Regulation of Pancreatic β-cell Life and Death in the Context of Type 2 Diabetes: Study of the Potential Implication of the Orphan Nuclear Receptor NR4A3/Nor1 and the NZF Transcription Factor ST18.

by

Anne-Françoise Close

A thesis submitted in partial fulfillment of the requirements for the degree of

Doctor of Philosophy

in

Nutrition and Metabolism

Department of Agricultural, Food & Nutritional Science
University of Alberta

Abstract

Type 2 diabetes is characterized by a progressive deterioration of functional β-cell mass. The mechanisms implicate the production of pro-inflammatory cytokines, elevated circulating fatty acids, disrupted mitochondrial function and dynamics as well as the subsequent up-regulation of β-cell apoptosis. To this day, the specific molecular pathways that govern β-cell life and death remain incompletely characterized. Identifying the factors that mediate the regulation of β-cell apoptosis and β-cell mass could lead to the development of new therapeutic approaches.

Nor1 is a member of the Nr4a subfamily of orphan nuclear receptors. The 2 other members of the family are Nur77 and Nurr1. Nor1 expression is modified in a tissue-specific manner in animal models of type 2 diabetes. Moreover, evidence suggests that Nor1 could trigger apoptosis in immune cells. Moreover, the unique DNA binding profile of Nor1 suggest specific biological functions compared to Nur77 and Nurr1. ST18 is a member of the NZF family of transcription factors. ST18 has been shown to be a tumor suppressor in breast cancer. Moreover, evidence suggests that ST18 mediates apoptosis induced by the pro-inflammatory cytokine TNFα. However, the biological roles of Nor1 and ST18 in β-cells remain largely unexplored. This prompted us to *i)* define the expression profile of *Nor1* and *ST18* in β-cells exposed to various nutrients and environmental stresses and *ii)* investigate a potential role for Nor1 and ST18 in the regulation of β-cell life and death.

We hypothesize that Nor1 and ST18 could act as pro-apoptotic agents in β-cells thereby potentially downregulating β-cell mass.

To test this hypothesis, we started by evaluating the effect of all 3 Nr4as (Nur77, Nurr1 and Nor1) on β-cell mass *in vivo*. We studied the expression of Nor1 and ST18 in β-cells exposed to

various pro-apoptotic factors such as high glucose, palmitate and pro-inflammatory cytokines. We also evaluated the effect of Nor1 and ST18 on apoptosis and proliferation in β-cells. Glucose stimulated insulin secretion was measured as well in β-cells overexpressing Nor1 or ST18. In addition, we performed a first exploration of the mechanisms of action of Nor1 in β-cells.

Nor1$^{-/-}$ mice but not Nur77$^{-/-}$ or Nurr1$^{-/-}$ animals presented elevated β-cell mass, higher β-cell proliferation, lower blood glucose, and better glucose tolerance compared to the controls. In the pancreas, Nor1 was predominantly expressed in the islets of Langerhans. In β-cells, pro-apoptotic cytokines significantly up-regulated the expression of Nor1. Interestingly, the expression of the other members of the *Nr4a* family was less affected by cytokines. Cytokines induced a swift translocation of Nor1 to the mitochondria, where it has been shown to exert pro-apoptotic actions. Overexpression of Nor1 induced beta-cell apoptosis. Conversely, silencing of *Nor1* blunted cytokine-induced apoptosis. Interestingly *Nor1* expression was significantly increased in the islets of type 2 diabetic patients. Our genomic study revealed that Nor1 did not alter the expression of canonical apoptotic genes, but rather down-regulated key genes encoded by the mitochondrial genome. This prompted us to further investigate the role of Nor1 at the mitochondria. Nor1 reduced glucose oxidation and ATP production in β-cells whereas lactate production was increased. Consistently, Nor1 also downregulated glucose stimulated insulin secretion. In addition, our data suggests that Nor1 modulates mitochondrial membrane potential. Electron microscopy images revealed that Nor1 induces mitochondrial fragmentation and increases mitophagy.

We also found that the expression and activity of ST18 was increased by palmitate and cytokines in β-cells. Furthermore, ST18 induced β-cell apoptosis and downregulated proliferation. Finally, our data suggests that ST18 blunts glucose stimulated insulin secretion.

Our results describe Nor1 as an important pro-apoptotic factor in β-cells through a mechanism potentially involving modulation of mitochondrial homeostasis. These results support our observation that Nor1 is a negative regulator of β-cell mass. In addition, our data on the role of ST18 in β-cells presents it as a mediator of palmitate and cytokines-induced apoptosis. The work presented in the present thesis therefore allowed the discovery of 2 new potential therapeutic targets in diabetes treatment.

Preface

This thesis is an original work by Anne-Françoise Close.

Chapter 3

This chapter is adapted from work re-submitted with reviews as Close A. F., Villela B., Dadheech N., Rouillard C. and Buteau J. (April 2017). The orphan nuclear receptor Nor1/Nr4a3 mediates cytokine-induced beta-cell death *in vivo* and regulates beta-cell mass in mice. *Endocrinology*.

Close A.F. was responsible for data collection, analysis, and manuscript composition. Villela B. and Dadheech N. assisted with data collection; in these instances, Close A.F. performed analyses. Rouillard C. kindly provided the knockout animals, contributed to the discussion, reviewed, and edited the manuscript. Buteau J. was the supervisory authority and was involved in concept formation and edits to the manuscript.

Chapter 4

This chapter is adapted from work submitted as Anne-Françoise Close, Claude Rouillard and Jean Buteau. (April 2017). Disruption of beta-cell mitochondrial networks by the orphan nuclear receptor Nor1/Nr4a3. *Molecular Metabolism.*

Close A.F was responsible for data collection, analysis, and manuscript composition. Rouillard C. kindly provided the Nor1-knockout animals. Buteau J. was the supervisory authority and was involved in concept formation and edits to the manuscript.

Chapter 5

This chapter is adapted from work published as Cyndi Henry, Anne-Françoise Close and Jean Buteau (2014) A critical role for the neural zinc factor ST18 in pancreatic β-cell apoptosis. *J Biol Chem* 289(12):8413-9.

C. Henry was responsible for data collection, analysis, and manuscript composition. A.F. Close was responsible for the data collection and analysis that was done on human islets in order to answer the reviewers. J. Buteau was the supervisory authority and was involved in concept formation and manuscript composition.

All Research work in this thesis received research ethics approval from the University of Alberta Animal Care and Use Committee: Health Sciences, protocol AUP00000504, and the Health Research Ethics Board, protocol Pro00036707.

For Cyanne

If you hear a voice within you saying, “you cannot paint “,
then by all means paint and that voice will be silenced.

--Vincent Van Gogh

Acknowledgements

I am extremely grateful to my supervisor, Dr. Jean Buteau, for welcoming me to his laboratory. His constant availability, his explanations, his rigor and his involvement in all the aspects of the progression of my laboratory work have enabled me to acquire high quality training. I also thank Dr. Buteau for letting me work independently which allowed me to gain an invaluable know-how.

Past and present members of the Buteau lab, thank you for your availability and the good times spent together. Your enthusiasm for lab work and life in general is communicative. Thanks to you, the atmosphere in the lab was always joyful. I am especially grateful to Wayne for his unshakable calm, his technical help and advice as well as for managing the practical aspects of lab life so well. Thanks for making everyone's life easier every day.

To the members of my supervisory committee, Dr. Catherine Chan and Dr. René Jacobs, Thank you for your priceless advice, expertise, and guidance during this process. Your input has always been constructive and invaluable. You genuinely helped making my project move forward.

I am deeply thankful to Dr. Hélène Lemieux for her expertise and precious ideas. Thank you also for giving me the opportunity to use your equipment and lab space.

Special thanks to Dr. Claude Rouillard for providing the knockout animals and editing the first manuscript. His comments greatly improved the quality of my work.

I would like to acknowledge the personal funding from Wallonie Brussels International, Alberta Innovates Health Solutions, Alberta Diabetes Institute and the department of AFNS, as well as the Canadian Institutes for Health Research for project funding.

To all the students and staff doing research on the 6th floor of the Li Ka Shing building, thank you. Sometimes, only knowing that other people are on the same boat with you can give you the courage to row some more even when your arms want to give up.

Finally, to Thomas: such strong roots no one could dream of. And to Cyanne, thank you for making everything shine so bright.

Table of contents

Abstract ii

Preface v

Chapter 3 v

Chapter 4 v

Chapter 5 v

Acknowledgements viii

Table of contents ix

List of Figures and Table xiii

List of abbreviations xvi

Chapter 1: Introduction 1

1.1 Epidemiology and diagnosis of diabetes 1

1.2 Pathophysiology of the endocrine pancreas 2

1.2.1 The endocrine pancreas 2

1.2.2 Glucose homeostasis 3

1.2.3 Insulin secretion 5

1.2.4 Pathogenesis of diabetes 7

1.2.4.1 Type 1 diabetes 8

1.2.4.2 Type 2 diabetes 8

1.3 β-cell mass 10

1.3.1 Definition 10

1.3.2 β-cell mass in diabetes 10

1.3.3 Physiopathological regulation of β-cell mass 12

1.3.3.1 β-cell mass variations through life and in response to high metabolic loads 12

1.3.3.2 Causes of β-cell mass reduction in diabetes 13

1.4 Apoptosis 15

1.4.1 Definition and pathways 15

1.4.1.1 Definition 15

1.4.1.2 Caspases 16
1.4.1.3 Apoptosis induction pathways 16
1.4.2 Techniques used to detect apoptosis 19
1.4.3 Mechanisms of apoptosis induction in diabetes 20
1.4.3.1 Pro-inflammatory cytokines 20
1.4.3.2 Mitochondrial function and dynamics 26
Mitochondrial ROS production 26
Mitochondrial morphology 27
Mitophagy 29
1.4.3.3 Lipotoxicity 31
1.5 Transcriptional regulation of β-cell mass 32
1.5.1 Pdx1 33
1.5.2 Nr4a orphan nuclear receptors 34
1.5.2.1 Structure and regulation 34
1.5.2.2 Role in glucose homeostasis 35
1.5.2.3 Role in the regulation of β-cell mass 38
Expression in response to diabetogenic factors 38
Effect on β-cell replication and apoptosis 40
Effect on mitochondrial function and dynamics 41
1.5.3 NZF transcription factors 42
Chapter 2: Research plan 44
2.1 Rationale 44
2.2 Hypothesis 45
2.3 Specific objectives 45
2.3.1 Objective 1 (Chapter 3) 45
2.3.2 Objective 2 (Chapter 4) 46
2.3.3 Objective 3 (Chapter 5) 46
Chapter 3: The orphan nuclear receptor Nor1/Nr4a3 mediates cytokine-induced β-cell death in vitro and regulates β-cell mass in mice 48
3.1 Introduction 48
3.2 Methods 50

3.3 Results 53
3.4 Discussion 63
3.5 Supplementary figure 66
3.6 References 67
3.7 Additional results 71
3.7.1 Nor1 promoter activity 71
Methods 71
Results 72
3.7.2 Nor1 expression in mice models of diabetes 73
Methods 73
Results 74
3.7.3 Insulin content 78
Methods 78
Results 79
Chapter 4: Disruption of β-cell mitochondrial networks by the orphan nuclear receptor Nor1/Nr4a3 80
4.1 Introduction 80
4.2 Material and Methods 82
4.3 Results 86
4.4 Discussion 95
4.5 References 97
4.6 Supplementary material 99
__4.6.1 Supplementary figures 99
4.6.2 Supplementary methods 102
4.7 Additional results: Respiratory flux 103
4.7.1 Methods 104
4.7.2 Results 107
Chapter 5: A critical role for the neural zinc factor ST18 in pancreatic β-cell apoptosis 109
5.1 Introduction 109
5.2 Experimental procedures 111
5.3 Results 114

5.4 Discussion 127
5.5 References 129
Chapter 6: Discussion and conclusions 131
6.1 Summary of research findings 131
Chapter 3: Evaluation of the effects of Nr4as on β-cell mass and exploration of their role and expression in β-cells. 132
Chapter 4: Mechanism of action of Nor1 133
Chapter 5: Expression profile and role of the transcription factor ST18 in β-cells. 134
6.2 Discussion 135
6.2.1 Nor1 downregulates β-cell mass 135
6.2.2 Nor1 impairs glucose stimulated insulin secretion 136
6.2.3 Nor1 as a mediator a diabetogenic stresses 137
6.2.4 Regulation of Nr4as 138
6.2.5 Mitochondrial effects of Nor1 140
6.2.6 The pro-apoptotic effects of ST18 142
6.3 Future directions 143
Nor1 143
ST18 144
6.4 Relevance of the work and conclusions 145
References 147
Annexe 1: T2D islets 158
Annexe 2: Plasmids 159
1. pCMV-Nor1-GFP 159
2. pCMV-Nor1-Flag 160

List of Figures and Table

Figure 1.1: Triple immunostaining on a human pancreatic section centered on an islet of Langerhans. 3

Figure 1.2: Effects of insulin on nutrient flow in peripheral tissues and blood. 4

Figure 1.3: Representation of the pathways of glucose stimulated insulin secretion 6

Figure 1.4: The phases of insulin secretion. 7

Figure 1.5: Schematic representation of the events leading to type 2 diabetes 9

Figure 1.6: β-cell mass in type 2 diabetes. 10

Figure 1.7: β-cell apoptosis in type 2 diabetes. 14

Figure 1.8: Morphological changes that characterize apoptosis 16

Figure 1.9: Schematic representation of the main pathways of apoptosis 17

Figure 1.10: Induction of the intrinsic pathway of apoptosis. 19

Figure 1.11: Expression of Il1β in human β-cells in pancreatic sections from a diabetic and a non-diabetic donor. 21

Figure 1.12: The number of islet macrophages is increased in type 2 diabetic patients 22

Figure 1.13: Macrophages recruitment and secretion of Il1β by β-cells in type 2 diabetes 23

Figure 1.14: Signalling mechanisms through which Il1β, IFNγ and TNFα induce apoptosis in β-cells 25

Figure 1.15: Regulation of mitochondrial fusion and fission 27

Figure 1.16: Palmitate-induced mitochondrial fragmentation in β cells 29

Figure 1.17: Mitophagy. 30

Figure 1.18: Effects of malonyl-CoA on fatty acid metabolism. 32

Figure 1.19: Schematic representation of Nr4a proteins and their DNA-binding specificities 35

Table 1: Reported biological effects of NR4A nuclear receptors in the control of glucose homeostasis. 37

Figure 1.20: Effect of 0.4 mM of palmitate on the mRNA levels of Nur77, Nurr1 and Nor1 in human islets 40

Figure 1.21: In a thymoma cell line, Il1β induces the PKCδ-mediated phosphorylation of Nor1. 41

Figure 3.1: Nor1-knockout mice display increased β-cell mass. 53

Figure 3.2: Invalidation of Nor1 improves glucose tolerance. 55

Figure 3.3: Nor1 expression in pancreatic islets. 56

Figure 3.4: Nor1 is upregulated by pro-inflammatory cytokines in INS and human beta-cells. .. 58

Figure 3.5: Nor1 plays a critical role in β-cell apoptosis. 60

Figure 3.6: Nor1 impairs glucose-stimulated insulin secretion. 61

Figure 3.7: Nor1 is upregulated in type 2 diabetic islets. 62

Suppl Fig 3.1: Relative expression of Nr4a members in β-cells and human islets. 66

Figure 3.8: Cytokines modulate Nor1 expression via an element located within the first 1000 base pairs of the Nor1 gene. 72

Figure 3.9: Evolution of the NOD mice glycaemia in mmol/L. 9 female NOD mice were received at 8 weeks old. Blood glucose was measured bi-weekly with FreeStyle Lite test strips. 75

Figure 3.10: Weights in g of C57BL/6J mice after 8 weeks of high fat diet 76

Figure 3.11: Nor1 expression in the β-cells of obese and diabetic mice. 77

Figure 3.12: Insulin expression is not modulated by Nor1. 79

Figure 4.1: Nor1 translocates to the mitochondria in response to cytokines in INS832/13-cells. 87

Figure 4.2: Nor1 impairs mitochondrial function in β-cells. 89

Figure 4.3: Nor1 induces mitochondrial fragmentation in β-cells. 91

Figure 4.4: Nor1 increases mitophagy in β-cells. 93

Figure 4.5: Nor1 does not alter mitochondrial content in β-cells. 94

Suppl Fig 4.1: RNA sequencing. 99

Suppl Fig 4.2: Nor1 does not colocalize with Bcl2 in beta-cells 100
Suppl Fig 4.3: Nor1 affects metabolic pathways in beta-cells 101
Figure 5.1: Tissue distribution of ST18. 114
Figure 5.2: ST18 expression is restricted to endocrine cells in the pancreas. 116
Figure 5.3: ST18 expression and activity are up-regulated by palmitate or cytokines in INS832/13 cells. 118
Figure 5.4: Nuclear localization of ST18 is increased in islets of diet-induced obese animals. 119
Figure 5.5: Manipulation of ST18 expression in INS cells. 121
Figure 5.6: ST18 induces β-cell apoptosis and suppresses proliferation in INS832/13 cells. 123
Figure 5.7: Pro-apoptotic action of ST18 in isolated human islet cells. 124
Figure 5.8: Nuclear localization of ST18 in β-cell apoptosis. 125
Figure 5.9: ST18 impairs insulin secretion. 126
Figure 6.1: Schematic representation of the pro-apoptotic effect of Nor1 in β-cells. 133

List of abbreviations

ADA	American Diabetes Association
AICAR	5-AminoImidazole-4-CArboxamide Ribonucleotide
AMPK	5’ AMP-activated Protein Kinase
Apaf-1	Apoptotic protease activating factor-1
ATP/ADP	Adenosine TriPhosphate/Adenosine DiPhosphate
Bcl-2	B-cell lymphoma-2
BMI	Body Mass Index
BrdU	BromodeoxyUridine
CDA	Canadian Diabetes Association
CPT1	Carnitine Palmitoyl Transferase 1
CRP	C Reactive Protein
DNA/RNA	Deoxyribonucleic Acid/Ribonucleic Acid
Drp1	Dynamin-related protein 1
ELISA	Enzyme-linked Immunosorbent Assay
EMSA	ElectroMobility Shift Assay
ERK	Extracellular signal-Regulated Kinase
ETS	Electron Transfer System
FADH2	Flavin Adenine Dinucleotide
FCCP	triFluoromethoxy Carbonyl Cyanide Phenylhydrazone
FOXO1	Forkhead BOx O1
GFP	Green Fluorescent Protein
GIP	Gastric Inhibitory Polypeptide
GK rats	Goto-Kakizaki rats

GLP-1	Glucagon-Like-Peptide 1
GLUT2/4	GLUcose Transporter 2/4
Gpx	Glutathione peroxidase
GSIS	Glucose Stimulated Insulin Secretion
HbA1c	Glycated Hemoglobin
HFD	High Fat Diet
HGH	Human Growth Hormone
H_2O_2	Hydrogen Peroxide
IAPP	Islet Amyloid PolyPeptide
IκB	Inhibitor of κB
IFNγ	Interferon γ
IGFI-II	Insulin like Growth FactorI-II
Il1β	Interleukin 1 β
IMS	mitochondrial Intermembrane Space
iNOS	inducible Nitric Oxide Synthase
ipGTT	intraperitoneal Glucose Tolerance Test
IRS-1	Insulin Receptor Substrate-1
JAK/STAT	Janus Kinase/Signal Transducers and Activators of Transcription
JNK	c-Jun N-terminal Kinase
KO	Knockout
LC3	microtubule-associated protein 1A/1B-Light Chain 3
LCSFA	Long Chain Saturated Fatty Acids
MafA	v-maf Musculoaponeurotic fibrosarcoma oncogene family, protein A
MAP/SAPKs	Mitogen and Stress-Activated Protein Kinase
Mfn	Mitofusin

