem* **283:** 469-475

Su Z, Xu P, Ni F (2004) Single phosphorylation of Tyr304 in the cytoplasmic tail of ephrin B2 confers high-affinity and bifunctional binding to both the SH2 domain of Grb4 and the PDZ domain of the PDZ-RGS3 protein. *Eur J Biochem* **271:** 1725-1736

Suda T, Takahashi T, Golstein P, Nagata S (1993) Molecular cloning and expression of the Fas ligand, a novel member of the tumor necrosis factor family. *Cell* **75:** 1169-1178

Sudhakar A, Ramachandran A, Ghosh S, Hasnain SE, Kaufman RJ, Ramaiah KV (2000) Phosphorylation of serine 51 in initiation factor 2 alpha (eIF2 alpha) promotes complex formation between eIF2 alpha(P) and eIF2B and causes inhibition in the guanine nucleotide exchange activity of eIF2B. *Biochemistry* **39:** 12929-12938

Sudhof TC, Rothman JE (2009) Membrane fusion: grappling with SNARE and SM proteins. *Science* **323:** 474-477

Sun XJ, Rothenberg P, Kahn CR, Backer JM, Araki E, Wilden PA, Cahill DA, Goldstein BJ, White MF (1991) Structure of the insulin receptor substrate IRS-1 defines a unique signal transduction protein. *Nature* **352:** 73-77

Sun XJ, Wang LM, Zhang Y, Yenush L, Myers MG, Jr., Glasheen E, Lane WS, Pierce JH, White MF (1995) Role of IRS-2 in insulin and cytokine signalling. *Nature* **377:** 173-177

Szegezdi E, Logue SE, Gorman AM, Samali A (2006) Mediators of endoplasmic reticulum stress-induced apoptosis. *EMBO Rep* **7:** 880-885

Tabas I, Ron D (2011) Integrating the mechanisms of apoptosis induced by endoplasmic reticulum stress. *Nat Cell Biol* **13:** 184-190

Takeuchi K, Yang H, Ng E, Park SY, Sun ZY, Reinherz EL, Wagner G (2008) Structural and functional evidence that Nck interaction with CD3epsilon regulates T-cell receptor activity. *J Mol Biol* **380:** 704-716

Takeuchi K, Sun ZY, Park S, Wagner G (2010) Autoinhibitory interaction in the multidomain adaptor protein Nck: possible roles in improving specificity and functional diversity. *Biochemistry* **49:** 5634-5641

Tamemoto H, Kadowaki T, Tobe K, Yagi T, Sakura H, Hayakawa T, Terauchi Y, Ueki K, Kaburagi Y, Satoh S, et al. (1994) Insulin resistance and growth retardation in mice lacking insulin receptor substrate-1. *Nature* **372:** 182-186

Tanaka E, Sabry J (1995) Making the connection: cytoskeletal rearrangements during growth cone guidance. *Cell* **83:** 171-176

Tanaka M, Lu W, Gupta R, Mayer BJ (1997) Expression of mutated Nck SH2/SH3 adaptor respecifies mesodermal cell fate in Xenopus laevis development. *Proc Natl Acad Sci U S A* **94:** 4493-4498

Tanaka N, Meineke B, Shuman S (2011) RtcB, a novel RNA ligase, can catalyze tRNA splicing and HAC1 mRNA splicing in vivo. *J Biol Chem* **286:** 30253-30257

Tanaka Y, Tran PO, Harmon J, Robertson RP (2002) A role for glutathione peroxidase in protecting pancreatic beta cells against oxidative stress in a model of glucose toxicity. *Proc Natl Acad Sci U S A* **99:** 12363-12368

Tang JG, Wang CC, Tsou CL (1988) Formation of native insulin from the scrambled molecule by protein disulphide-isomerase. *Biochem J* **255:** 451-455

Taniguchi CM, Emanuelli B, Kahn CR (2006) Critical nodes in signalling pathways: insights into insulin action. *Nat Rev Mol Cell Biol* **7:** 85-96

Teodoro T, Odisho T, Sidorova E, Volchuk A (2012) Pancreatic beta-cells depend on basal expression of active ATF6alpha-p50 for cell survival even under nonstress conditions. *Am J Physiol Cell Physiol* **302:** C992-1003

Thorens B (1992) Molecular and cellular physiology of GLUT-2, a high-Km facilitated diffusion glucose transporter. *Int Rev Cytol* **137:** 209-238