MMP	Mitochondrial outer Membrane Permeability
MODY	Maturity Onset Diabetes of the Young
NAD(H)	Nicotinamide Adenine Dinucleotide
ND	Non-Diabetic
NeuroD1	Neurogenic Differentiation 1
NF-κB	Nuclear Factor κ-light-chain-enhancer of activated B cells
NO	Nitric Oxide
NOD mice	Non-Obese Diabetic
NZF	Neural specific Zinc Finger
PARP	PolyADP Ribose Polymerase
Pdx1	Pancreatic duodenal homeobox 1
PINK1	PTEN-Induced putative Kinase 1
PKC	Protein Kinase C
PTH	ParaThyroid Hormone
PTPC	Permeability Transition Pore Complex
ROS	Reactive Oxigen Species
ROX	Residual Oxigen fluX
RSK	Ribosomal S6 Kinase
RT-qPCR	quantitative Reverse Transcription Polymerase Chain Reaction
RXR	Retinoid X Receptor
SEM	Standard Error of the Mean
siRNA	small interfering RNA
SOD	SuperOxide Dismutase
SNP	Single-Nucleotide Polymorphism
ST18	Suppression of Tumorigenicity 18

T2DM/TTDM	Type 2 Diabetes Mellitus
TEM	Transmission Electron Microscopy
TG	TriGlyceride
TNF	Tumor Necrosis Factor
TUNEL	Terminal deoxynucleotidyl transferase dUDP Nick-End Labelling
WHO	World Health Organization
ZDF rat	Zucker Diabetic Fatty rat
$\Delta\psi M$	mitochondrial trans-membrane potential

Chapter 1: Introduction

1.1 Epidemiology and diagnosis of diabetes

Diabetes mellitus is a chronic metabolic disease that has a significant impact on morbidity and mortality and is increasing in prevalence. Indeed, according to the World Health Organization (WHO) 8.5% of the world's adult population had been diagnosed with diabetes [1]. This represents a 100% increase since 1980 and the elevation of diabetes prevalence is not predicted to halt by 2025 as planned by WHO. This is particularly concerning because of the heavy burden that diabetes represents for patient's health. For example, diabetic retinopathy could be accounted for 2.6% of the cases of blindness worldwide in 2010 and 12-55% of end stage renal disease is caused by diabetes [2, 3]. Moreover, diabetic adults present a 2-3 times higher rate of cardio-vascular disease compared to non-diabetic subjects [4]. Diabetes strongly increases the risk of lower limb amputation due to non-treated infected foot ulcers [5]. Finally, diabetes is predicted to become the 7th leading cause of death by 2030 [1, 6]. The main biological sign of diabetes mellitus is elevated blood glucose (hyperglycaemia), and the diagnosis is therefore based on 2 fasting glucose tests higher or equal to 126 mg/dL (7 mmol/L), a non-fasting blood glucose of 200 mg/dL or higher (11.1 mmol/L) in a symptomatic patient, a blood glucose greater than or equal to 200 mg/dL at the 120th minute of an oral glucose tolerance test or blood glycated hemoglobin (HbA1c) greater or equal to 6.5% (48 mmol/mol) [Diagnostic criteria according to the American Diabetes Association (ADA 2016) and the Canadian Diabetes Association (CDA 2013)) [6, 7].

The purpose of my PhD work was to understand elements of the pathogenesis of diabetes and identify factors that participate to the development of the disease and would therefore constitute interesting therapeutic targets. Diabetes is a disease that affects glucose homeostasis that is regulated by 2 main hormones, insulin and glucagon, secreted by pancreatic endocrine cells. I will therefore start by a brief review of the anatomy and physiology of the endocrine pancreas as well as the regulation of glucose homeostasis. I will then provide up to date evidence concerning the factors that lead to dysregulation and diabetes.

1.2 Pathophysiology of the endocrine pancreas

1.2.1 The endocrine pancreas

The pancreas is a mixed exocrine and endocrine gland. The acinar cells of the exocrine part secrete an alkaline juice rich in enzymes (proteases, ribonucleases, lipases, amylases) in the duodenum through the Wirsung canal. The endocrine cells are grouped in islets of Langerhans, distributed among the exocrine acini. These islets, which can measure 50 to 400 μm in diameter and have 500 to 5000 cells, represent 1-2% of the pancreas mass but receive 10 to 20% of the pancreatic blood flow. Although there is a strong correlation between body weight and pancreas weight, the size of the islets is similar in rodents and humans. It seems, however, that the number of islets and the proportion of large islets is greater in man [8]. The islets of Langerhans consist of endothelial cells, nerve cells and fibroblasts as well as 5 types of endocrine cells each secreting a specific hormone: 60% to 80% of insulin secreting β cells, 25% of glucagon secreting α cells, 2 to 8% of δ cells secreting somatostatin, 1 to 2% of PP cells secreting Pancreatic Polypeptide and less than 0.5% ε cells secreting ghrelin (Fig 1.1). In rodents, β-cells are found in the center of the islet, while in humans, the cell distribution is more heterogeneous [9]. The pancreatic endocrine cells secrete the hormones they produce in the portal venous system.

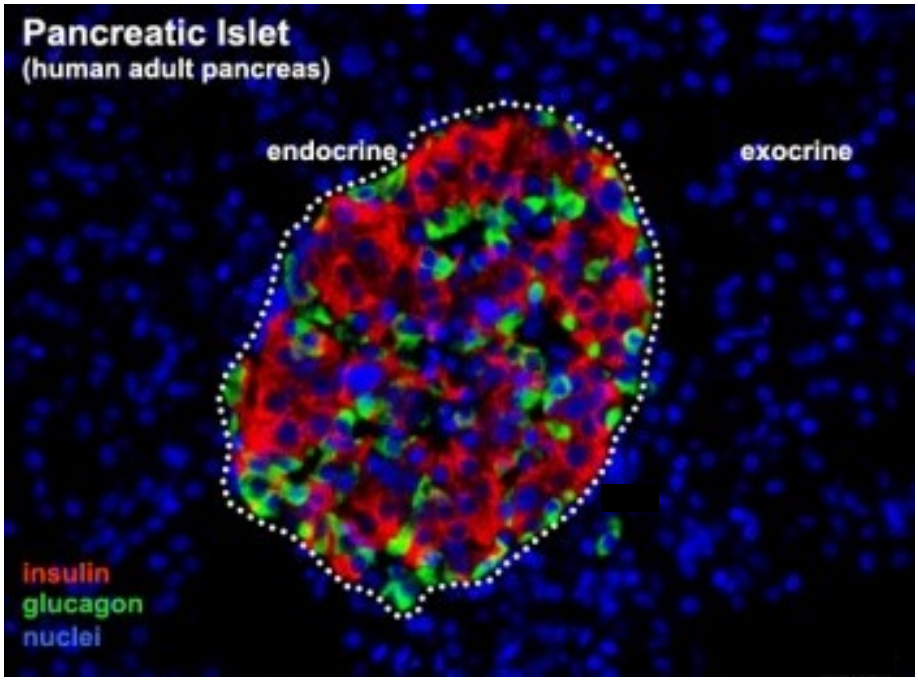


Figure 1.1: Triple immunostaining on a human pancreatic section centered on an islet of Langerhans. β-cells (insulin) appear in red and α-cells (glucagon) in green. Adapted from [10]. Used with permission.

1.2.2 Glucose homeostasis

Maintaining the plasma glucose concentration (glycaemia) between 4 and 6 mmol/L (72 and 108 mg/dL) is essential for the proper functioning of the body. Hypoglycaemia (<3 mmol/L) is harmful to organs that use glucose almost exclusively as a source of energy such as the brain, red blood cells, retina and renal tubular cells. In addition, prolonged hyperglycaemia can cause chronic complications due to non-enzymatic glycation of proteins and an accumulation of sorbitol and fructose in some tissues. An effective hormone system regulates energy homeostasis. That includes the regulation of carbohydrate, amino acid and lipid homeostasis [11]. For the purpose of this thesis, I will focus on the hormonal regulation of glucose homeostasis.

The postprandial increase in blood glucose is followed by the secretion of insulin by pancreatic β-cells. Insulin, by acting on its various target organs, exerts a hypoglycaemic and anabolic effect (Fig 1.2):

- In the liver, insulin stimulates the storage of glucose in the form of glycogen and its use through glycolysis. It inhibits glycogenolysis, ketogenesis and gluconeogenesis.
- In muscle cells, insulin receptor stimulation results in the translocation of the GLUcose Transporter-4 (GLUT4) to the plasma membrane allowing for glucose uptake. This is one of the mechanisms that allows insulin to stimulate glycogenesis in myocytes.
- In the adipose tissue, insulin increases lipogenesis and inhibits lipolysis.
- In all tissues expressing the insulin receptor, it stimulates protein synthesis, DNA synthesis and cell division and inhibits proteolysis [12].

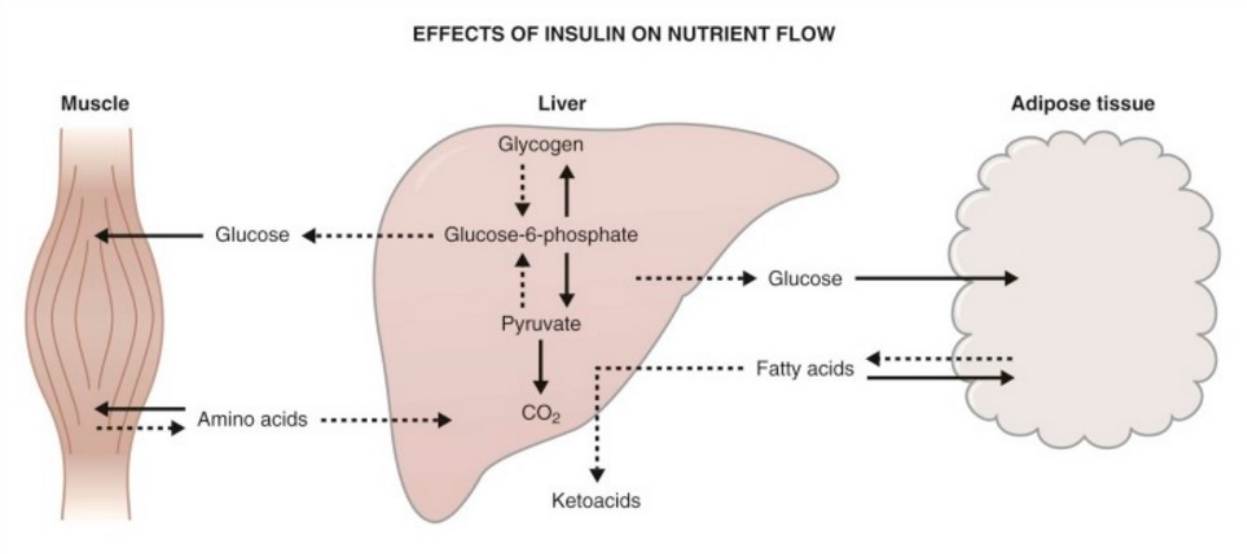


Figure 1.2: Effects of insulin on nutrient flow in peripheral tissues and blood. *Solid arrows* indicate that the step is stimulated; *dashed arrows* indicate that the step is inhibited. Figure adapted from [13]. Used with permission.

When blood glucose decreases to values below 3.7 mmol/L, counter-regulatory hormones are secreted: glucagon and catecholamines are responsible for a rapid rise in blood glucose, whereas regulation due to cortisol and Human Growth Hormone (HGH) is slower. Glucagon increases hepatic glucose production by stimulating glycogenolysis and gluconeogenesis and inhibiting glycolysis and glycogenogenesis. In the adipose tissue, glucagon stimulates lipolysis, an effect shared with catecholamines [11].

1.2.3 Insulin secretion

The stimulation of insulin secretion by glucose takes place via two pathways requiring glucose metabolism: the triggering pathway and the amplifying pathway. These two pathways are altered in animal models of type 2 diabetes [14]. The successive steps of the triggering pathway are the following (Fig 1.3):

- Transport of extracellular glucose to the cytoplasm by the GLUcose Transporter 2 (GLUT2) of low affinity (Km ~ 15 mmol/L) and of high capacity.
- Phosphorylation of glucose to glucose-6-phosphate by glucokinase, a low-affinity hexokinase (Km ~ 11 mmol/L) which is not inhibited by glucose-6-phosphate.
- Production of ATP from glucose-6-phosphate by glycolysis followed by oxidative phosphorylation in the mitochondria.
- Elevation of the cytoplasmic ATP/ADP ratio leading to the closure of ATP-sensitive potassium channels (K^+ ATP) in the plasma membrane. This leads to the depolarization of the cell membrane which causes the opening of the voltage-dependent calcium channels and an increase in intracellular calcium concentration ($[Ca^{2+}]_i$). This last event is the signal triggering the exocytosis of the insulin-containing secretion granules.

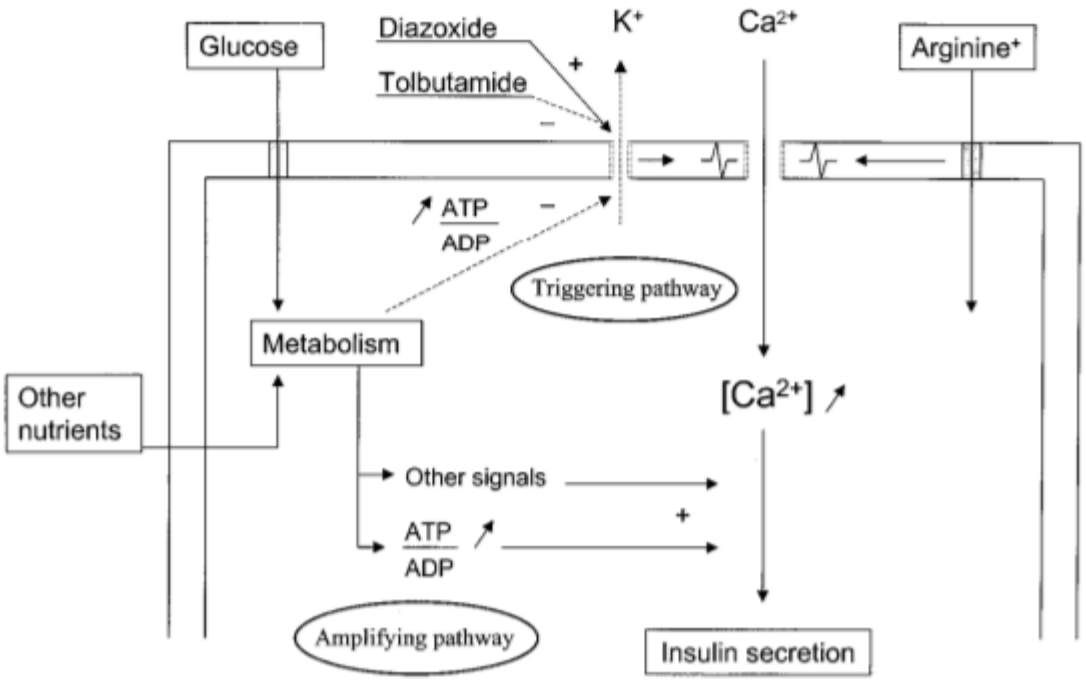


Figure 1.3: Representation of the pathways of glucose stimulated insulin secretion: triggering and amplifying pathways [14]. Used with permission.

The amplifying pathway provides sustained insulin secretion in the presence of glucose if and only if the triggering pathway is activated beforehand. The amplifying pathway enhances the secretion of insulin caused by an elevation of $[Ca^{2+}]_i$. It is independent from modifications of the opening of K^+ ATP channels [14].

Certain nutrients (amino acids, fatty acids) and other molecules (incretin hormones and parasympathetic neurotransmitters) can activate the amplifying pathway and thus increase glucose stimulated insulin secretion. At therapeutic doses, sulfonylureas such as tolbutamide cause partial closure of the K^+ ATP channels, which results in an amplification of insulin secretion in response to glucose. At higher doses, they can lead to insulin secretion independently from the extracellular concentration of glucose. In contrast, catecholamines and diazoxide, a pharmacological agent that opens K^+ ATP channels and repolarizes the membrane, inhibit insulin secretion stimulated by glucose [15]. In humans, the relationship between insulin secretion and extracellular glucose concentration follows a sigmoid curve with a secretion threshold at 3-4 mmol/L glucose and maximum stimulation at ~ 15 mmol/L glucose [15]. In rats and mice, the threshold values are 6 and 7 mmol/L glucose, while the maximum values are 20 and 30 mmol/L glucose.

In vitro, the acute stimulation of insulin secretion by a rapid increase in the extracellular glucose concentration (1 to 15 mmol/L) presents a biphasic profile (Fig 1.4). The first phase consists of a rapid and marked increase in the secretion of insulin and lasts only ~ 10 minutes, whereas in the second phase, the secretion is slower and more sustained (~ 30 minutes) until it gradually decreases (third phase) [15].

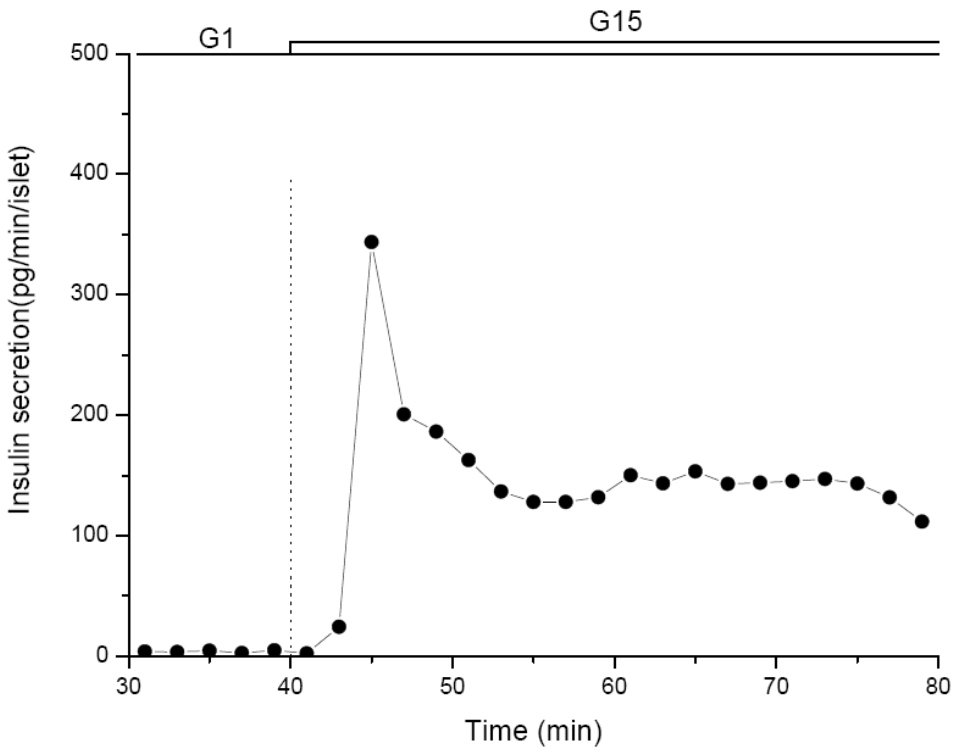


Figure 1.4: The phases of insulin secretion. Effect of a rapid increase in the extracellular concentration of glucose (from 1 to 15 mmol/L) on the insulin secretion in mouse islets cultured for 24 h in the presence of 10 mmol/L of glucose and then 30 min in the presence of 1 mmol/L glucose; Nizar Mourad, unpublished results. Used with permission.