Thurmond DC, Gonelle-Gispert C, Furukawa M, Halban PA, Pessin JE (2003) Glucose-stimulated insulin secretion is coupled to the interaction of actin with the t-SNARE (target membrane soluble

N-ethylmaleimide-sensitive factor attachment protein receptor protein) complex. *Mol Endocrinol* **17:** 732-742

Tiganis T, Bennett AM (2007) Protein tyrosine phosphatase function: the substrate perspective. *Biochem J* **402:** 1-15

Tirasophon W, Welihinda AA, Kaufman RJ (1998) A stress response pathway from the endoplasmic reticulum to the nucleus requires a novel bifunctional protein kinase/endoribonuclease (Ire1p) in mammalian cells. *Genes Dev* **12:** 1812-1824

Tomasevic N, Jia Z, Russell A, Fujii T, Hartman JJ, Clancy S, Wang M, Beraud C, Wood KW, Sakowicz R (2007) Differential regulation of WASP and N-WASP by Cdc42, Rac1, Nck, and PI(4,5)P2. *Biochemistry* **46:** 3494-3502

Tsai B, Ye Y, Rapoport TA (2002) Retro-translocation of proteins from the endoplasmic reticulum into the cytosol. *Nat Rev Mol Cell Biol* **3:** 246-255

Tschopp O, Yang ZZ, Brodbeck D, Dummler BA, Hemmings-Mieszczak M, Watanabe T, Michaelis T, Frahm J, Hemmings BA (2005) Essential role of protein kinase B gamma (PKB gamma/Akt3) in postnatal brain development but not in glucose homeostasis. *Development* **132:** 2943-2954

Tsukumo Y, Tsukahara S, Furuno A, Iemura S, Natsume T, Tomida A (2014) TBL2 is a novel PERK-binding protein that modulates stress-signaling and cell survival during endoplasmic reticulum stress. *PLoS One* **9:** e112761

Tsuruzoe K, Emkey R, Kriauciunas KM, Ueki K, Kahn CR (2001) Insulin receptor substrate 3 (IRS-3) and IRS-4 impair IRS-1- and IRS-2-mediated signaling. *Mol Cell Biol* **21:** 26-38

Tu BP, Weissman JS (2004) Oxidative protein folding in eukaryotes: mechanisms and consequences. *J Cell Biol* **164:** 341-346

Tu Y, Li F, Wu C (1998) Nck-2, a novel Src homology2/3-containing adaptor protein that interacts with the LIM-only protein PINCH and components of growth factor receptor kinase-signaling pathways. *Mol Biol Cell* **9:** 3367-3382

Tu Y, Liang L, Frank SJ, Wu C (2001) Src homology 3 domain-dependent interaction of Nck-2 with insulin receptor substrate-1. *Biochem J* **354:** 315-322

Tuttle RL, Gill NS, Pugh W, Lee JP, Koeberlein B, Furth EE, Polonsky KS, Naji A, Birnbaum MJ (2001) Regulation of pancreatic beta-cell growth and survival by the serine/threonine protein kinase Akt1/PKBalpha. *Nat Med* **7:** 1133-1137

Tyagi R, Shahani N, Gorgen L, Ferretti M, Pryor W, Chen PY, Swarnkar S, Worley PF, Karbstein K, Snyder SH, Subramaniam S (2015) Rheb Inhibits Protein Synthesis by Activating the PERK-eIF2alpha Signaling Cascade. *Cell Rep*

Unger RH, Grundy S (1985) Hyperglycaemia as an inducer as well as a consequence of impaired islet cell function and insulin resistance: implications for the management of diabetes. *Diabetologia* **28:** 119-121

Unger RH (1995) Lipotoxicity in the pathogenesis of obesity-dependent NIDDM. Genetic and clinical implications. *Diabetes* **44:** 863-870

Urano F, Wang X, Bertolotti A, Zhang Y, Chung P, Harding HP, Ron D (2000) Coupling of stress in the ER to activation of JNK protein kinases by transmembrane protein kinase IRE1. *Science* **287:** 664-666

Uruno A, Furusawa Y, Yagishita Y, Fukutomi T, Muramatsu H, Negishi T, Sugawara A, Kensler TW, Yamamoto M (2013) The Keap1-Nrf2 system prevents onset of diabetes mellitus. *Mol Cell Biol* **33:** 2996-3010

Usui M, Yamaguchi S, Tanji Y, Tominaga R, Ishigaki Y, Fukumoto M, Katagiri H, Mori K, Oka Y, Ishihara H (2012) Atf6alpha-null mice are glucose intolerant due to pancreatic beta-cell failure on a high-fat diet but partially resistant to diet-induced insulin resistance. *Metabolism: clinical and experimental* **61:** 1118-1128

van Huizen R, Martindale JL, Gorospe M, Holbrook NJ (2003) P58IPK, a novel endoplasmic reticulum stress-inducible protein and potential negative regulator of eIF2alpha signaling. *J Biol Chem* **278:** 15558-15564

Van Lommel L, Janssens K, Quintens R, Tsukamoto K, Vander Mierde D, Lemaire K, Denef C, Jonas JC, Martens G, Pipeleers D, Schuit FC (2006) Probe-independent and direct quantification of insulin mRNA and growth hormone mRNA in enriched cell preparations. *Diabetes* **55:** 3214-3220

Vattem KM, Wek RC (2004) Reinitiation involving upstream ORFs regulates ATF4 mRNA translation in mammalian cells. *Proc Natl Acad Sci U S A* **101:** 11269-11274

Vaxillaire M, Froguel P (2008) Monogenic diabetes in the young, pharmacogenetics and relevance to multifactorial forms of type 2 diabetes. *Endocr Rev* **29:** 254-264

Vaxillaire M, Veslot J, Dina C, Proenca C, Cauchi S, Charpentier G, Tichet J, Fumeron F, Marre M, Meyre D, Balkau B, Froguel P, Group DS (2008) Impact of common type 2 diabetes risk polymorphisms in the DESIR prospective study. *Diabetes* **57:** 244-254

Vaxillaire M, Bonnefond A, Froguel P (2012) The lessons of early-onset monogenic diabetes for the understanding of diabetes pathogenesis. *Best Pract Res Clin Endocrinol Metab* **26:** 171-187

Verma R, Wharram B, Kovari I, Kunkel R, Nihalani D, Wary KK, Wiggins RC, Killen P, Holzman LB (2003) Fyn binds to and phosphorylates the kidney slit diaphragm component Nephrin. *J Biol Chem* **278:** 20716-20723

Verma R, Kovari I, Soofi A, Nihalani D, Patrie K, Holzman LB (2006) Nephrin ectodomain engagement results in Src kinase activation, nephrin phosphorylation, Nck recruitment, and actin polymerization. *J Clin Invest* **116:** 1346-1359

Voight BF, Scott LJ, Steinthorsdottir V, Morris AP, Dina C, Welch RP, Zeggini E, Huth C, Aulchenko YS, Thorleifsson G, McCulloch LJ, Ferreira T, Grallert H, Amin N, Wu G, Willer CJ, Raychaudhuri S, McCarroll SA, Langenberg C, Hofmann OM, Dupuis J, Qi L, Segre AV, van Hoek M, Navarro P, Ardlie K, Balkau B, Benediktsson R, Bennett AJ, Blagieva R, Boerwinkle E, Bonnycastle LL, Bengtsson Bostrom K, Bravenboer B, Bumpstead S, Burtt NP, Charpentier G, Chines PS, Cornelis M, Couper DJ, Crawford G, Doney AS, Elliott KS, Elliott AL, Erdos MR, Fox CS, Franklin CS, Ganser M, Gieger C, Grarup N, Green T, Griffin S, Groves CJ, Guiducci C, Hadjadj S, Hassanali N, Herder C, Isomaa B, Jackson AU, Johnson PR, Jorgensen T, Kao WH, Klopp N, Kong A, Kraft P, Kuusisto J, Lauritzen T, Li M, Lieverse A, Lindgren CM, Lyssenko V, Marre M, Meitinger T, Midthjell K, Morken MA, Narisu N, Nilsson P, Owen KR, Payne F, Perry JR, Petersen AK, Platou C, Proenca C, Prokopenko I, Rathmann W, Rayner NW, Robertson NR, Rocheleau G, Roden M, Sampson MJ, Saxena R, Shields BM, Shrader P, Sigurdsson G, Sparso T, Strassburger K, Stringham HM, Sun Q, Swift AJ, Thorand B, Tichet J, Tuomi T, van Dam RM, van Haeften TW, van Herpt T, van Vliet-Ostaptchouk JV, Walters GB, Weedon MN, Wijmenga C, Witteman J, Bergman RN, Cauchi S, Collins FS, Gloyn AL, Gyllensten U, Hansen T, Hide WA, Hitman GA, Hofman A, Hunter DJ, Hveem K, Laakso M, Mohlke KL, Morris AD, Palmer CN, Pramstaller PP, Rudan I, Sijbrands E, Stein LD, Tuomilehto J, Uitterlinden A, Walker M, Wareham NJ, Watanabe RM, Abecasis GR, Boehm BO, Campbell H, Daly MJ, Hattersley AT, Hu FB, Meigs JB, Pankow JS, Pedersen O, Wichmann HE, Barroso I, Florez JC, Frayling TM, Groop L, Sladek R, Thorsteinsdottir U, Wilson JF, Illig T, Froguel P, van Duijn CM, Stefansson