In vivo, only the intravenous glucose perfusion makes it possible to observe a biphasic profile of secretion comparable to that described above. In animals as well as in humans, insulin is secreted into the systemic blood flow in a pulsatile manner that has been described as oscillations [16-18]. The inter-pulse time can range from 4 to 20 minutes.

1.2.4 Pathogenesis of diabetes

There are 4 classes of diabetes: type 1, type 2, gestational diabetes and other specific types including genetic defects of insulin secretion such as the Maturity Onset Diabetes of the Young (MODY 1-4) and mitochondrial DNA 3243 mutation. The two main classes of the disease are type 1 diabetes and type 2 diabetes [19].

1.2.4.1 Type 1 diabetes

Type 1 diabetes results from an absolute deficiency in insulin following the progressive autoimmune destruction of pancreatic β-cells. Treatment is therefore insulin replacement therapy either by regular injection of insulin or by β-cell or islet transplantation [20, 21]. This form of diabetes is most often characterized by a sudden onset and mainly affects people under 30 years of age whose BMI is between 20 and 25 [22, 23].

1.2.4.2 Type 2 diabetes

In the context of type 2 diabetes, the combination of genetic and environmental factors leads to a relative deficiency in insulin by a process involving insulin resistance and/or a partial defect in insulin secretion by β-cells [24, 25]. This type of diabetes affects 85-90% of diabetics and begins most frequently insidiously after 40 years. 60-90% of people with type 2 diabetes suffer from obesity and visceral fat mass overload appears to be involved in the pathogenesis of this disease [26].

Indeed, in a context of visceral obesity, a subtle inflammatory syndrome, an increase in circulating free fatty acids and an ectopic accumulation of triglycerides lead to peripheral resistance to insulin. First, β-cells adapt and maintain normoglycaemia by secreting more insulin (Fig 1.5). This compensation phenomenon may be explained by an increase in insulin synthesis, an increase in insulin secretion in response to nutrient stimulation, and an increase in β-cell mass [27, 28].

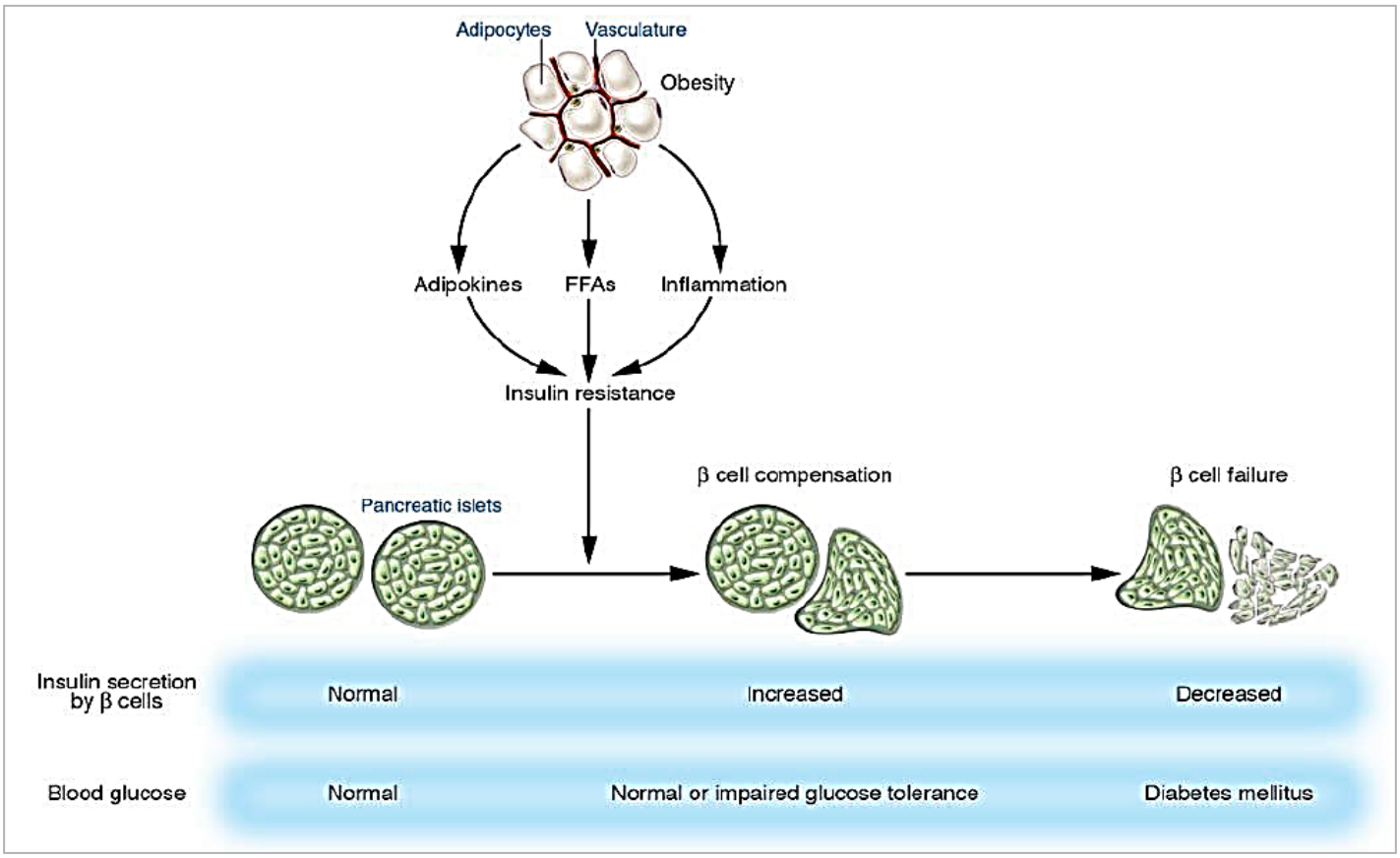


Figure 1.5: Schematic representation of the events leading to type 2 diabetes [26]. Used with permission.

Most patients remain at this stage and never become diabetic. Unfortunately, about 20% of people evolve towards a state of β-cell decompensation. Following various mechanisms (Mitochondrial dysfunction, oxidative stress, endoplasmic reticulum stress, lipotoxicity, chronic hyperglycaemia, certain cytokines), the β-cells of these patients lose their ability to secrete enough insulin to compensate for insulin resistance [27]. Moreover, the first phase of glucose stimulated insulin secretion as well as the pulsatile nature of insulin secretion are lost in type 2 diabetic patients indicating a significant disruption of β-cell function [29, 30]. This relative deficit of insulin secretion results in progressively increasing blood glucose levels until they eventually reach the numbers that constitute the diagnosis of diabetes [26]. Pancreatic β-cell decompensation might be attributed to a reduction of the number of β-cells or β-cell mass [31]. The fluctuations and regulations of β-cell mass in physiological and pathological circumstances including type 2 diabetes will therefore be reviewed here.

1.3 β-cell mass

1.3.1 Definition

Immunohistochemistry on pancreatic sections is commonly used to evaluate β-cell mass [32]. To do so, β-cells first need to be identified by immunostaining of a β-cell specific protein. Several proteins have been tested but insulin is the most commonly used. The area covered by β-cells on the sections is then measured and normalized on pancreas weight [33]. In healthy adults, β-cell mass reaches 1-2 g [31].

1.3.2 β-cell mass in diabetes

It has been well established that type 1 diabetes results from an auto-immune destruction of β-cells that occurs over several years [31]. However, the role that β-cell loss plays in the pathogenesis of type 2 diabetes deserves further examination.

Teams from Europe, Korea and the US have shown that type 2 diabetic patients present a 15-65% deficit of β-cell mass compared to non-diabetic subjects (Fig 1.6) [32-34].

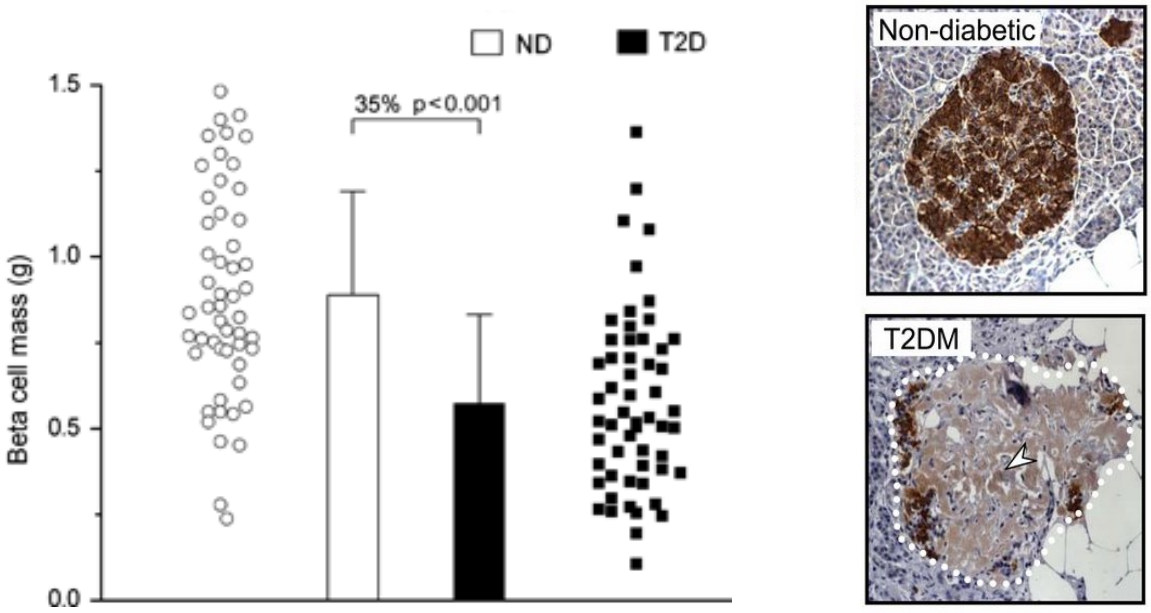


Figure 1.6: β-cell mass in type 2 diabetes. Left: β-cell mass in the pancreas of 52 non-diabetic (ND) and 57 type 2 diabetic (T2D) subjects [32]. Right: Human islets from a non-diabetic subject and a patient with Type 2 Diabetes (T2DM) stained for insulin (brown). Magnification 40X [35]. Used with permission.

However, β-cell function is also altered in type 2 diabetes. The relative contribution of β-cell dysfunction and loss of β-cell mass to the progression of type 2 diabetes remains to be determined.

Several pieces of evidence imply that β-cell loss alone cannot account for the development of diabetes and that an independent direct insult on β-cell function exists. Indeed, first phase insulin secretion is more impaired than second phase in type 2 diabetes [36, 37]. Both would be equally affected if the loss of function was solely due to a reduction of the number of β-cells. Moreover, bariatric surgery, Very Low Calories Diet, and intense drug therapy all induce the resolution of β-cell defects within weeks [38-41]. Because of the low turnover rate of β-cells, this is too fast to be attributed to a restoration of β-cell mass [42]. Furthermore, Hanley et al showed that the loss of β-cell mass they observed (15%) could not account for the reduction of insulin content (55%) they measured in their diabetic patient's pancreata [43]. Finally, it has been reported that β-cell mass and glucose tolerance do not necessarily correlate [44].

Nevertheless, some advocate for a preponderant and essential role of β-cell loss in the pathogenesis of type 2 diabetes. First, Meier et al showed an inverse correlation between β-cell area and fasting glucose, HbA1c levels, insulin, C-peptide and glucose tolerance in their cohort of diabetic and non-diabetic patients [45]. Secondly, evidence suggests that the reduction of β-cell mass is an early event in the story of the disease and is sufficient for its development. Indeed, patients with impaired fasting glucose, a state that can be considered as pre-diabetes, have been shown to present a 40% deficit of β-cell volume, a surrogate to β-cell mass [34]. Also, in an animal model of diabetes, an initial loss of β-cell mass can recapitulate the phenotype of type 2 diabetes [46]. Furthermore, pancreatectomy results in diabetes if β-cell area is reduced by 50-65% [47]. Lastly, β-cell loss entails an increased workload for the remaining β-cells. This puts them in a situation of stress which can in turn result in altered function [48]. This could explain the discrepancy between the number of β-cells lost and the decrease of insulin content reported by Hanley et al.

Altogether, this data suggests that the loss of β-cell mass is a trigger point for the development of type 2 diabetes. Understanding the mechanisms that regulate β-cell mass and how to modulate them might allow us to slow down or prevent the reduction of β-cell mass and the progression of diabetes.

1.3.3 Physiopathological regulation of β-cell mass

The regulation of β-cell mass is governed by the balance between 5 processes. The major determinants of β-cell mass are β-cell ***replication*** as well as increases (hypertrophy) and decreases (atrophy) in ***β-cell size***. It has been estimated that in pancreata from human donors, ~0.5% of β-cells were proliferating [49] . However, new β-cells can also be produced by ***neogenesis*** which is the differentiation of stem cell precursors or the trans-differentiation of acinar cells or cells from the pancreatic ductal epithelium [50]. Indeed, in humans, small clusters of insulin positive cells have been identifying within or protruding from the pancreatic ducts [51]. On the other hand, ***apoptosis*** results in β-cell loss. In addition, it has been suggested that mature β-cells can lose their specific gene expression profile, which corresponds to a ***loss of differentiation*** [52]. Recently, Talchai et al. suggested that in a mouse model of type 2 diabetes, the reduction of β-cell mass was due to a return to an immature β-cell phenotype rather than to β-cell death [53].

1.3.3.1 β-cell mass variations through life and in response to high metabolic loads

Modulation of these 5 processes enables a certain degree of β-cell mass plasticity that allows for variation and adaptation to life stages and changes in metabolic demand. During late embryogenesis and a short period after birth, β-cell mass expands because of a transient burst of β-cell replication followed by an increase of neogenesis [28]. Then, β-cell mass stays mostly constant throughout adulthood with a ~ 0.5% rate of apoptosis balanced by replication [31, 50]. In the elderly, Rahier et al observed an 18% reduction of β-cell mass [32]. This could be due to increased apoptosis that finally outweighs replication and neogenesis in this population.

Situations that are accompanied by an elevated metabolic load entail an increase of β-cell mass. Pregnancy is a state of high metabolic demand. Woman present insulin resistance to help ensure adequate glucose supply to the foetus. As a response, the number of β-cells increases. It has been suggested that β-cell mass doubles in pregnancy [50]. This could be attributed to upregulated β-cell replication. Shortly after delivery, replication is downregulated and apoptosis as well as atrophy contribute to the restoration of pre-conceptional β-cell mass [28]. Another situation associated with insulin resistance and elevated nutrient concentration in the blood stream is obesity. Again, β-cell mass is subsequently increased to respond to the high demand in insulin.

Indeed, it has been shown that β-cell mass can be raised by 50-100% in obese individuals compared to control subjects [32, 34, 43]. This growth of β-cell mass is due to a rise of replication and neogenesis as well as hypertrophy [34, 43].

Numerous factors drive the changes of β-cell mass; glucose appears to be the most prominent. Part of the increase of the number of β-cells in obesity could be attributed to short-lived postprandial elevations of blood glucose. Indeed, short-time exposure to high glucose concentration in rodents induces a rapid upregulation of β-cell replication as well as an increase of β-cell volume [52, 54, 55]. Moreover, glucose has been shown to trigger β-cell neogenesis and reduce apoptosis in rats [50]. Insulin itself might be implicated in the regulation of β-cell mass. Insulin resistance-induced hyperglycaemia results in an increase of β-cell mass and insulin receptor knockout mice present reduced β-cell mass [50, 56]. Circulating free fatty acids are elevated in obesity [26]. It is therefore noteworthy that acute culture in the presence of free fatty acids increases β-cell insulin secretion. Gut hormones such as GLP-1 and GIP, both increased in obesity in response to the high intestinal nutrient load, also upregulate β-cell replication and prevent against apoptosis [57]. Other factors that have been suggested to improve β-cell proliferation are IGFI-II, cholecystokinine, PTH related protein, ghrelin, menin, leptin, visfatin, Bone Morphogenic Proteins, placental lactogen and prolactin [50]. The last 2 factors are responsible for β-cell expansion in pregnancy. Finally, the parasympathetic nervous system might be involved in β-cell compensatory growth as well [27].

1.3.3.2 Causes of β-cell mass reduction in diabetes

In some cases, β-cell mass is too low from birth to ever compensate for increased metabolic loads. Indeed, some patients have insufficient peri-natal islet formation due to a lack of nutrients during gestation and early life or due to polymorphisms [48]. Moreover, type 2 diabetes could result from the deleterious effects of genetic defects and environmental stresses on β-cell replication and neogenesis [31].

However, the main cause of β-cell defect in type 2 diabetes is the reduction of β-cell mass due to elevated apoptosis. Indeed, type 2 diabetic patients have a 2-10 fold increased rate of β-cell apoptosis that surpasses replication and neogenesis (Fig 1.7) [34, 43].

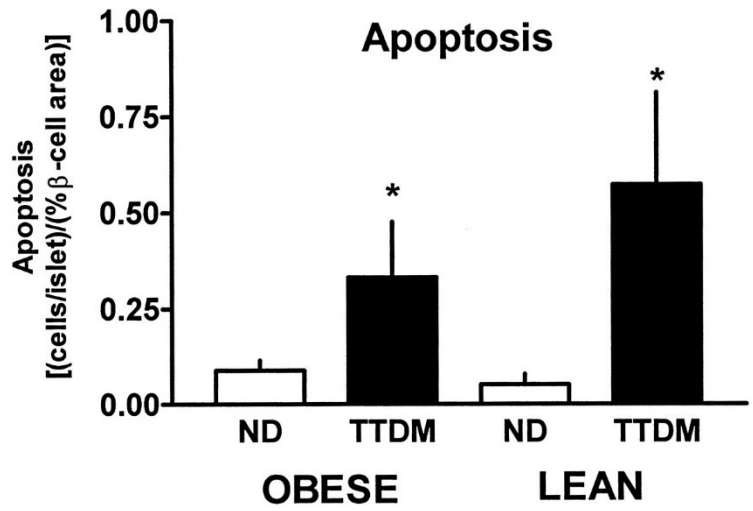


Figure 1.7: β-cell apoptosis in type 2 diabetes. β-cell apoptosis occurrence normalized to relative β-cell volume. ND, nondiabetic; TTDM, type 2 diabetes [34]. Used with permission.

The increase in apoptosis rate precedes changes in glycemic control in rodents prone to diabetes [58]. Moreover, apoptosis prevents adequate expansion of β-cell mass in Zucker Diabetic Fatty (ZDF) rats which results in diabetes despite increased proliferation [50]. Zucker rats, on the other hand only have increased β-cell proliferation and therefore can expand their β-cell mass and adapt to obesity without developing diabetes.

These results highlight the key role that apoptosis plays in the regulation of β-cell mass in type 2 diabetic subjects. Understanding the signalling pathways that regulate apoptosis as well as the factors that induce them in diabetes could lead to the production of interesting therapeutic targets.