K, Altshuler D, Boehnke M, McCarthy MI, investigators M, Consortium G (2010) Twelve type 2 diabetes susceptibility loci identified through large-scale association analysis. *Nat Genet* **42:** 579-589

Voisin L, Larose L, Meloche S (1999) Angiotensin II stimulates serine phosphorylation of the adaptor protein Nck: physical association with the serine/threonine kinases Pak1 and casein kinase I. *Biochem J* **341 (Pt 1):** 217-223

Vorobieva N, Protopopov A, Protopopova M, Kashuba V, Allikmets RL, Modi W, Zabarovsky ER, Klein G, Kisselev L, Graphodatsky A (1995) Localization of human ARF2 and NCK genes and 13 other NotI-linking clones to chromosome 3 by fluorescence in situ hybridization. *Cytogenet Cell Genet* **68:** 91-94

Wahren J, Johansson BL, Wallberg-Henriksson H (1994) Does C-peptide have a physiological role? *Diabetologia* **37 Suppl 2:** S99-107

Wang J, Takeuchi T, Tanaka S, Kubo SK, Kayo T, Lu D, Takata K, Koizumi A, Izumi T (1999) A mutation in the insulin 2 gene induces diabetes with severe pancreatic beta-cell dysfunction in the Mody mouse. *J Clin Invest* **103:** 27-37

Wang L, Chen Y, Sternberg P, Cai J (2008) Essential roles of the PI3 kinase/Akt pathway in regulating Nrf2-dependent antioxidant functions in the RPE. *Invest Ophthalmol Vis Sci* **49:** 1671-1678

Wang X, Proud CG (2006) The mTOR pathway in the control of protein synthesis. *Physiology (Bethesda)* **21:** 362-369

Wang XZ, Harding HP, Zhang Y, Jolicoeur EM, Kuroda M, Ron D (1998) Cloning of mammalian Ire1 reveals diversity in the ER stress responses. *EMBO J* **17:** 5708-5717

Wang Z, Oh E, Thurmond DC (2007) Glucose-stimulated Cdc42 signaling is essential for the second phase of insulin secretion. *J Biol Chem* **282:** 9536-9546

Wang Z, Thurmond DC (2009) Mechanisms of biphasic insulin-granule exocytosis - roles of the cytoskeleton, small GTPases and SNARE proteins. *J Cell Sci* **122:** 893-903

Wang Z, Oh E, Clapp DW, Chernoff J, Thurmond DC (2011) Inhibition or ablation of p21-activated kinase (PAK1) disrupts glucose homeostatic mechanisms in vivo. *J Biol Chem* **286:** 41359-41367

Weir GC, Bonner-Weir S (1990) Islets of Langerhans: the puzzle of intraislet interactions and their relevance to diabetes. *J Clin Invest* **85:** 983-987

Welch MD, Mullins RD (2002) Cellular control of actin nucleation. *Annu Rev Cell Dev Biol* **18:** 247-288

Welihinda AA, Kaufman RJ (1996) The unfolded protein response pathway in Saccharomyces cerevisiae. Oligomerization and trans-phosphorylation of Ire1p (Ern1p) are required for kinase activation. *J Biol Chem* **271:** 18181-18187

Wellbrock C, Karasarides M, Marais R (2004) The RAF proteins take centre stage. *Nat Rev Mol Cell Biol* **5:** 875-885

Wenzel J, Sanzenbacher R, Ghadimi M, Lewitzky M, Zhou Q, Kaplan DR, Kabelitz D, Feller SM, Janssen O (2001) Multiple interactions of the cytosolic polyproline region of the CD95 ligand: hints for the reverse signal transduction capacity of a death factor. *FEBS Lett* **509:** 255-262

Weston CR, Davis RJ (2007) The JNK signal transduction pathway. *Curr Opin Cell Biol* **19:** 142-149

White MF (1998) The IRS-signalling system: a network of docking proteins that mediate insulin action. *Mol Cell Biochem* **182:** 3-11

Whyte JR, Munro S (2002) Vesicle tethering complexes in membrane traffic. *J Cell Sci* **115:** 2627-2637

Wierup N, Svensson H, Mulder H, Sundler F (2002) The ghrelin cell: a novel developmentally regulated islet cell in the human pancreas. *Regul Pept* **107:** 63-69

Winckler W, Weedon MN, Graham RR, McCarroll SA, Purcell S, Almgren P, Tuomi T, Gaudet D, Bostrom KB, Walker M, Hitman G, Hattersley AT, McCarthy MI, Ardlie KG, Hirschhorn JN, Daly MJ, Frayling TM, Groop L, Altshuler D (2007) Evaluation of common variants in the six known maturity-onset diabetes of the young (MODY) genes for association with type 2 diabetes. *Diabetes* **56:** 685-693

Winnay JN, Boucher J, Mori MA, Ueki K, Kahn CR (2010) A regulatory subunit of phosphoinositide 3-kinase increases the nuclear accumulation of X-box-binding protein-1 to modulate the unfolded protein response. *Nat Med* **16:** 438-445

Wiser O, Trus M, Hernandez A, Renstrom E, Barg S, Rorsman P, Atlas D (1999) The voltage sensitive Lc-type Ca2+ channel is functionally coupled to the exocytotic machinery. *Proc Natl Acad Sci U S A* **96:** 248-253

Withers DJ, Gutierrez JS, Towery H, Burks DJ, Ren JM, Previs S, Zhang Y, Bernal D, Pons S, Shulman GI, Bonner-Weir S, White MF (1998) Disruption of IRS-2 causes type 2 diabetes in mice. *Nature* **391:** 900-904

Wojtaszewski JF, Richter EA (2006) Effects of acute exercise and training on insulin action and sensitivity: focus on molecular mechanisms in muscle. *Essays Biochem* **42:** 31-46

Wolcott CD, Rallison ML (1972) Infancy-onset diabetes mellitus and multiple epiphyseal dysplasia. *J Pediatr* **80:** 292-297

Wright J, Birk J, Haataja L, Liu M, Ramming T, Weiss MA, Appenzeller-Herzog C, Arvan P (2013) Endoplasmic reticulum oxidoreductin-1alpha (Ero1alpha) improves folding and secretion of mutant proinsulin and limits mutant proinsulin-induced endoplasmic reticulum stress. *J Biol Chem* **288:** 31010-31018

Wu CL, Buszard B, Teng CH, Chen WL, Warr CG, Tiganis T, Meng TC (2011) Dock/Nck facilitates PTP61F/PTP1B regulation of insulin signalling. *Biochem J* **439:** 151-159

Wunderlich L, Farago A, Downward J, Buday L (1999) Association of Nck with tyrosine-phosphorylated SLP-76 in activated T lymphocytes. *Eur J Immunol* **29:** 1068-1075

Xi Y, Liu S, Bettaieb A, Matsuo K, Matsuo I, Hosein E, Chahed S, Wiede F, Zhang S, Zhang ZY, Kulkarni RN, Tiganis T, Haj FG (2015) Pancreatic T cell protein-tyrosine phosphatase deficiency affects beta cell function in mice. *Diabetologia* **58:** 122-131

Xie L, Zhu D, Kang Y, Liang T, He Y, Gaisano HY (2013) Exocyst sec5 regulates exocytosis of newcomer insulin granules underlying biphasic insulin secretion. *PLoS One* **8:** e67561

Xie Z, Klionsky DJ (2007) Autophagosome formation: core machinery and adaptations. *Nat Cell Biol* **9:** 1102-1109

Yagishita Y, Fukutomi T, Sugawara A, Kawamura H, Takahashi T, Pi J, Uruno A, Yamamoto M (2014) Nrf2 protects pancreatic beta-cells from oxidative and nitrosative stress in diabetic model mice. *Diabetes* **63:** 605-618