1.4 Apoptosis

1.4.1 Definition and pathways

1.4.1.1 Definition

The term apoptosis comes from the ancient Greek word for “falling off” like the leaves of a tree in the fall. It is an ATP-dependent, highly regulated programmed cell death that targets one cell at a time and is not accompanied by any inflammatory reaction. It therefore differs greatly from necrosis, the other most occurrent form of cell death, that is an accidental event involving a group of cells and resulting in an inflammatory reaction [59].

Apoptosis plays multiple roles. During embryonic development, it eliminates developmentally defective cells such as auto-reactive thymocytes as well as cells in excess. Apoptosis is also the mechanism that allows the removal of cells that have no function or have lost their function such as embryo’s interdigital skin and mammary glands epithelial cells after lactation. Moreover, apoptosis helps discard injured cells that suffered unrepairable DNA damage or are infected by viruses. Finally, chemotherapeutic agents work by triggering apoptosis to eliminate cancer cells [59]. Given its central role in tissue homeostasis, dysregulation of apoptosis can lead to the development of diseases. After a stroke for example, the cells surrounding the initial ischemic injury necrosis, die by apoptosis. Furthermore, apoptosis is involved in the neuronal degeneration in fetal-alcohol syndrome and general anesthesia. Finally apoptosis plays a role in the weakening of the organism’s defenses during sepsis [60].

Apoptosis is characterized by a specific sequence of morphological changes (Fig 1.8). First, chromatin condensation as well as aggregation to the nuclear membrane can be observed. This is followed by shrinking of the cytoplasm. Then, the following series of modifications occur at the plasma membrane even though the membrane stays intact throughout the entire process:

- Membrane blebbing (protrusion of the plasma membrane).
- Cell fractionation into membrane enclosed apoptotic bodies containing intact organelles.
- Loss of asymmetry between the phospholipids of the membrane bilayer. Phosphatidylserine appears on the outer layer of the membrane.

In the meantime, the DNA undergoes fragmentation including pathognomonic double strands breaks. Finally, the apoptotic bodies will be removed by local phagocytes [59].

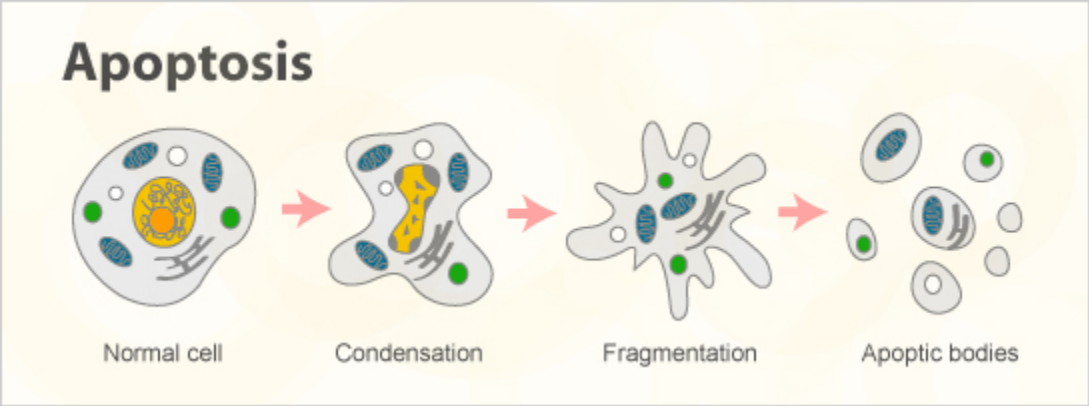


Figure 1.8: Morphological changes that characterize apoptosis [61]. Used with permission.

1.4.1.2 Caspases

The alterations that occur during the course of apoptosis are driven by a family of proteins called caspases. Caspases are cysteine aspartases, proteases bearing a cysteine residue in the active site that cleave proteins on the C-terminal side of aspartate. There are 4 initiator caspases (caspases 2, 8, 9 and 10) and 3 effector caspases (caspases 3, 6, 7). Caspases target cytoskeleton proteins such as gelsolin and cytokeratin, which results in the collapse of subcellular components and subsequent cell shrinkage. They also activate nucleases (caspase Activated DNAse or CAD) and inhibit DNA repair proteins (Poly ADP Ribose polymerase or PARP) therefore leading to DNA strand breaks. Finally, effector caspases activate themselves as well as initiator caspases in a positive feedback loop [62].

1.4.1.3 Apoptosis induction pathways

Caspases intervene in the 2 different pathways that trigger apoptosis: the extrinsic and intrinsic pathways (Fig 1.9). The extrinsic pathway of apoptosis is primarily a response to death signals coming from other cells than the one undergoing apoptosis. It is used for the negative selection of lymphocytes and by cytotoxic T lymphocytes. The extrinsic pathway of apoptosis therefore involves the activation of transmembrane receptors by extracellular ligands. These receptors are of the Tumor Necrosis Factor (TNF) receptor family [63]. They all have cytoplasmic domains of

80 amino acids called Death Domains. During activation of the receptor, an adaptive protein is recruited at the level of the Death Domain. This adaptive protein is then associated with pro-caspase 8 that constitutes the Death Inducing Signalling Complex (DISC). As a result of this complex formation, caspase 8 is dimerized and self-cleaved which enables its activation as well as the subsequent cleavage and activation of caspase 3 [64]. Caspase 3 is an effector caspase that will act on its targets in order to trigger the characteristic cascade of apoptosis.

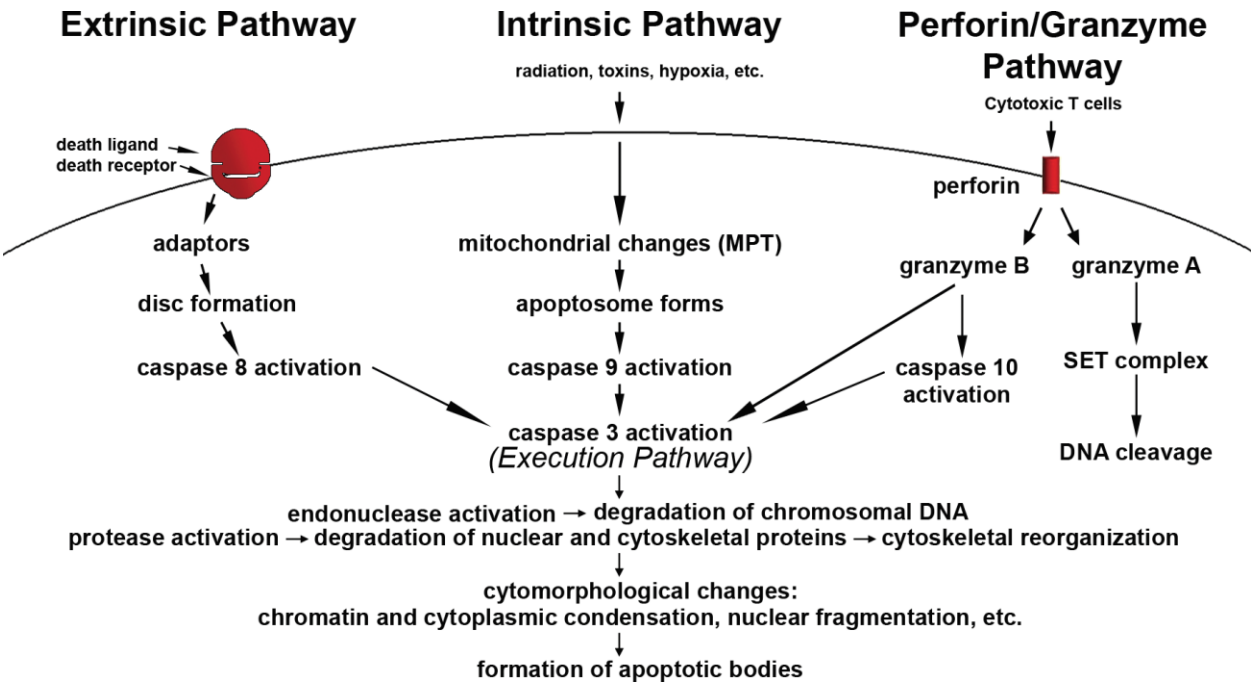


Figure 1.9: Schematic representation of the main pathways of apoptosis [62]. Used with permission.

The intrinsic or mitochondrial pathway of apoptosis is predominant over the extrinsic pathway in vertebrates [59]. It is triggered mostly by intracellular surveillance mechanisms when the cell's homeostasis and integrity are threatened. Examples of situations that trigger the intrinsic pathway are the accumulation of intra-cellular Reactive Oxygen Species (ROS), the Unfolded Protein Response (UPR), DNA damage, lack of nutrients and reduction of pro-survival signals [59]. The initial phase of the intrinsic pathway of apoptosis is regulated by proteins of the bcl-2

family [65]. These proteins are characterized by the presence of short conserved protein sequences called Bcl-2 Homology (BH) domains. The number and type of BH domains varies between the members of the bcl-2 family:

- Bcl-2 and bcl-xL contain 4 BH domains and have a protective effect against apoptosis.
- Bak and Bax have 3 BH domains and actively participate to the induction of cell death.
- Bad, Bid, Bim and PUMA only bear 1 BH domain (BH3) and promote cell death by interfering with protectors and activating killer bcl-2 proteins [65].

For the intrinsic pathway to be initiated, outer mitochondrial membrane permeabilization needs to occur so as to allow the release of intermembrane space proteins (IMS) such as cytochrome c into the cytosol. In physiological conditions, mitochondrial transmembrane potential ($\Delta\psi M$) is high and outer membrane permeability (MMP) is low [66]. The permeability transition pore complex (PTPC) in the outer membrane is quickly oscillating or flickering between opened and closed state to ensure the exchange of metabolites between the mitochondria and the cytosol. Upon pro-apoptotic signalling, MMP will be increased following 1 of 4 processes (Fig 1.10) [67]:

1) Destabilization of the membrane lipids resulting in pore formation.
2) PTPC closure that leads to a transient transmembrane hyperpolarization followed by an osmotic imbalance between the mitochondrial matrix and the intermembrane space. This imbalance causes the matrix to swell and eventually rupture the outer membrane thereby creating an opening for the IMS to be released [68].
3) Long lasting opening of the PTPC.
4) Migration of the cytosolic protein Bax to the outer mitochondrial membrane where, upon lifting of the inhibitory effect of bcl-2, it can form a pore with the outer mitochondrial membrane protein Bak.

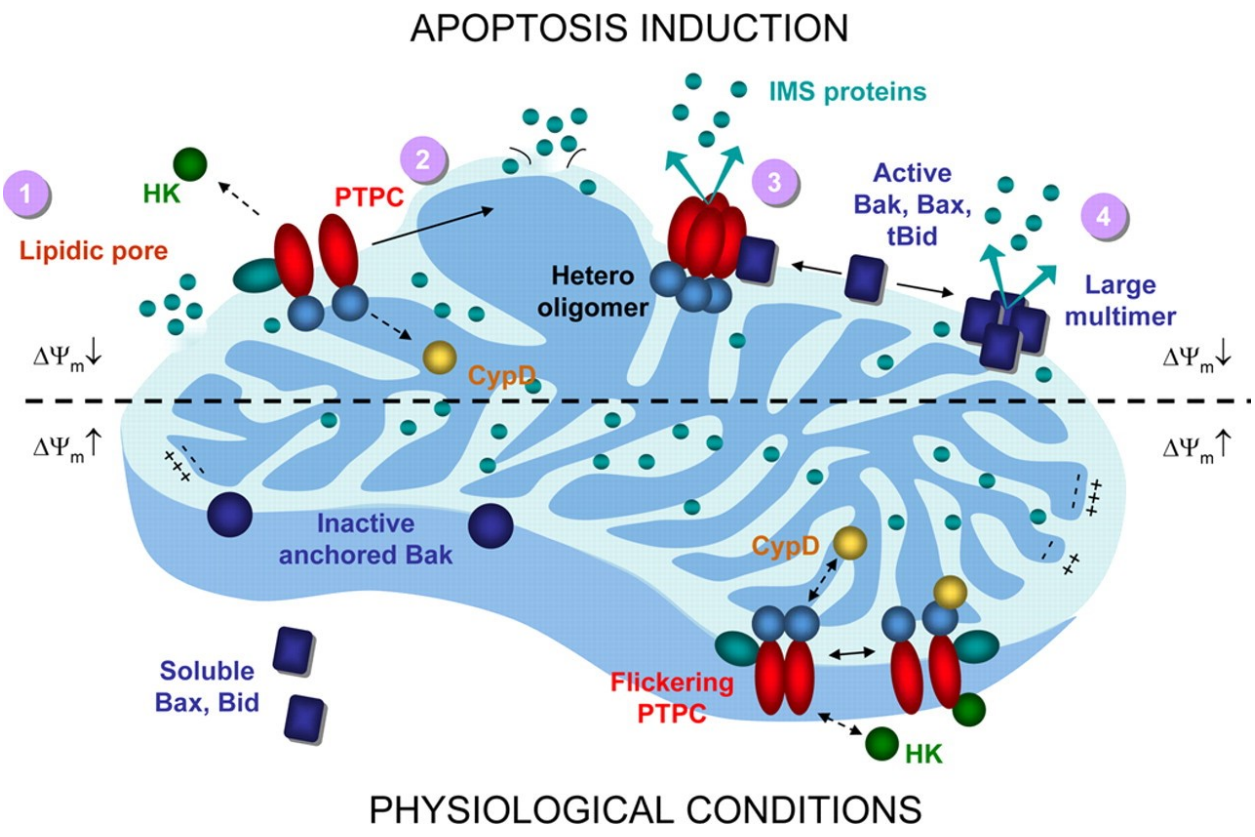


Figure 1.10: Induction of the intrinsic pathway of apoptosis. Schematic representation of the mechanisms leading to mitochondrial membrane permeabilization and the release of intermembrane space proteins (IMS) in the intrinsic pathway of apoptosis [67]. Used with permission.

Once released to the cytosol, cytochrome c will form a complex called apoptosome with Apoptotic Protease Activating Factor 1 (Apaf-1) and 7 procaspase-9 monomers. Apaf-1 will induce the dimerization and activation of caspase-9 that will in turn activate caspase-3 [60].

1.4.2 Techniques used to detect apoptosis

Numerous techniques can be used to detect apoptosis. Because the features of apoptosis and necrosis can overlap, it is of the utmost importance to use at least 2 of these techniques to assess apoptosis. Morphological manifestations of apoptosis can be revealed by hematoxylin/eosin staining or Transmission Electron Microscopy (TEM), which is the gold standard. DNA fragmentation, a late-stage event in apoptosis, can be objectified by DNA laddering or TUNEL assay. Moreover, the expression, activation or sub-cellular localization of the molecules involved

in apoptosis can be assayed. Finally, the changes in membrane phospholipids can be studied using Annexin V [62].

1.4.3 Mechanisms of apoptosis induction in diabetes

β-cell apoptosis is elevated in the islets of type 2 diabetic patients compared to non-diabetic subjects (Fig 1.7) [32, 34]. This increase in apoptosis may be due to various aggressions (lipotoxicity, glucotoxicity, oxidative stress, ER stress, hypoxia, islet amyloid deposits, infiltration by pro-inflammatory cytokines and mitochondrial dysfunction and fragmentation) to which the pancreatic β-cell is subjected to in type 2 diabetes [27].

The role of cytokines and changes in mitochondrial function and dynamics as initiating factors of β-cell apoptosis will be the main theme of this thesis. However, the contribution of free fatty acids to β-cell demise in type 2 diabetes will be briefly discussed as well.

1.4.3.1 Pro-inflammatory cytokines

Type 1 diabetes is the result of an auto-immune destruction of β-cells mediated by pro-inflammatory cytokines [69]. Cytokines could also contribute to β-cell apoptosis and the subsequent reduction of β-cell mass that leads to a deficit of insulin secretion and the development of type 2 diabetes in patients suffering from obesity and/or insulin resistance. Type 2 diabetic patients have increased circulating biomarkers of inflammation such as sialic acid, α1 acid glycoprotein [70, 71], serum amyloid A, C Reactive protein (CRP), cortisol and cytokines [72-75]. Moreover, the fact that increased serum levels of Il1β, Il6 and CRP are predictive of type 2 diabetes implies that cytokines might play an important role in the pathogenesis of the disease [76]. Furthermore, the islets of type 2 diabetic patients as well as animal models of obesity and diabetes are subject to inflammation with fibrosis and the presence of amyloid deposits [77]. The pro-inflammatory cytokines Il1β, TNFα and IFNγ are present in the islets of type 2 diabetic patients and not in non-diabetic subjects [78-81]. In addition, it has been shown that Il1β mRNA and protein levels respectively were increased in the β-cells of type 2 diabetic patients *versus* healthy controls (Fig 1.11) [71, 81].

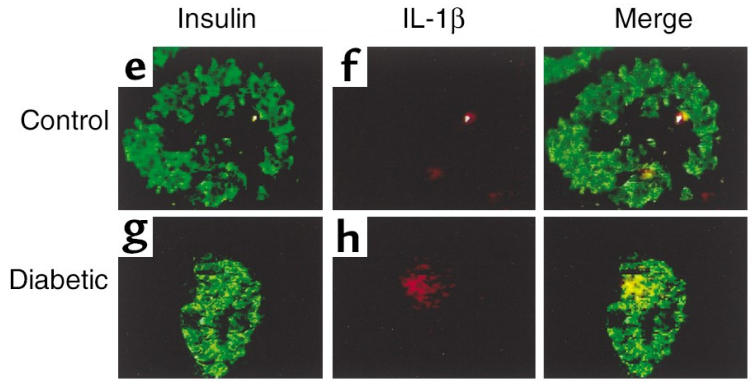


Figure 1.11: Expression of Il1β in human β-cells in pancreatic sections from a diabetic and a non-diabetic donor. Double immunostaining for IL-1β (red in f and h) and insulin (green in e and g) [81]. Used with permission.

Macrophages are constitutively present in islets and are necessary during embryonic development. Indeed, the osteopetrotic (op/op) mice, who bear a deficiency of macrophages due to an inactivating mutation in the Colony-Stimulating Factor-1 (CSF-1) gene, present decreased β-cell mass at birth and throughout adult life compared to controls [69, 82]. However, an accumulation of macrophages is a sign of inflammation. It is therefore noteworthy that the number of macrophages has been shown to be up to a 2 fold higher in the islets of type 2 diabetic patients compared to non diabetic individuals (Fig 1.12) [70, 71, 83]. Interestingly, diabetes is also accompanied by a shift of the phenotype of islets macrophages. In physiological conditions, the resident macrophages of islets are M2-type macrophages. They intervene in wound healing and modulate the immune response. In the islets of db/db diabetic mice however, the number of classically activated M1-type macrophages is elevated. These macrophages produce pro-inflammatory cytokines such as Il1β and TNFα [84].

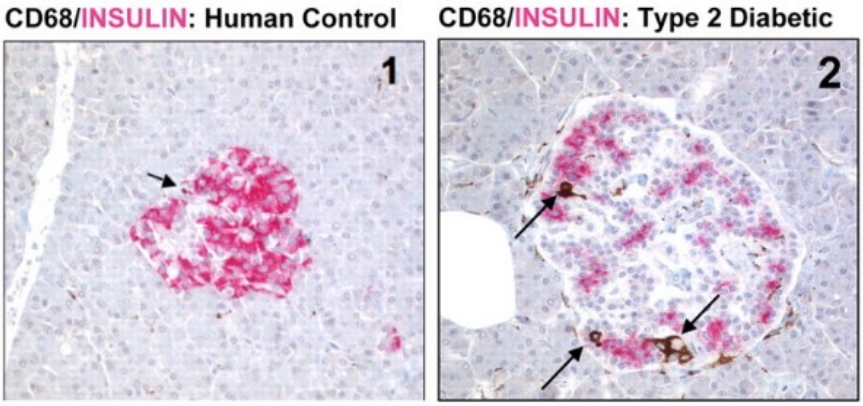


Figure 1.12: The number of islet macrophages is increased in type 2 diabetic patients (2) *versus* controls (1). Double immunostaining for Insulin (Red) and the macrophage lineage marker CD68 (brown, arrows) [83]. Used with permission.