Yamamoto K, Sato T, Matsui T, Sato M, Okada T, Yoshida H, Harada A, Mori K (2007) Transcriptional induction of mammalian ER quality control proteins is mediated by single or combined action of ATF6alpha and XBP1. *Dev Cell* **13:** 365-376

Yamani L, Latreille M, Larose L (2014) Interaction of Nck1 and PERK phosphorylated at Y(5)(6)(1) negatively modulates PERK activity and PERK regulation of pancreatic beta-cell proinsulin content. *Mol Biol Cell* **25:** 702-711

Yan W, Frank CL, Korth MJ, Sopher BL, Novoa I, Ron D, Katze MG (2002a) Control of PERK eIF2alpha kinase activity by the endoplasmic reticulum stress-induced molecular chaperone P58IPK. *Proc Natl Acad Sci U S A* **99:** 15920-15925

Yan W, Gale MJ, Jr., Tan SL, Katze MG (2002b) Inactivation of the PKR protein kinase and stimulation of mRNA translation by the cellular co-chaperone P58(IPK) does not require J domain function. *Biochemistry* **41:** 4938-4945

Yang YL, Reis LF, Pavlovic J, Aguzzi A, Schafer R, Kumar A, Williams BR, Aguet M, Weissmann C (1995) Deficient signaling in mice devoid of double-stranded RNA-dependent protein kinase. *EMBO J* **14:** 6095-6106

Ye J, Rawson RB, Komuro R, Chen X, Dave UP, Prywes R, Brown MS, Goldstein JL (2000) ER stress induces cleavage of membrane-bound ATF6 by the same proteases that process SREBPs. *Mol Cell* **6:** 1355-1364

Yorimitsu T, Nair U, Yang Z, Klionsky DJ (2006) Endoplasmic reticulum stress triggers autophagy. *J Biol Chem* **281:** 30299-30304

Yoshida H, Haze K, Yanagi H, Yura T, Mori K (1998) Identification of the cis-acting endoplasmic reticulum stress response element responsible for transcriptional induction of mammalian glucose-regulated proteins. Involvement of basic leucine zipper transcription factors. *J Biol Chem* **273:** 33741-33749

Yoshida H, Okada T, Haze K, Yanagi H, Yura T, Negishi M, Mori K (2000) ATF6 activated by proteolysis binds in the presence of NF-Y (CBF) directly to the cis-acting element responsible for the mammalian unfolded protein response. *Mol Cell Biol* **20:** 6755-6767

Yoshida H, Matsui T, Yamamoto A, Okada T, Mori K (2001a) XBP1 mRNA is induced by ATF6 and spliced by IRE1 in response to ER stress to produce a highly active transcription factor. *Cell* **107:** 881-891

Yoshida H, Okada T, Haze K, Yanagi H, Yura T, Negishi M, Mori K (2001b) Endoplasmic reticulum stress-induced formation of transcription factor complex ERSF including NF-Y (CBF) and activating transcription factors 6alpha and 6beta that activates the mammalian unfolded protein response. *Mol Cell Biol* **21:** 1239-1248

Yu J, Zhang Y, McIlroy J, Rordorf-Nikolic T, Orr GA, Backer JM (1998) Regulation of the p85/p110 phosphatidylinositol 3'-kinase: stabilization and inhibition of the p110alpha catalytic subunit by the p85 regulatory subunit. *Mol Cell Biol* **18:** 1379-1387

Zeng R, Cannon JL, Abraham RT, Way M, Billadeau DD, Bubeck-Wardenberg J, Burkhardt JK (2003) SLP-76 coordinates Nck-dependent Wiskott-Aldrich syndrome protein recruitment with Vav-1/Cdc42-dependent Wiskott-Aldrich syndrome protein activation at the T cell-APC contact site. *J Immunol* **171:** 1360-1368

Zhang DD, Lo SC, Cross JV, Templeton DJ, Hannink M (2004) Keap1 is a redox-regulated substrate adaptor protein for a Cul3-dependent ubiquitin ligase complex. *Mol Cell Biol* **24:** 10941-10953

Zhang K, Wang S, Malhotra J, Hassler JR, Back SH, Wang G, Chang L, Xu W, Miao H, Leonardi R, Chen YE, Jackowski S, Kaufman RJ (2011) The unfolded protein response transducer IRE1alpha prevents ER stress-induced hepatic steatosis. *EMBO J* **30:** 1357-1375

Zhang P, McGrath B, Li S, Frank A, Zambito F, Reinert J, Gannon M, Ma K, McNaughton K, Cavener DR (2002a) The PERK eukaryotic initiation factor 2 alpha kinase is required for the development of the skeletal system, postnatal growth, and the function and viability of the pancreas. *Mol Cell Biol* **22:** 3864-3874

Zhang P, McGrath BC, Reinert J, Olsen DS, Lei L, Gill S, Wek SA, Vattem KM, Wek RC, Kimball SR, Jefferson LS, Cavener DR (2002b) The GCN2 eIF2alpha kinase is required for adaptation to amino acid deprivation in mice. *Mol Cell Biol* **22:** 6681-6688

Zhang W, Feng D, Li Y, Iida K, McGrath B, Cavener DR (2006) PERK EIF2AK3 control of pancreatic beta cell differentiation and proliferation is required for postnatal glucose homeostasis. *Cell Metab* **4:** 491-497

Zhao B, Shah P, Budanov AV, Qiang L, Ming M, Aplin A, Sims DM, He YY (2014) Sestrin2 protein positively regulates AKT enzyme signaling and survival in human squamous cell carcinoma and melanoma cells. *J Biol Chem* **289:** 35806-35814

Zhou J, Liu CY, Back SH, Clark RL, Peisach D, Xu Z, Kaufman RJ (2006) The crystal structure of human IRE1 luminal domain reveals a conserved dimerization interface required for activation of the unfolded protein response. *Proc Natl Acad Sci U S A* **103:** 14343-14348

Zhou Y, Lee J, Reno CM, Sun C, Park SW, Chung J, Lee J, Fisher SJ, White MF, Biddinger SB, Ozcan U (2011) Regulation of glucose homeostasis through a XBP-1-FoxO1 interaction. *Nat Med* **17:** 356-365

Zhu J, Attias O, Aoudjit L, Jiang R, Kawachi H, Takano T (2010) p21-activated kinases regulate actin remodeling in glomerular podocytes. *Am J Physiol Renal Physiol* **298:** F951-961

Zimmermann K, Baldinger J, Mayerhofer B, Atanasov AG, Dirsch VM, Heiss EH (2015) Activated AMPK boosts the Nrf2/HO-1 signaling axis-A role for the unfolded protein response. *Free Radic Biol Med*

Zimmet P, Alberti KG, Shaw J (2001) Global and societal implications of the diabetes epidemic. *Nature* **414:** 782-787

Zipper LM, Mulcahy RT (2002) The Keap1 BTB/POZ dimerization function is required to sequester Nrf2 in cytoplasm. *J Biol Chem* **277:** 36544-36552