It has been suggested that the M1-type macrophages are recruited to the islets by chemokines secreted by β-cells. Free fatty acids such as palmitate accumulate in the islets of type 2 diabetic patients. These fatty acids exert an indirect action on Toll Like Receptors TLR2 and TLR4 that results in an activation of NF-κB and a subsequent release of additional chemokines such as CCL2, CCL3, CXCL8 and CXC11 (Fig 1.13) [76].

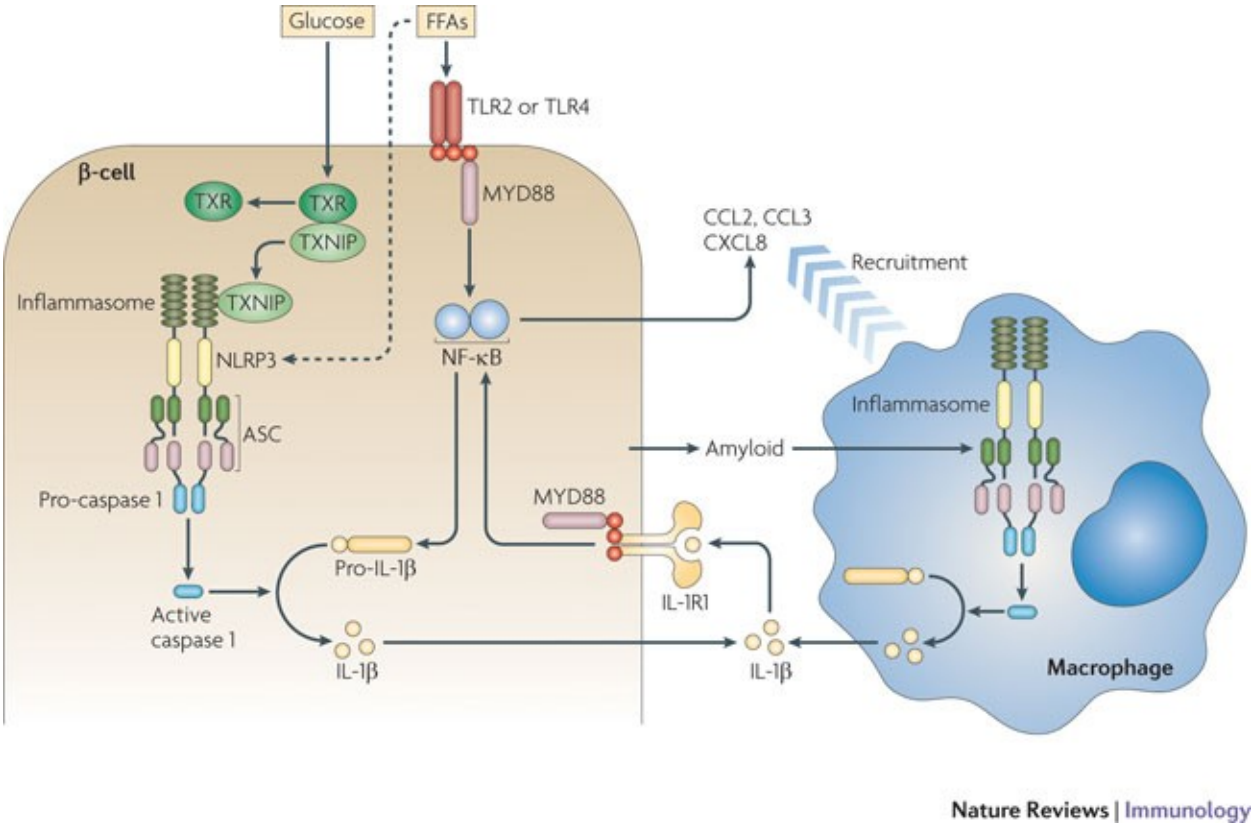


Figure 1.13: Macrophages recruitment and secretion of Il1β by β-cells in type 2 diabetes [76]. Used with permission.

The presence of the macrophages could explain the presence of Il1β and TNFα in diabetic islets. Nevertheless, Il1β could be secreted by β-cells themselves in response to glucose. Indeed, elevated glucose induces the assembly of a multiprotein immune complex called the inflammasome in β-cells (Fig 1.13). This inflammasome activates pro-caspase 1 which in turn cleaves pro- Il1β into Il1β [85]. The inflammasome can also be activated by ROS and the soluble Islet Amyloid PolyPeptide (IAPP). The newly produced Il1β can then recruit more macrophages to the site. This highlights the interplay between β-cells and macrophages in type 2 diabetes.

The accumulation of cytokines is detrimental for β-cell function and survival [86]. Indeed, short-term exposure (6h) to 5-10 ng/mL of Il1β, TNFα and IFNγ induces β-cell apoptosis in human and mouse primary islets [87, 88]. Interestingly, Il1β alone is sufficient to induce a significant increase of apoptosis in human β-cells. The addition of TNFα and/or IFNγ potentiates this effect [89].

The signalling pathways through which cytokines induce cell death are multiple (Fig 1.14). The binding of Il1β to its receptor on β-cells induces the activation of IκB kinase [89, 90]. This kinase phosphorylates IκB in the cytosol. This results in the release of the p65 subunit of NFκB. p65 then translocates to the nucleus where it regulates the expression of target genes. One of the genes that are upregulated by Il1β induced activation of NFκB is Fas, a death receptor that triggers the extrinsic pathway of apoptosis upon ligand binding [85]. Another target of NFκB is iNOS, which catalyzes the production of NO from L-arginine. This results in ER stress, a reduction of bcl2 expression and apoptosis [78, 91]. Il1β also activates the MAP/SAPKs pathway, comprising ERK, p38 and JNK. This contributes to cytokines-induced apoptosis. Indeed, JNK inhibitors protect β-cells against Il1β-induced death [89]. Moreover, even though MAP/SAPKs have been shown to activate NFκB, they have been independently implicated in the induction of β-cell apoptosis [90]. Finally, it has been suggested that Il1β could promote apoptosis by activating PKCδ and 12,15-lipooxygenases as well as by increasing cytosolic calcium concentrations [92]. The pro-apoptotic effect of TNFα has been attributed to its activation of the MAP/SAPKs and NFκB pathways [89]. When IFNγ binds its receptor, it activates the JAK/STAT pathway. The fact that IFNγ activates a different pathway then Il1β could account for its potentiating effect on the pro-apoptotic action of Il1β [89].

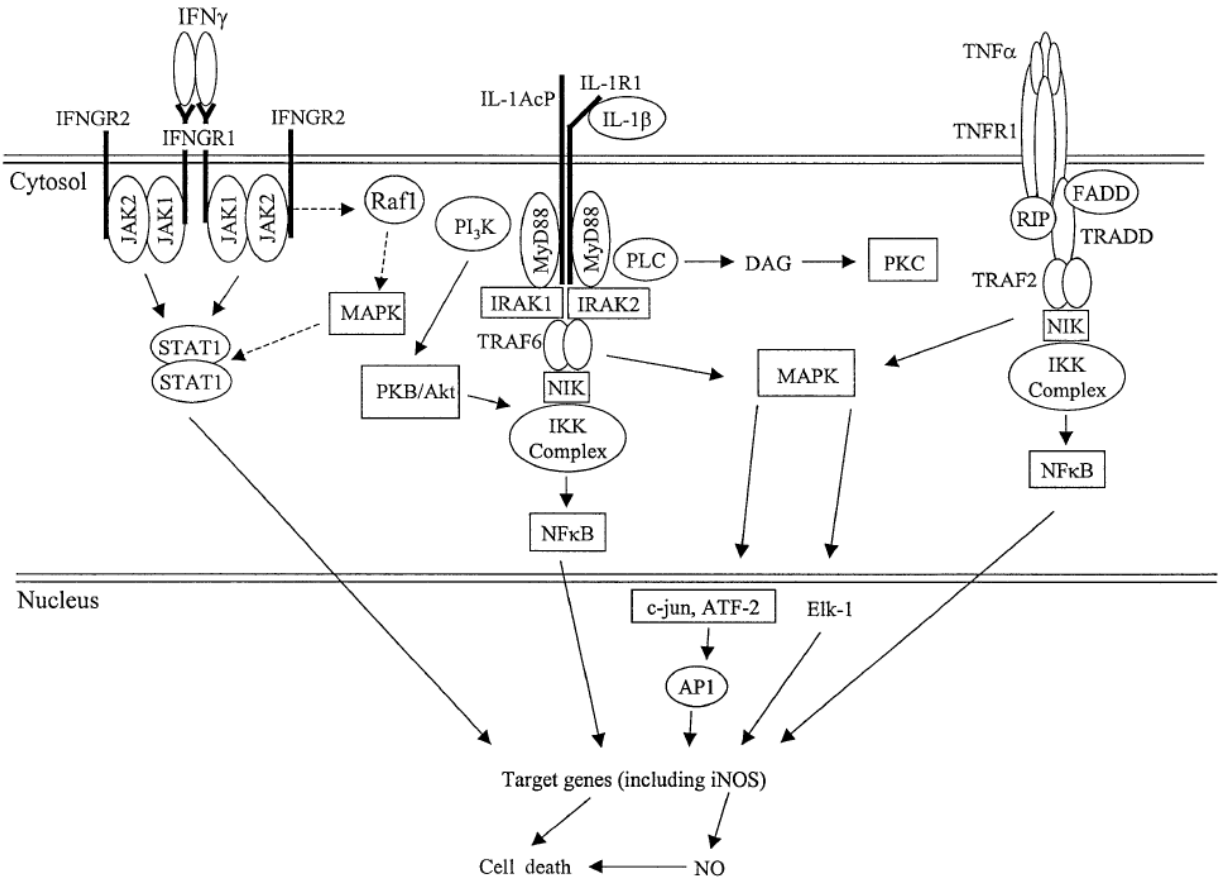


Figure 1.14: Signalling mechanisms through which Il1β, IFNγ and TNFα induce apoptosis in β-cells [78]. Used with permission.

Because of the key role played by pro-inflammatory cytokines in the induction of β-cell apoptosis in diabetes, the potential beneficial effect of anti-inflammatory drugs on the development and the progression of the disease is currently being evaluated. Il1β antagonists such as Anakinra reduce inflammation and macrophage infiltration in the islets of Goto-Kakizaki (GK) diabetic rats and db/db mice [84, 85]. These antagonists have also been shown to reduce HbA1c and improve insulin secretion in diabetic patients. Moreover, the recombinant humanized monoclonal Il1β antibody Gerokizumab induces a decrease of HbA1c and CRP as well as an increase of serum C-peptide in type 2 diabetic patients [69]. Finally, Salsalate, a prodrug of salicylic acid that targets NFκB also exerts positive effects on glycaemic control in diabetes [69, 85].

1.4.3.2 Mitochondrial function and dynamics

Mitochondrial ROS production

The main endogenous source of superoxide anion ($O_2^{\cdot -}$) is the mitochondrial electron transfer chain. Indeed, 0.1 to 0.5% of the electrons in the chain combine prematurely with oxygen to generate $O_2^{\cdot -}$ in place of H_2O [93]. In response to hyperglycaemia and elevated free fatty acids, mitochondrial nutrient oxidation increases thereby driving additional $O_2^{\cdot -}$ production [94, 95]. $O_2^{\cdot -}$ is a reactive oxygen species (ROS) and thus has the capacity to oxidize other molecules and generate other ROS such as H_2O_2. The main antioxidant enzymes are superoxide dismutase (SOD), which transforms the superoxide anion into hydrogen peroxide, catalase and glutathione peroxidase (Gpx), both of which degrade H_2O_2. However, β-cells have only small amounts of these enzymes. Indeed, the expression of the SOD is only 30% and that of catalase and Gpx 5% compared to what is observed in the liver [96]. Therefore, when $O_2^{\cdot -}$ and ROS production increases consequently to nutrient surfeit, the antioxidant defense capacities of β-cells are rapidly exceeded. This results in a situation of oxidative stress. Indeed, in the presence of high concentrations of glucose, the concentration of H_2O_2 in cultured human and mouse islets increases [97].

In the case of oxidative stress, the ROS surplus will have adverse effects on the cellular components: lipid peroxidation, protein oxidation, DNA alterations and interference with signaling pathways. Finally, ROS induce an increase in apoptosis following an activation of the intrinsic pathway of apoptosis induced by the transcription factor p53 [98] and an activation of c-Jun N-terminal Kinase [99]. In the pancreas of patients with type 2 diabetes, there is a correlation between the decrease in the mass of β cells and the increase of the 8-OHdG oxidative stress marker (8-hydroxy-2'-deoxyguanosine) [100]. ROS also have beneficial effects on the β-cell. H_2O_2 derived from glucose metabolism is one of the metabolic signals of insulin secretion in response to glucose [101]. In addition, ROS induce the expression of the oxidative stress response gene c-Myc [102]. Temporary activation of the stress response system is probably necessary for the adaptation and survival of β cells whereas persistent activation leads to their death [103].

Mitochondrial morphology

A strain on mitochondrial function with subsequent elevated ROS production is not the only way through which nutrients affect mitochondria to induce apoptosis in type 2 diabetes. It has indeed been proposed that mitochondrial dynamics were an important factor in the regulation of cell death.

Mitochondria are dynamic organelles that undergo changes in size and shape through fusion, fission and branching [104]. The morphology and number of mitochondria depends on the balance between fusion and fission. In healthy cells, the balance leans towards fusion and mitochondria are organized in a network but when fission takes over, the number of mitochondria increases and small, separated mitochondria prevail [105]. Fusion involves both the outer and inner mitochondrial membranes and is governed by GTPases located on the outer membrane and called mitofusins (Mfn1 and 2) (Fig 1.15). Optic Atrophy 1 (OPA1) also intervenes in mitochondrial fusion as it controls the structure of the inner membrane [106, 107]. Fission is regulated by a cytosolic GTPase called Dynamin-related protein 1 (Drp1) and its receptors on the outer mitochondrial membrane (Fis1, MFF, MiD49 and MiD51) as well as by endophilin B1 [108].

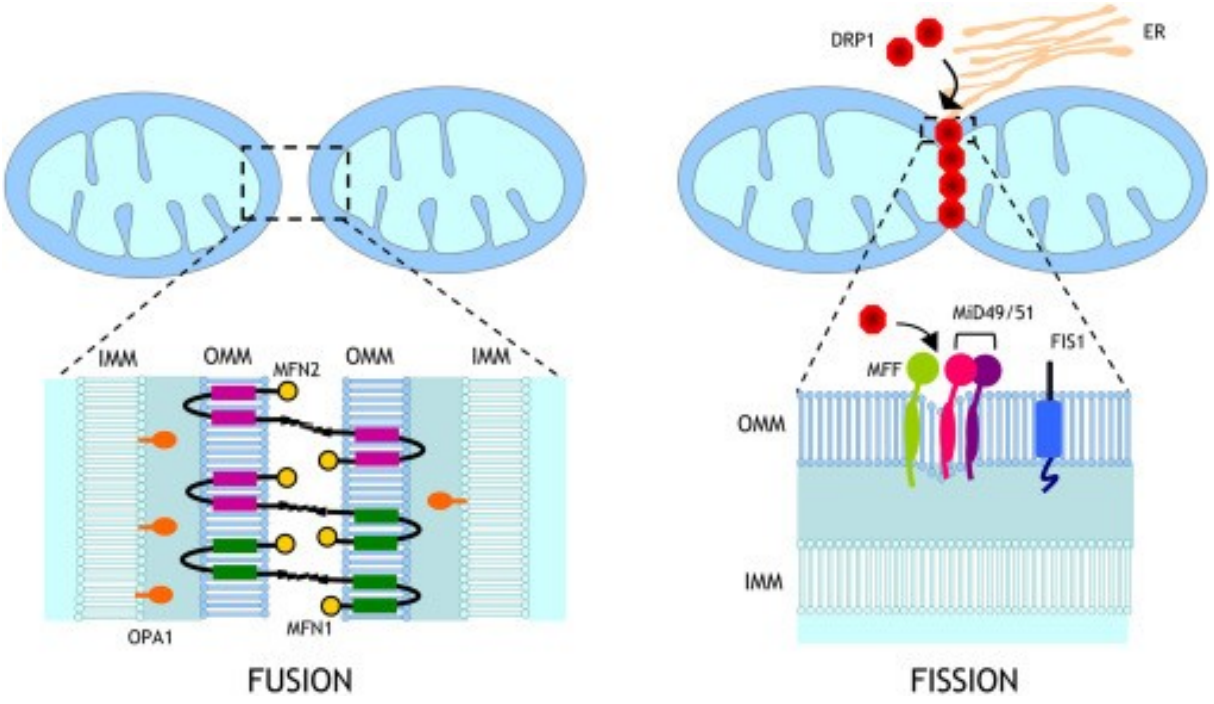


Figure 1.15: Regulation of mitochondrial fusion and fission [109]. Used with permission.

The maintenance of the mitochondrial network is critical for mitochondrial homeostasis. Indeed, the network is necessary for mitochondrial DNA distribution, oxidative phosphorylation and ATP production [110]. A reduction of fusion has been shown to induce a decrease of glucose oxidation in myotubes [111]. The mitochondrial network is also crucial to repair or eventually eliminate damaged organelles. Indeed, connected mitochondria will exchange components but if an injury is too extensive to be repaired, the damaged organelle will be segregated by fission from the network, encapsulated by autophagosomes and degraded by lysosomes [112].

In addition to playing a role in mitochondrial function and maintenance, mitochondrial morphology might be involved in apoptosis. Apoptosis is always associated with a disruption of the network that occurs prior to cytochrome c release [113]. Moreover, in response to pro-apoptotic signalling, Drp1 translocates to the outer mitochondrial membrane where it interacts with the pro-apoptotic bcl2 protein Bax [114]. It has also been suggested that downregulating Drp1 inhibited cytochrome c release and cell death [115]. Furthermore, Molina et al reduced Fis1 expression using RNAi in β-cells. This prevented mitochondrial fragmentation and had a protective effect against high glucose and palmitate induced apoptosis [116]. This protective effect might be due to the fact that fission is necessary for glucose and palmitate-induced production of ROS [117]. Data from experiments of fusion manipulation seems to confirm that changes in mitochondrial morphology have a causative effect on apoptosis. Indeed, inhibiting fusion by silencing Mfn1 or Mfn2 promotes apoptosis while overexpressing the mitofusins delays cytochrome c release and cell death [118, 119]. These results imply that mitochondrial fragmentation is a prerequisite for apoptosis. However, if fission impairment affects the release of cytochrome c, it does not interfere with the release of other Intermembrane space proteins such as Smac/Diablo. This might explain why some authors observe a delay but no complete blockage of apoptosis upon inhibition of fusion [120, 121].

Interestingly, Mfn2 expression and mitochondrial fusion are decreased in obesity and type 2 diabetes [122]. Furthermore, the mitochondria of type 2 diabetic patients are smaller than healthy controls [123]. These changes in mitochondrial morphology might be attributed to the excess nutrient environment in obesity and type 2 diabetes. Indeed, high glucose and palmitate induce mitochondrial fragmentation in β-cells (Fig 1.16) [117]. Also, hyperglycaemia and palmitate

decrease fusion and inhibit oxygen consumption in the β-cell mitochondria of type 2 diabetic patients [116].

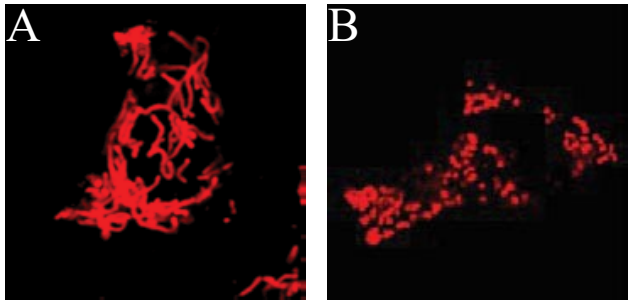


Figure 1.16: Palmitate-induced mitochondrial fragmentation in β cells [116]. Confocal images of INS1 cells incubated during 4 hours in the presence (B) or in the absence (A) of 0.4 mM of palmitate. Mitochondria were labeled in red. Used with permission.

In conclusion, the regulation of mitochondrial dynamics might play a role in the induction of β-cell apoptosis in type 2 diabetes. The mediators of mitochondrial fragmentation in diabetes could therefore represent promising therapeutic targets.

Mitophagy

As I already mentioned, malfunctioning mitochondria are separated from the network by fission [109]. That fission precedes and is necessary for mitophagy. It is due to the degradation of the mitofusins and OPA1. The damaged mitochondrion thus isolated is characterized by a depolarization that causes the accumulation of PTEN-induced putative kinase 1 (PINK1). PINK1 then phosphorylates PARKIN which mediates the ubiquitination of the outer mitochondrial membrane. The mitochondrial membrane thus marked will be recognized by p62 which binds to microtubule-associated protein 1A/1B-light chain 3 (LC3) in the double membrane of an autophagosome. The autophagosome will encapsulate the mitochondria and merge with a lysosome in which the damaged mitochondria will be degraded and its components recycled (Fig 1.17) [124].

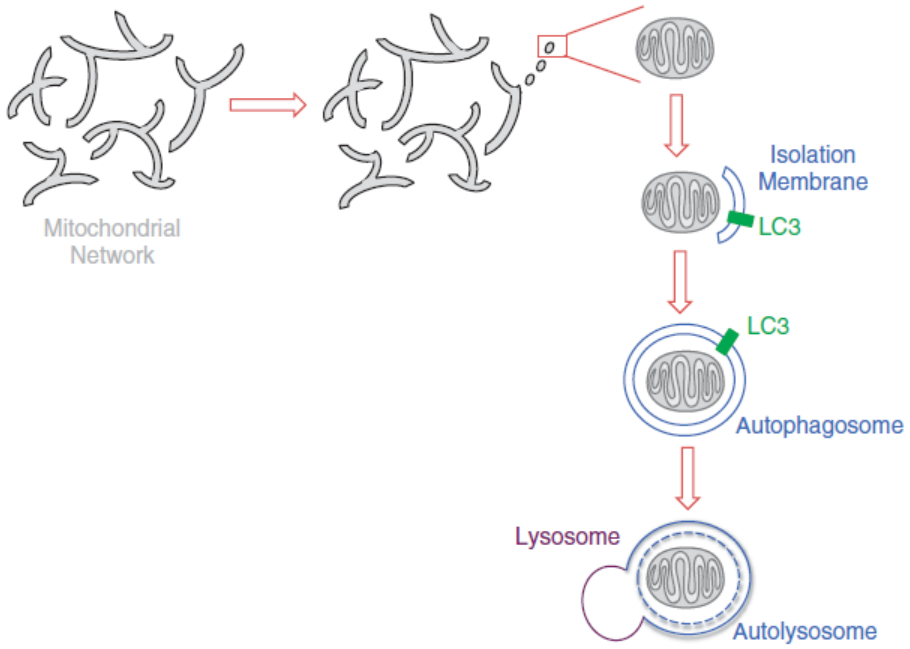


Figure 1.17: Mitophagy. Steps from segregation of damaged mitochondria by fission to encapsulation by autophagosome and lysosomal degradation (mitophagy) [125]. Used with permission.

This mitochondrion-specific form of autophagy is called mitophagy [126]. The removal of damaged mitochondria is crucial for cell survival. Mitophagy can have a protective effect against apoptosis by eliminating the malfunctioning, depolarized mitochondria that otherwise would have triggered the intrinsic pathway of apoptosis [67]. Charley Choe et al showed that activation of mitophagy prior to apoptosis induction reduces caspase activation [127]. On the other hand, autophagy is associated to cell death. However, it is not known whether it contributes to apoptosis, accompanies it or represents an independent cell death pathway [128]. Indeed, a potential new form of cell death has recently been unveiled. It is called autophagic cell death and is characterized by an accumulation of double-membraned vacuoles in the cytosol without any perturbation of chromatin organization [129]. The number of autophagosomes is significantly increased in the β-cells of type 2 diabetic patients compared to non-diabetic donors [130]. This form of cell death could therefore be relevant to the loss of β-cell mass in type 2 diabetes.

1.4.3.3 Lipotoxicity

Hyperlipidemia is a feature of obesity and free fatty acids might be mediators of β-cell apoptosis and the subsequent reduction of β-cell mass that leads to the development of type 2 diabetes. Indeed, diabetes is accompanied by an accumulation of triglycerides in the islets (islet steatosis) [131, 132]. Moreover, Long Chain Saturated fatty Acids (LCSFA) such as palmitate have been shown to induce β-cell apoptosis in animal models of diabetes as well as in human islets [133-135].

In healthy β-cells, Carnitine-Palmitoyl-Transferase 1 (CPT1) allows fatty acids to enter the mitochondria where they are oxidized to generate ATP. In physiological conditions, fatty acids are thus an essential source of energy [136]. They also participate to the amplifying pathway of glucose stimulated insulin secretion. However, when β-cells are chronically exposed to high glucose as well as elevated LCSFA, the fatty acids are diverged from oxidation and esterification into long chain fatty acyl-CoA increases [136-138]. This shift away from oxidation and towards esterification could be responsible for the pro-apoptotic effect of LCSFA. Indeed, the long chain fatty acyl-CoA activates nPKC. This novel Protein Kinase C isoform induces the Serine/Threonine phosphorylation and subsequent degradation of the promoter of cell survival IRS-2 [28]. Moreover, restoring fatty acid oxidation by activating AMPK using Metformin, AICAR or exercise inhibits high glucose and LCSFA induced β-cell apoptosis [138]. Also, promoting fatty acid oxidation by activation of AMPK prevents or delays type 2 diabetes in ZDF rats [139]. This highlights the important role that fatty acid metabolism plays in β-cells demise and the progression of type 2 diabetes. The cause of the impairment of fatty acid oxidation in states of impaired glucose tolerance and diabetes could be elevated blood glucose. Indeed, high glucose induces the activation of malonyl CoA due to increased citrate production. Malonyl CoA inhibits CPT1 and thus favors the esterification rather than oxidation of fatty acids (Fig 1.18) [136]. It has been shown that there is no β-cell toxicity induced by LCSFA in the absence of high glucose [138].

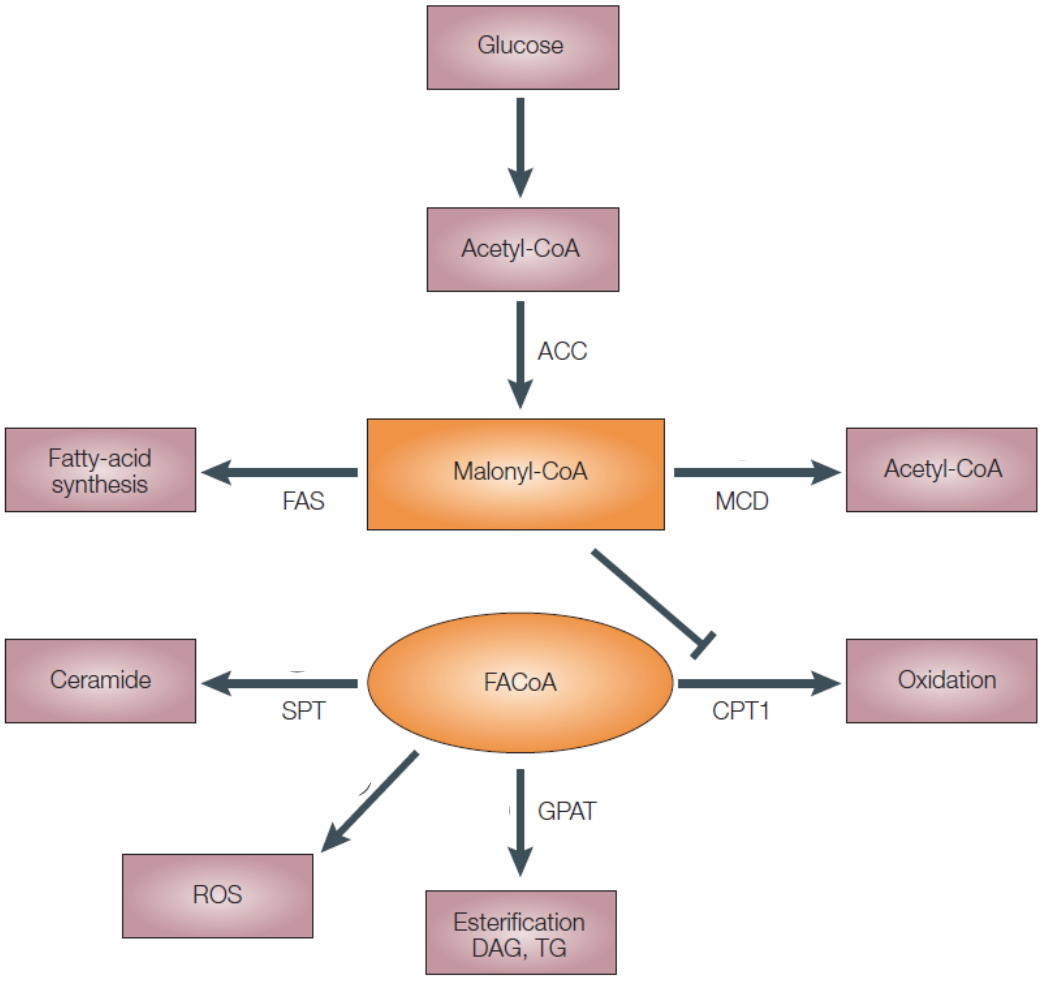


Figure 1.18: Effects of malonyl-CoA on fatty acid metabolism. Adapted from [139]. Used with permission.

Other mechanisms could explain the pro-apoptotic effect of LCSFA in β-cells. First, ceramides can be generated from palmitate which induces the intrinsic pathway of apoptosis [28, 135]. Secondly, the accumulation of LCSFA in β-cells has been suggested to result in the reduction of the anti-apoptotic bcl2 mRNA levels [140].

1.5 Transcriptional regulation of β-cell mass

So far, I have provided evidence that in conditions of nutrient excess and low grade inflammation, pancreatic β-cells die by apoptosis until β-cell mass is significantly decreased and the remaining β-cells can no longer secrete enough insulin to maintain glucose homeostasis

thereby causing the patient to develop diabetes. In this context, identifying the mediators of environmental stress-induced β-cell apoptosis could lead to the development of new therapeutic targets. Our lab specifically studies the transcription factors that regulate β-cell life and death in diabetogenic conditions. The most prominent and extensively studied of these factors whose expression is decreased in type 2 diabetes is Pdx1 [141].

1.5.1 Pdx1

Pancreatic duodenal homeobox 1 (Pdx1) is a transcription factor. It therefore exerts its actions almost exclusively in the nucleus by modulating gene transcription [142]. Pdx1 is mostly expressed in β-cells but also in 20% of the islet's α cells and in the gut endocrine cells [141]. Pdx1 is essential for the normal development of the pancreas during embryogenesis. Indeed, Pdx1 null mice as well as humans with homozygous mutations of Pdx1 present pancreas agenesis [143, 144]. Later throughout adult life, Pdx1 participates to the maintenance of β-cell phenotype by promoting the expression of genes that enable β-cell's specialized function such as insulin, glucokinase and IAPP [145]. Pdx1 is a key regulator of insulin expression. Indeed, heterozygous mutations in the Pdx1 locus in humans do not affect pancreas formation but leads to a deficit of insulin secretion and one of the forms of Maturity Onset Diabetes of the Young (MODY4) [142]. Moreover, Pdx1 is a mediator of the pro-survival effects of Insulin and IGF1. Insulin and IGF1 induce the Akt-mediated phosphorylation of FOXO1 which provokes the translocation of FOXO1 from the nucleus to the cytoplasm. This lifts the inhibition of FOXO1 on Pdx1 [146]. This pathway has been accounted for β-cell replication in response to insulin resistance as well as the protective effect of insulin on low serum-induced apoptosis [142, 147, 148]. In addition, the pro-survival effect of GLP1 has also been attributed to Pdx1 [142].

At the opposite, chronic hyperglycaemia and palmitate might exert their pro-apoptotic effects on β-cells through an inhibition of Pdx1. Indeed, chronic hyperglycaemia decreases Pdx1 protein expression and DNA binding activity [141, 149]. Palmitate reduces Pdx1's transcriptional activity by decreasing its nuclear localization. Such a reduction of Pdx1 activity appears to have a pro-apoptotic effect because the islets of mice with a 50% reduction of Pdx1 are more susceptible to apoptosis and present a significantly increased age-related reduction of β-cell mass compared to controls [145].

Other transcription factors that have been implicated in the regulation of β-cell life and death are CREB, NFAT, FoxM1, Pax4 and PPARα. Pdx1 and these other factors could be promising therapeutic targets if the wide range of their effects did not entail heavy side effects for the patients. There is therefore still extensive ongoing research concerning the transcription factors that modulate β-cell mass. The main purpose of my thesis is the study of Nor1, a member of the Nr4a family of orphan nuclear receptors. As a side project, I also studied the expression and role of ST18, an NZF transcription factor that was upregulated in a microarray conducted in the laboratory on INS1 cells exposed to palmitate.

1.5.2 Nr4a orphan nuclear receptors

In 2013, at the beginning of my PhD, I wrote a review about the role of Nr4as in glucose homeostasis that has been published in Diabetes and Metabolism[1] [150]. Here are the updated main points of that review.

1.5.2.1 Structure and regulation

Nr4a nuclear receptors compose a family of immediate-early response genes whose expression is regulated by a variety of extracellular stimuli. The Nr4a family includes 3 members in mammalian cells: Nr4a1 (also known as NGFIB, Nur77 TR3), Nr4a2 (Nurr1, NOT) and Nr4a3 (Nor1, MINOR) [151]. All three Nr4a members share a high degree of homology and a common structure consisting of a ligand-independent activation-function (AF)-1 transactivation domain in the N-terminal region, a DNA-binding domain composed of two zinc fingers and a ligand-dependent AF-2 transactivation domain in its C-terminal region [152, 153].

To date, the natural ligands for the Nr4a nuclear receptors remain unidentified and, consequently, the Nr4as are designated as “orphan receptors”. Moreover, determination of the structure of the ligand binding domain of Nurr1 by crystallography revealed two distinct structural characteristics that distinguish Nr4as from the rest of the nuclear receptors superfamily: *(i)* the absence of both a ligand binding cavity and *(ii)* a classical binding site for coactivators [16]. These observations point to a ligand-independent function for Nr4a nuclear receptors, suggesting

[1] A version of section 1.5.2 has been published as: Close, A.F., C. Rouillard, and J. Buteau, *NR4A orphan nuclear receptors in glucose homeostasis: a minireview.* Diabetes Metab, 2013. **39**(6): p. 478-84.

that their activity could be instead regulated by expression and posttranslational modifications. Consistently, Nurr1 has been shown to be constitutively active [154, 155] and its transcriptional activity dovetails with its proteasome-dependent turnover [156]. Additionally, all three Nr4as can be phosphorylated by a selection of kinases including JNK, RSK and MAPK [157-159]. However, the consequences of these regulatory posttranslational modifications have not been investigated extensively. Finally, Nr4as are subject to sumoylation, which has been proposed to repress their transcriptional activity [160, 161].

Upon activation, Nr4as bind the consensus sequence AAAGGTCA, known as the NGFIB responsive element (NBRE), either as monomers or homodimers [162]. Additionally, Nur77 and Nurr1 can bind the Nur responsive element (NurRE), composed of an everted repeat of the octanucleotide AAAT(G/A)(C/T)CA separated by 6 base pairs, as homodimers [153, 163, 164]. In addition, both Nur77 and Nurr1, but not Nor1, can form heterodimers or interact with retinoid X receptors (RXRs) to target DR5 elements (Fig 1.19) [19]. The fact that they exert different binding affinities for specific responsive elements and cofactors suggests non-redundant biological functions for the three Nr4a family members [165, 166].

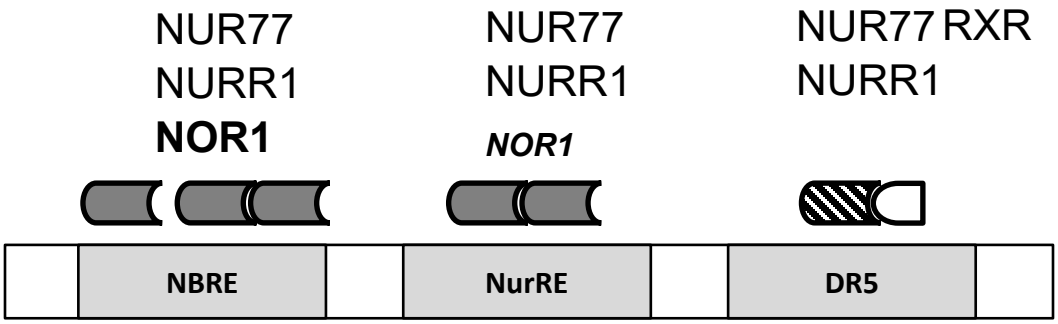


Figure 1.19: Schematic representation of Nr4a proteins and their DNA-binding specificities [150].

1.5.2.2 Role in glucose homeostasis

Several pieces of evidence hint at an important role for Nr4as in the pathogenesis of diabetes. These include *(i)* changes in Nr4a expression in animal models of insulin resistance, diabetes and/or obesity compared to their respective controls; *(ii)* the fact that both Nr4a expression and

activity are under nutritional control; and *(iii)* recent reports demonstrating important roles for Nr4a members in the regulation of glucose transport, glucose utilization, insulin sensitivity, gluconeogenesis and insulin secretion [167-169]. These putative roles in metabolic tissues are summarized in table 1.

Tissue	Biological effects	NR4A subfamily members implicated	References
Skeletal Muscle	↑ glucose uptake	NR4A1 and 2	[170, 171]
	↑ glycolytic activity	NR4A1	[172]
	↑ mitochondrial function	NR4A1 and 3	[173, 174]
	↑ HFD-induced insulin resistance	NR4A1	[172]
	↓ TG and DAG accumulation	NR4A1	[172]
Adipose tissue	↑ glucose uptake	NR4A3	[168, 175]
Liver	↓ insulin sensitivity	NR4A1	[172]
	↑ glucose output	NR4A1	[167]
	↓ TG and cholesterol accumulation	NR4A1	[172, 176, 177]
Beta-cells	↑ GSIS in INS1 cells	NR4A3	[178, 179] [180, 181] [182]
	↓ GSIS in Min6 cells	NR4A1, NR4A3	

	↑ insulin secretion in humans	NR4A3 (SNP)	
Brain	↑ food intake	NR4A3	[183]

Table 1: Reported biological effects of NR4A nuclear receptors in the control of glucose homeostasis. Adapted from [150].