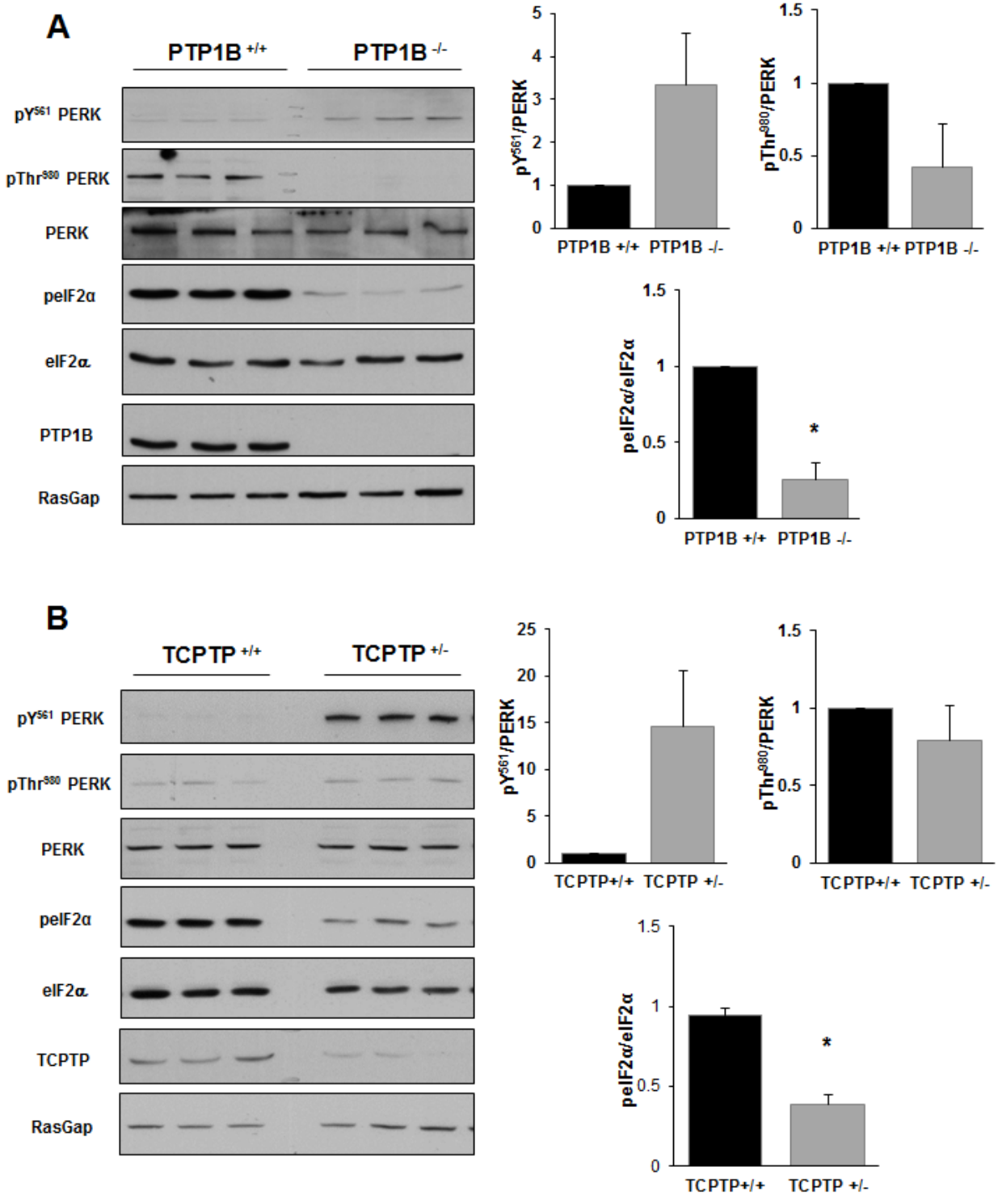
Zito E, Chin KT, Blais J, Harding HP, Ron D (2010) ERO1-beta, a pancreas-specific disulfide oxidase, promotes insulin biogenesis and glucose homeostasis. *J Cell Biol* **188:** 821-832

Zou W, Chen C, Zhong Y, An J, Zhang X, Yu Y, Yu Z, Fu J (2013) PI3K/Akt pathway mediates Nrf2/ARE activation in human L02 hepatocytes exposed to low-concentration HBCDs. *Environ Sci Technol* **47:** 12434-12440

Appendix 1

pY^{561} PERK levels are increased in protein tyrosine phosphatase 1B (PTP1B)-deficient MEFs, and T cell protein tyrosine phosphatase (TCPTP) heterozygous MEFs

Figure appendix 1. Levels of pY^{561} PERK are modulated by the non-receptor protein tyrosine phosphatases PTP1B and TC-PTP. Total cell lysates from indicated MEF genotypes were analyzed by immunoblotting using indicated antibodies. Images and bar charts represent three independent experiments. (A) Levels of pY^{561} PERK in *PTP1B*$^{+/+}$ and *PTP1B* $^{-/-}$ mouse embryonic fibroblasts. (B) Levels of pY^{561} PERK in *TCPTP*$^{+/+}$ and *TCPTP*$^{+/-}$ mouse embryonic fibroblasts.


A
PTP1B +/+
PTP1B -/-
pY561 PERK
pThr980 PERK
PERK
peIF2α
eIF2α
PTP1B
RasGap
pY561/PERK
PTP1B +/+ PTP1B -/-
pThr980/PERK
PTP1B +/+ PTP1B -/-
peIF2α/eIF2α
PTP1B +/+ PTP1B -/-
B
TCPTP +/+
TCPTP +/-
pY561 PERK
pThr980 PERK
PERK
peIF2α
eIF2α
TCPTP
RasGap
pY561/PERK
TCPTP+/+ TCPTP +/-
pThr980/PERK
TCPTP+/+ TCPTP +/-
peIF2α/eIF2α
TCPTP+/+ TCPTP +/-