Nr4as expression has been shown to vary in a tissue-dependent fashion in animal models of insulin resistance, diabetes and obesity compared to controls. Indeed, whereas all three *Nr4as* were increased in the liver of streptozotocin-treated and *db/db* diabetic mice [167, 168], *Nur77* and *Nor1* mRNA levels were decreased in the skeletal muscle and adipose tissue of Zucker diabetic fatty rats, *ob/ob* and *db/db* mice compared to controls [167, 168]. Moreover, *Nur77* expression was reduced by 50% in the skeletal muscle of rats fed a high fat diet compared to standard chow-fed specimens [169].

In humans, *Nr4as* expression seems to be differently influenced by body weight and fat mass. Veum and al. recorded significantly elevated *Nr4a1-3* mRNA levels in the subcutaneous adipose tissue of severely obese patients compared to lean subjects, and this expression was normalized one year after bariatric surgery and fat loss [184]. Furthermore, the same team observed upregulated *Nr4as* expression in the omental fat of the obese patients compared to the subcutaneous fat. Such a difference was not observed in lean subjects.

In the pancreas *Nr4as* expression has been shown to be restricted to pancreatic islet beta-cells [180]. *Nur77* overexpression in the clonal MIN6 beta-cell line impaired glucose-stimulated insulin secretion [180]. This effect was associated with a decrease in the expression of several transcription factors regulating insulin gene transcription such as *MafA*, *Pdx-1* and *NeuroD1*. Consequently, insulin expression and protein content were also reduced in MIN6 cells overexpressing *Nur77*. In the same paper, both Nur77 and Nurr1 modulated the expression of genes involved in cation homeostasis, including zinc transporters. This resulted in an elevation of

free intracellular zinc content, thereby providing another mechanism by which Nr4as could influence insulin secretion since intracellular zinc levels have been shown to play a role in glucose-stimulated insulin secretion, both *in vivo* and *in vitro* [185-189]. Likewise, Gao et al showed that the adenoviral overexpression of *Nor1* in MIN6 cells downregulated genes implicated in the regulation of insulin biosynthesis and decreased glucose stimulated insulin secretion [181]. Conversely, other reports suggest that Nor1 might have a beneficial effect on insulin secretion. Indeed, silencing *Nor1* has been shown to hinder insulin secretion via modulation of exocytosis genes and direct downregulation of *Ins1* and *Ins2* genes transcription in β-cells [178]. Moreover, Reynolds et al suggested that silencing *Nur77* or *Nor1* but not *Nurr1* significantly reduced insulin secretion [179]. Interestingly, common polymorphisms located within the *Nor1* locus were associated with significantly higher insulin secretion in a clinical trial involving non diabetic subjects and confirmed by a larger study involving diabetic as well as non diabetic male patients [182]. However, in this study, no association with either diabetes or glucose tolerance status could be established [182].

1.5.2.3 Role in the regulation of β-cell mass

Recently, evidence has started to accumulate suggesting that Nr4as might play a role in the regulation of β-cell mass as well. Indeed, *(i)* the expression of Nr4as is controlled by well established inducers of β-cell death such as palmitate, glucose and pro-inflammatory cytokines; *(ii)* Nr4as have been suggested to modulate cell replication and apoptosis in a manner dependent on their subcellular localization; *(iii)* Nr4as have been shown to exert an effect on mitochondrial function and dynamics.

Expression in response to diabetogenic factors

Saturated long chain fatty acids have been found to up-regulate *Nr4as* expression *in vitro* and *in vivo* [180, 190, 191]. Indeed, infusion of clonal rat beta-cells and isolated rat islets with palmitate (C16:0) and oleate (C18:1, ω9) caused a rapid increase of *Nur77* gene transcription in a dose-dependent manner [191], an effect that was implicated in the deleterious effect of free fatty acids on glucose-stimulated insulin secretion [180]. Moreover, palmitate has been shown to increase *Nor1* mRNA and protein levels in the MIN6 β-cell line as well as in mouse islets [181, 192]. In addition, Nur77 sub-cellular localization was found to be cytoplasmic in the clonal

MIN6 β-cells but stimulation with either palmitate or oleate provoked its translocation to the nucleus [180]. If long chain fatty acids increase *Nr4as* mRNA levels in hepatocytes [190] and β-cells, this action appears to be tissue-specific. Indeed, the free fatty acids palmitate, oleate and linoleate failed to alter *Nur77* expression in skeletal muscle [169]. The deleterious effects of fatty acids are related to their accumulation in cells. It is therefore noteworthy that Nr4as regulate triglyceride (TG) accumulation. *Nur77* knockdown in C2C12 myoblasts induced the expression of *sterol regulatory element binding protein-1c* (*Srebp-1c*), a major regulator of lipogenesis [193], thereby predicting a role for Nur77 in triglyceride accumulation. Indeed, high fat diet fed *Nur77*-null mice showed higher intramuscular TG content [172]. In that same model, lipid and cholesterol accumulation was observed in the liver, an effect that could be attributed at least in part to increased *Srebp-1c* mRNA levels [172, 176]. These findings might be of systemic significance since female *Nur77*-deficient mice presented higher fasting blood glucose levels and insulin resistance compared to controls [176]. However, their glucose tolerance, assessed by ipGTT was not impaired. Consistently with a role of Nur77 in the regulation of fat accumulation, overexpression of *Nur77* in mice reduced hepatic TG levels possibly via down-regulation of *Srebp-1c* and its canonical target genes (*Scd1*, *Gpam* and *Fas*) [177]. Therefore, Nr4as might have a protective effect against hepatic fat accumulation [177]. In order to further explore the mechanism by which Nr4as could regulate lipid metabolism, their effect on *carnitine palmitoyltransferase1* (*Cpt1*) has been studied. CPT1 regulates the entry of long chain fatty acylCoA into the mitochondria and is a rate-limiting enzyme in fatty acid β-oxidation [194, 195]. Muscle specific overexpression of *Cpt1* in rats improved high fat diet-induced insulin resistance [196]. Moreover, when fed a high fat diet, *Cpt1* knockout mice developed a more severe insulin resistance and impaired glucose tolerance than their wild type littermates [197]. Knocking down *Nur77* in skeletal muscle cells stunted *Cpt1* expression [193]. In addition, both Nur77 and Nurr1 were found to mediate 9-cis retinoic acid and HX600-induced expression of *Cpt1-a* in HEK293 kidney-derived cells [198], thereby supporting the possibility that Nr4as could promote β-oxidation to limit lipid accumulation.

Glucose acted synergistically with GLP-1 and PACAP-38, two cAMP raising hormones of the secretin/glucagon family, to increase *Nur77* mRNA expression in β-cell lines [199]. Cytokines and adenosine induce *Nr4as* expression [180, 200]. The pro-inflammatory cytokines Il1β, TNFα and IFNγ have been shown to increase Nr4as mRNA levels in MIN6 cells as well as mouse and

human islets (Fig 1.20) [180]. Taken together these studies suggest that both *Nr4a* expression and activity are under the control of pro-apoptotic factors. Whether they mediate the pro-apoptotic effects of fatty acids, high glucose and pro-inflammatory cytokines and their deleterious effects on β-cell mass remains to be elucidated.

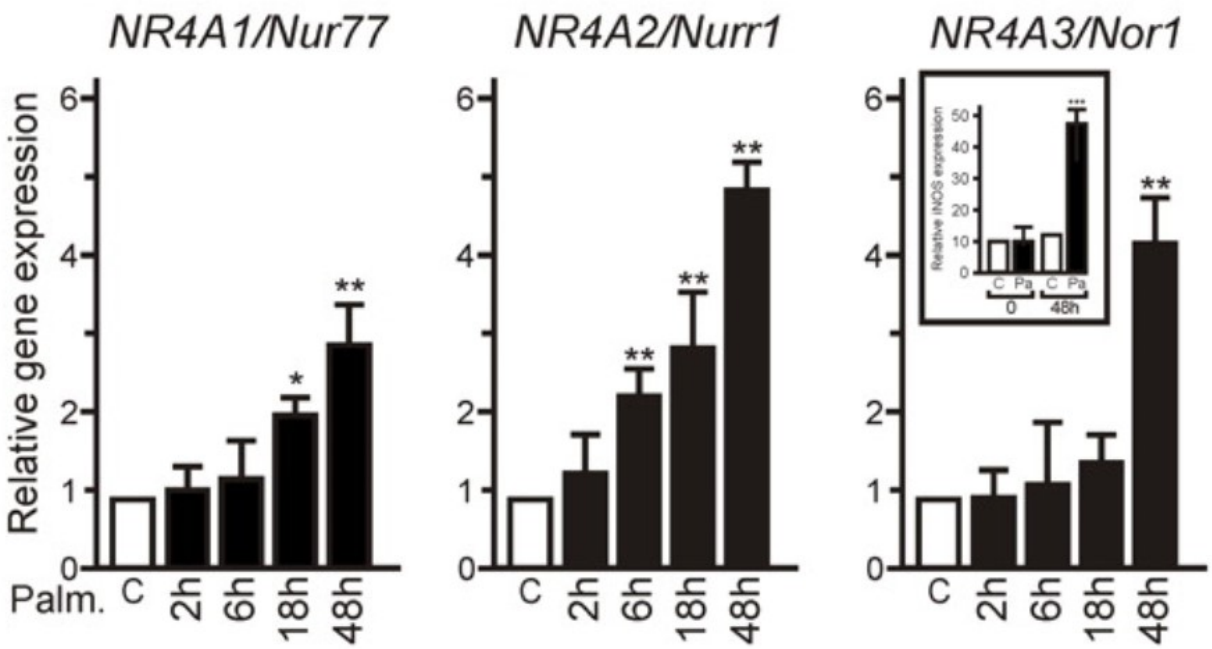


Figure 1.20: Effect of 0.4 mM of palmitate on the mRNA levels of Nur77, Nurr1 and Nor1 in human islets [180]. Used with permission.

Effect on β-cell replication and apoptosis

Nur77 and Nor1 have been suggested to increase β-cell proliferation in rat and human islets through modulation of the expression of genes involved in cell cycle regulation such as *Ube2c* and *p21* [201]. In agreement with these findings, the same team reported a decrease of β-cell mass in *Nur77* knockout animals. Unfortunately, the effects of a deletion of *Nor1 in vivo* were not evaluated.

In immune cells and cancer cells, Nur77 and Nor1 induce apoptosis [202-206]. This action is dependent on their cellular localisation. Indeed, it is the translocation of Nur77 or Nor1 to the mitochondria followed by their interaction with bcl2 and subsequent exposure of the BH3 death domain that allows cytochrome c release (Fig 1.21). If Nur77 is located in the nucleus however,

it has the opposite effect and promotes cancer cell growth [207]. Curiously, evidence on the effect of Nr4as on apoptosis in β-cells is scarce. Nur77 overexpression has been shown to protect MIN6 cells against palmitate and thapsigargin-induced apoptosis [192]. In this study, Nur77 acts via nuclear translocation and modulation of the transcription of ER stress genes as well as protective genes such as *GADD34*, *bcl2* and *survivin*. The role that all Nr4as play in the induction of apoptosis by environmental stressors such as palmitate, high glucose and cytokines in β-cells remains to be determined as well as their subcellular localization in the same conditions.

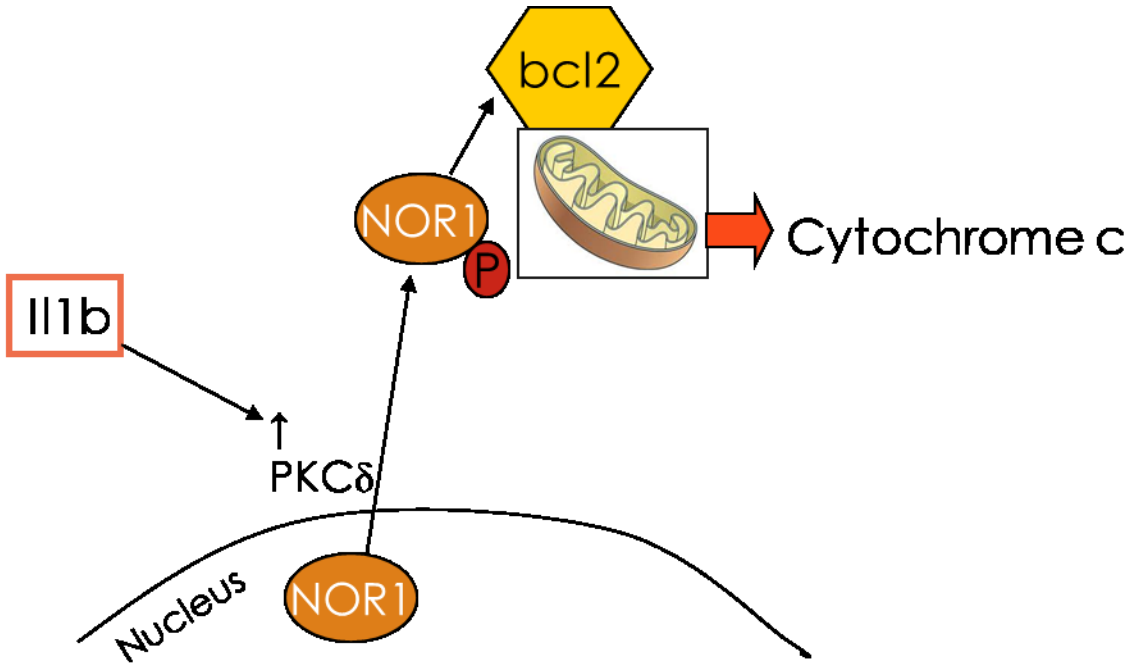


Figure 1.21: In a thymoma cell line, Il1β induces the PKCδ-mediated phosphorylation of Nor1. This induces the translocation of Nor1 from the nucleus to the mitochondria where it interacts with bcl2, exposing its BH3 domain and thus triggering the release of cytochrome c. Adapted from [206]. Used with permission.

Effect on mitochondrial function and dynamics

I have described above the interplay between mitochondrial function, mitochondrial dynamics and apoptosis. It is thus interesting to note that Nur77 and Nor1 have been suggested to exert a positive effect on mitochondrial fusion and function. Indeed muscle specific Nur77 or Nor1

transgenic mice present increased mitochondrial fusion [170]. In addition, silencing *Nur77* or *Nor1* in β-cells has been suggested to reduce oxygen consumption and ATP production [179]. Finally, in melanoma cells, Nur77 has been shown to reduce mitochondrial membrane potential and thus induce mitophagy upon translocation to the mitochondria [208].

1.5.3 NZF transcription factors

The NZF family of transcription factors comprises 3 members: Neural specific Zinc Finger factor 1 (NZF1), Myelin Transcription factor 1 (MyT1 or NZF2) and Suppression of tumorigenicity 18 (ST18 or NZF3) [209, 210]. There exists a high degree of homology between the members of the family. They are zinc finger transcription factors containing 6 Cy5-X5-Cys-X12-His-X4-Cys (C2HC) type zinc fingers arranged in 2 main clusters that can each bind DNA sequences independently. NZFs are also characterized by an acidic amino-terminal domain and a Serine/Threonin-rich domain in the region between the 2 zinc finger clusters [209, 211]. All 3 members of the family bind the AAAGTTT core motif. NZF1 and MyT1 can both activate as well as repress transcription but ST18 is an exclusive repressor of promoter activity. The repressor activity of ST18 can be attributed to the region located between the zinc fingers clusters in combination with either one of the 2 clusters [209].

NZF1, MyT1 and ST18 are expressed in developing neuronal cells and MyT1 has been suggested to play a role in neuronal differentiation [212, 213]. MyT1 is also expressed in endocrine progenitors and differentiated islet cells [212, 214]. Moreover, MyT1 might be necessary for α and β cell development in the embryonic pancreas. Indeed, mouse embryos expressing a dominant negative MyT1 show a reduced number of insulin and glucagon positive cells at embryonic day 14.5 compared to controls [214]. Furthermore, pancreas specific $MyT1^{-/-}$ mice embryos present abnormal multihormonal cells within their islets [212]. In adulthood, these mice also present reduced GLUT2 expression and glucose stimulated insulin secretion as well as impaired glucose tolerance suggesting that MyT1 plays a role in islet function in adult life.

One of the members of the NZF family, ST18, might be implicated in the induction of apoptosis by diabetogenic factors. ST18 is a breast cancer tumor suppressor gene. In breast cancer cell line and the majority of primary breast tumors, ST18 mRNA is significantly decreased. This could be attributed to hypermethylation of the ST18 promoter in breast cancer cells [210]. Moreover,

overexpressing ST18 in the MCF-7 breast cancer cell line inhibits colony formation *in vitro* and tumor formation in a xenograft mouse model [210]. This suggests that ST18 might exert pro-apoptotic or anti-proliferative actions in these cells. Another more direct evidence suggesting that ST18 might mediate the pro-apoptotic effects of diabetogenic stressors has been provided by Yang et al [211]. They determined that TNFα induces a quick and significant increase of ST18 DNA binding activity and mRNA levels in human adult dermal fibroblasts. Moreover, they showed that ST18 promotes the expression of pro-apoptotic genes including genes of the TNF receptor, Fas, intrinsic apoptosis and ceramide pathways. Finally, they showed that ST18 induces apoptosis in fibroblasts and is a mediator of TNFα-induced apoptosis.

Chapter 2: Research plan

2.1 Rationale

Diabetes is a serious disease that entails multiple complication risks for patients and is one of the 10 leading causes of death in the world [1-5]. It is therefore particularly alarming that the prevalence of diabetes is rapidly increasing worldwide [1][6]. Type 2 diabetes is the most frequent form of diabetes [26]. Chronic exposure to free fatty acids and pro-inflammatory cytokines, both circulating and locally in the islets of Langerhans, has been suggested to be an important etiologic factor for type 2 diabetes [27]. Indeed, fatty acids and cytokines cause β-cell apoptosis [89, 134, 135]. When the rate of apoptosis outweighs replication and neogenesis, β-cell mass decreases. The reduction of β-cell mass impedes sufficient insulin production to maintain glucose homeostasis. Consequently, type 2 diabetes develops [31]. Moreover, mitochondrial dysfunction and fragmentation, both occurring in type 2 diabetes, also contribute to β-cell demise and the onset of the disease [98, 99, 104, 122]. The exact pathways and mediators of diabetogenic factor-induced β-cell apoptosis in type 2 diabetes have not been entirely characterized. ***It is thus important to identify the mediators of stress-induced apoptosis in β-cells since this could lead to the development of new therapeutic targets against type 2 diabetes.***

NR4As are a family of orphan nuclear receptors with a suspected role in the regulation of glucose homeostasis. Indeed, Nr4as expression is modified in animal models of diabetes and in obese patients, they exert effects on insulin secretion as well as glucose uptake, production and glycolysis in peripheral tissues and their expression is under nutritional control [150]. ***Moreover, recent evidence suggests that NR4As might participate to the regulation of β-cell mass.*** Indeed, the expression of Nr4as is controlled by well established inducers of β-cell death such as palmitate, glucose and pro-inflammatory cytokines [150], Nr4as have been suggested to modulate cell replication and apoptosis in a manner dependent on their subcellular localization [201, 206] and finally Nr4as have been shown to exert an effect on mitochondrial function and dynamics [173, 179]. If evidence suggesting a potential role in β-cell mass regulation exists for all members of the Nr4a family, special attention should be given to Nr4a3/Nor1. Indeed, the

DNA binding profile of Nor1 is different from Nur77 and Nurr1 which suggests unique biological functions [165, 166]. ***Moreover, Nor1 is the least studied member of the family and its expression and role in β-cells remains largely unknown.***

The tumor suppressor ST18, a member of the NZF family of transcription factors, might be implicated in the environmental stress-induced apoptosis in β-cells as well. Indeed, Yang et al determined that ST18 is a mediator of TNFα-induced apoptosis [211]. TNFα is one of the pro-inflammatory cytokines that is present in the islets of type 2 diabetic patients [71-73]. ***However, the expression profile and role of ST18 in β-cells has, to our knowledge, never been evaluated.***

2.2 Hypothesis

We hypothesize that Nr4a nuclear receptors, and more specifically Nor1, as well as ST18, are novel regulators of β-cell mass which makes them key factors in the pathogenesis of type 2 diabetes. More specifically, we hypothesize that Nor1 and ST18 are mediators of diabetogenic factors-induced β-cell apoptosis.

We expect that the effects of Nor1 and ST18 are dependent on their subcellular localization.

To test this hypothesis, we fixed several specific objectives.

2.3 Specific objectives

2.3.1 Objective 1 (Chapter 3)

To determine the expression profile and role of Nr4as and specifically Nor1 in beta-cells exposed to control and diabetogenic environments.

<u>To achieve this objective, we:</u>

- Evaluated Nor1 expression in mice pancreata by immunofluorescence.
- Evaluated Nr4a's expression in β-cells exposed to palmitate, high glucose or cytokines (Il1β and IFNγ).

- Conducted Nor1 gain and loss of function experiments *in vitro* and *in vivo* and studied the effect on β-cell proliferation and apoptosis.
- Measured β-cell mass in Nur77, Nurr1 and Nor1 knockout mice *versus* control.
- Determined non-fasting blood glucose, insulin secretion and glucose tolerance (Oral Glucose Tolerance Test) in Nur77, Nurr1 and Nor1 knockout mice *versus* control.

2.3.2 Objective 2 (Chapter 4)

To determine the mechanism of action of Nor1 in β-cells.

To achieve this objective, we:

- Performed RNA sequencing to determine the genomic profile of β-cells overexpressing Nor1 *versus* cells transfected with a control vector.
- Studied the subcellular localisation of an Nor1-GFP vector in response to a pro-apoptotic stimulus (cytokines) in β-cells.
- Studied the colocalization of Nor1 with bcl-2 in β-cells.
- Evaluated the effect of Nor1 overexpression on mitochondrial function in β-cells by measuring glucose oxidation and ATP production.
- Determined the effect of Nor1 overexpression on mitochondrial morphology in β-cells by Transmission Electron Microscopy (TEM).
- Studied the effect of Nor1 on mitophagy by assessing the colocalization of LC3-GFP with mitochondria and by determining the number of autophagosomes targeting the mitochondria by TEM in β-cells overexpressing Nor1.

2.3.3 Objective 3 (Chapter 5)

To elucidate the expression profile and activity of ST18, as well as its effects on β-cell function and survival.

To achieve this objective, we:

- Evaluated the tissue distribution of ST18 in mice.
- Evaluated the distribution of ST18 in mice pancreata by immunofluorescence.

- Measured the expression of ST18 in β-cells in response to environmental cues such as high glucose, palmitate, oleate, cytokines and GLP-1.
- Assessed the subcellular localization of ST18 in the islets of mice fed a high fat diet.
- Evaluated the DNA binding activity of ST18 by electrophoretic mobility shift assay in β-cells exposed to palmitate and cytokines.
- Determined the effect of ST18 overexpression on insulin secretion.
- Studied the effect of ST18 gain and loss of function on β-cell apoptosis and proliferation.

Chapter 3: The orphan nuclear receptor Nor1/Nr4a3 mediates cytokine-induced β-cell death *in vitro* and regulates β-cell mass in mice

A version of this chapter has been revised following reviews and re-submitted to Endocrinology in April 2017: Close, A. F., Villela B., Dadheech N., Rouillard C. and Buteau J. (2017). The orphan nuclear receptor Nor1/Nr4a3 mediates cytokine-induced beta-cell death *in vitro* and regulates beta-cell mass in mice.

3.1 Introduction

Type 2 diabetes is characterized by a progressive deterioration of both beta-cell mass and function, resulting in a relative deficit in insulin secretion [1, 2]. Autopsy studies have indeed reported significant reductions in β-cell mass in patients with type 2 diabetes [3, 4], an effect that has been largely attributed to an increase in β-cell apoptosis [3, 5]. Several environmental factors might contribute to β-cell death in diabetes [5], including elevated circulating levels of long chain fatty acids, chronic hyperglycemia and the production of pro-inflammatory cytokines [6]. Despite recent progress in this field, the instructive signals and the precise intra-cellular pathways that orchestrate β-cell death remain elusive.

The Nr4a family of orphan nuclear receptors comprises 3 members in mammalian cells: Nur77/Nr4a1, Nurr1/Nr4a2 and Nor1/Nr4a3 [7]. To date, no ligand has been identified for Nr4as [8]. Thus, their activity appears to be regulated at the level of expression (consistent with their designation as immediate early genes), as well as through post-translational modifications and proteasome-dependent degradation [9-15]. Nr4as have been implicated in numerous pathologies such as Parkinson disease, cancer and inflammatory diseases [16, 17]. Importantly, the potential involvement of Nr4as in metabolic diseases has received increasing attention (reviewed in [16]). This stems from several key observations: *i)* Nr4as expression varies in animal models of obesity and/or diabetes [18, 20], *ii)* their expression is increased in the adipose tissue of obese patients compared to lean subjects [21], *iii)* Nr4as expression are modulated by physical activity and

nutritional interventions [16, 22-24], and *iv)* Nr4as have been shown to exert tissue-specific functions resulting in the regulation of glucose homeostasis [18, 20, 25-32].

The biological roles of Nr4as in pancreatic β-cells remain relatively unexplored but have recently attracted interest. A previous publication has suggested that Nr4as expression in the pancreas was restricted to endocrine cells [26]. Further, Nur77 was induced by cytotoxic concentrations of free fatty acids to mediate some of the deleterious effects of lipotoxicity in MIN6 cells [26]. Importantly, Nur77 and Nor1 were found to regulate different transcriptional targets in MIN6 cells, suggesting non-redundant functions for the two Nr4a members [26]. More recently, the homeodomain transcription factor Nkx6.1 has been characterized as a transcriptional regulator of *Nur77* and *Nor1* [33]. The authors went on to show that the proliferative action of Nkx6.1 was blunted in Nur77-KO animals.

We herein sought to investigate the potential role of Nr4as in pancreatic β-cell mass. We thus examined cross-sectional β-cell area in animals with genetic deletion of *Nur77*, *Nurr1* or *Nor1*. Surprisingly, β-cell mass was significantly increased in Nor1-KO mice. We thus sought to further characterize the function of Nor1 in β-cells by studying its regulation and potential implication in β-cell death.

3.2 Methods

Animals ─ All animal studies were conducted in accordance with the Canadian Council on Animal Care Guidelines and Policies, with approval from the Animal Care and Use Committee of our Institutions. Efforts were made to minimize animal suffering and to reduce the number of animals used. *Nur77*$^{-/-}$ mice were graciously provided by Dr. Jeff Milbrandt (University of Washington, MO) [34]. *Nurr1*$^{+/-}$ mice were from Dr. Thomas Perlmann (Karolinska Institute, Stockholm) [35]. *Nor1*$^{-/-}$ mice were from Dr. Yves Labelle (St-François d'Assise Hospital, Quebec City). *Nur77*$^{-/-}$ and *Nurr1*$^{+/-}$ mice were maintained on a C57/B6 background. *Nor1*$^{-/-}$ mice were maintained on a 129S4/SV background. For all tested parameters, wild type animals of both backgrounds gave indiscernible results and were pooled for simplicity. 6 month-old male mice were used in the present study. The mice were housed in a temperature-controlled environment, on a 12h-light/dark cycle with *ad libitum* access to food and water. *Access to food was removed 12h prior to glucose challenge.* Blood glucose was measured with FreeStyle Lite test strips (Abbott, Alameda, CA). Plasma insulin was measured using a commercial Elisa kit (Millipore, Etobicoke, ON).

Immunostaining ─ Pancreata were fixed and embedded in paraffin. 5-10 µm sections were deparaffinized and rehydrated before microwave antigen retrieval in citrate buffer. Slides were then blocked in 0.5% BSA, 4% donkey serum in 0.1% PBST for 1h at room temperature followed by overnight incubation at 4ºC with mouse anti-Nor1 (Abnova, Taiwan; 1:100), guinea-pig anti-insulin (Dako, Burlington, ON; 1:800), rabbit anti-Ki67 (Millipore; 1:50) or rabbit anti-glucagon (Dako; 1:800) antibodies. The next day, slides were incubated for 1h at room temperature with anti-mouse Alexa Fluor568, anti-guinea-pig Alexa Fluor488, anti-rabbit Alexa Fluor568 or anti-rabbit Alexa Fluor488 secondary antibodies (1:250, Life Technologies, Burlington, ON). Slides were mounted with fluoromount-G® containing DAPI (Southern-Biotech, Birmingham, AL). Images were analyzed using a Spinning Disk ERS confocal microscope (PerkinElmer, Guelph, ON) and the Volocity imaging software.

Reagents ─ All cell culture reagents were purchased from Life Technologies.

Cell Culture ─ INS832/13 cells [36] (passage 52-78) were grown in RPMI 1640 medium supplemented with 10 mM HEPES, 10% heat-inactivated fetal bovine serum, 2 mM L-glutamine, 1 mM sodium pyruvate, and 50 µM β-mercaptoethanol at 37ºC in a humidified 5%

CO_2 atmosphere. Cells at 80% confluence were treated in serum-free RPMI with a cocktail of cytokines (IL-1β and IFNγ, both at 10 ng/ml) or with 25 mM glucose.

Human islets ─ Human islets were purchased from the Alberta Diabetes Institute Islet Core at the University of Alberta with the assistance of the Human Organ Procurement and Exchange Program and the Trillium Gift of Life Network who provide donor pancreata for research. For mRNA expression in response to cytokines and apoptosis assays, islets from 7 different healthy lean donors between 48 and 74 years of age were used. Islets were cultured in 11 mM glucose DMEM medium supplemented with 10 mM HEPES, 10% heat-inactivated fetal bovine serum, 2 mM L-glutamine, 1 mM sodium pyruvate, 1% penicillin-streptomycin and 50 µM β-mercaptoethanol at 37ºC in a humidified 5% CO_2 atmosphere. Our study was approved by the Human Research Ethics Board at the University of Alberta.

qPCR ─ RNAs were extracted using RNeasy Mini Kit (Qiagen, Germantown, MD) and reverse-transcribed using SuperScript First-Strand Synthesis System (Life Technologies). For real time PCR we used RT^2 SYBR Green ROX qPCR Mastermix (Qiagen) and an ABI 7900HT instrument (Life Technologies). Results were analysed using SDS software 2.3. Actin was chosen as gene of reference. Results are expressed as fold change ($2^{(-\Delta\Delta Ct)}$) compared to the indicated control condition.

Western Blot ─ Cells were lysed in a buffer containing 50 mM Tris (pH 8.0), 150 mM NaCl, 1% Triton X-100 and protease inhibitors (cOmpleteMini, Roche Applied Sciences, Laval, QC). Protein concentrations were determined by BCA protein assay. Equal amounts of heat-denaturated proteins from each treatment group were run on Novex gels (Life Technologies) and transferred onto nitrocellulose membranes. After blocking, membranes were incubated overnight with a mouse anti-Nor1 antibody (1:500) (R&D systems H7833). The next day, membranes were incubated with Horseradish-Peroxidase linked secondary antibodies and exposed to ECL reagents. Band density was determined using ChemiDoc MP Imaging System and Image Lab software 4.1 (Bio-Rad, Mississauga, ON). Membranes were stripped and incubated with an anti-Actin antibody (Santa Cruz, Dallas, TX) to confirm equal loading. Blots were developed on film and band density was determined using ImageJ software.

Plasmid Transfection ─ pCMV-Nor1-GFP and pCMV-Nor1-Flag were obtained from Origene (Rockville, MD). pCMV-mCherry was a kind gift from Dr. Peter Light (University of Alberta, Edmonton, AB). INS832/13 cells were transfected via nucleofection (Lonza, Allendale, NJ).

Human islet cells were dispersed using 0.05% trypsin-EDTA and transfected the following day using Lipofectamine 2000 (Life Technologies) according to manufacturer's protocol. Cells were assayed 24h post-transfection.

siRNA-mediated Knockdown ─ Nor1-specific siRNAs were from Life Technologies. The sequences used were RSS329251 (herein called siNor1-A) and RSS367608 (siNor1-B). siNor1 and control siRNAs were transfected using Lipofectamine RNAiMax. Briefly, cells were plated in 24 wells plates and incubated 24h to obtain adherence. For each well, 100 pmol of siRNA was mixed with 4 µL of Lipofectamine RNAiMax in RPMI 1640 medium containing no additive. Experiments were conducted 48h after siRNA transfection.

Apoptosis ─ Apoptosis was assessed by TUNEL assay using either TMR red (human islets) or fluorescein green (INS cells) *In Situ* Cell Death Detection Kits (Roche Applied Science), following manufacturer's protocol. Images were captured at 40X magnification using a Zeiss COLIBRI Inverted Fluorescence Microscope. At least 2000 cells were analyzed for each experimental condition in at least 3 independent experiments. In human islet studies, the results are presented as the percentage of apoptotic cells (red nuclei) in the transfected population only (green, GFP-positive cells).

Cytochrome c release – Cells were fractionated into nuclear, cytosolic and mitochondrial fractions using a Cell Fractionation Kit (Abcam, Toronto, ON). Cytochrome c release from the mitochondria to the cytoplasm was subsequently evaluated using a commercial ELISA kit (Abcam) and an EnVision 2104 Multilabel reader (PerkinElmer).

Insulin secretion – Cells were pre-incubated in KRBH medium containing 2 mM glucose for 30 min and then incubated at 2 mM glucose, 16 mM glucose or 35 mM KCl for 45 min. Media were collected and insulin concentrations were measured by ELISA (Alpco, Salem, NH). Cells were lysed in acidic ethanol for determination of insulin content. Secretion data were normalized to insulin content.

Statistical analysis ─ Data are presented as means ± S.E.M. Statistical analyses were performed with GraphPad Prism® using analysis of variance methods followed by Bonferroni post-test.

3.3 Results

Nor1-knockout mice display increased beta-cell mass and improved glucose homeostasis compared to wild types

To investigate the potential roles of Nr4a members in the regulation of β-cell mass, we measured the cross-sectional β-cell areas in pancreata harvested from wild type control animals and whole body *Nor1* or *Nur77* knockout mice. Because homozygous deletion of Nurr1 is lethal, we examined pancreata harvested from *Nurr1*$^{+/-}$ mice. Figure 3.1A shows typical immunohistochemistry images for each phenotype. Our morphological study indicated that *Nor1*$^{-/-}$ mice have increased β-cell mass compared to their wild type littermates (Fig 3.1B). Indeed, β-cell area was ~2.5-fold greater in *Nor1*$^{-/-}$ mice versus control animals. This phenotype was unique to *Nor1*$^{-/-}$ animals as *Nur77*$^{-/-}$ and *Nurr1*$^{+/-}$ islets did not present any significant difference compared to their respective controls. In addition, *Nor1*$^{-/-}$ animals consistently displayed a 50% increase in the number of replicating (Ki67-positive) β-cells compared to control animals (Fig 3.1C).

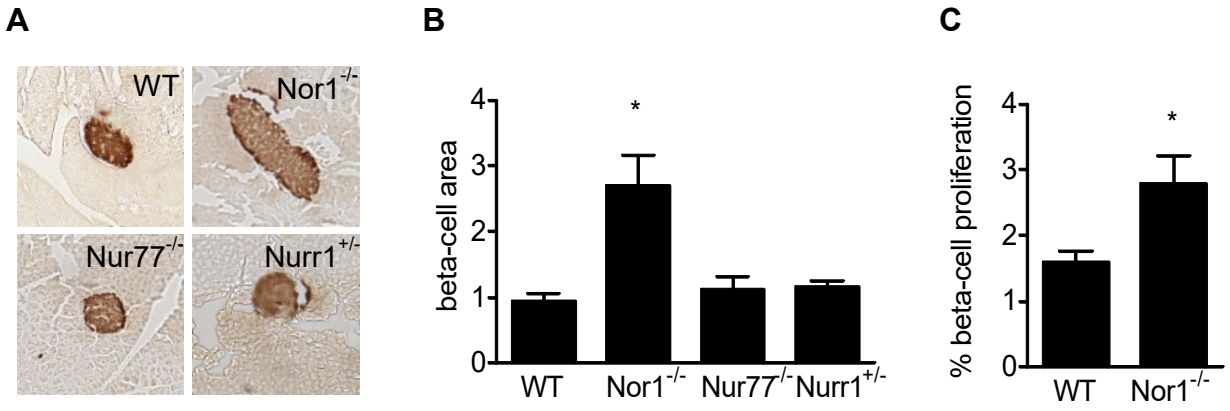


Figure 3.1: Nor1-knockout mice display increased β-cell mass. A) Images show representative insulin immunostaining in wild type, *Nor1*$^{-/-}$, *Nur77*$^{-/-}$ and *Nurr1*$^{+/-}$ pancreata. B) Quantification of cross-sectional β-cell areas. Results are represented as fold-change over wild type animals. n=6 for each genotype. C) β-cell proliferation was evaluated by counting the number of Ki67

positive nuclei. n=4 for each genotype. All results are represented as means +/- SEM; *, $p < 0.05$ versus wild type animals.[2]

We next sought to test whether the increase in β-cell mass in *Nor1*$^{-/-}$ animals translated into improved glucose tolerance. We thus measured blood glucose and performed i.p. glucose tolerance test in *Nor1*$^{-/-}$ mice versus controls. Non-fasting blood glucose was significantly lower in *Nor1*$^{-/-}$ mice with an average of 4.7 mM compared to 5.5 mM in wild type mice (Fig 3.2A). Also, mice with genetic deletion of *Nor1* displayed improved glucose tolerance, an effect that was not observed in *Nur77*$^{-/-}$ mice (Fig 3.2B). Indeed, blood glucose was significantly lower 15, 30 and 45 min following glucose injection in *Nor1*$^{-/-}$ versus wild type mice. After 120 min, the area under the curve was significantly smaller in *Nor1*$^{-/-}$ mice versus controls (Fig 3.2C). This improved glucose tolerance was accompanied by lower plasma insulin in *Nor1*$^{-/-}$ mice compared to controls at 60 min (Fig 3.2D). The effect of Nor1 on islet mass and glucose homeostasis could not be accounted for by any differences in body weight (Fig 3.2E) since this parameter was similar in *Nor1*$^{-/-}$ versus control mice.

These results highlight a hitherto uncharacterized role for Nor1 in the regulation of β-cell mass and glucose homeostasis.

[2] The knockout animals were kindly provided by Dr. Claude Rouillard.

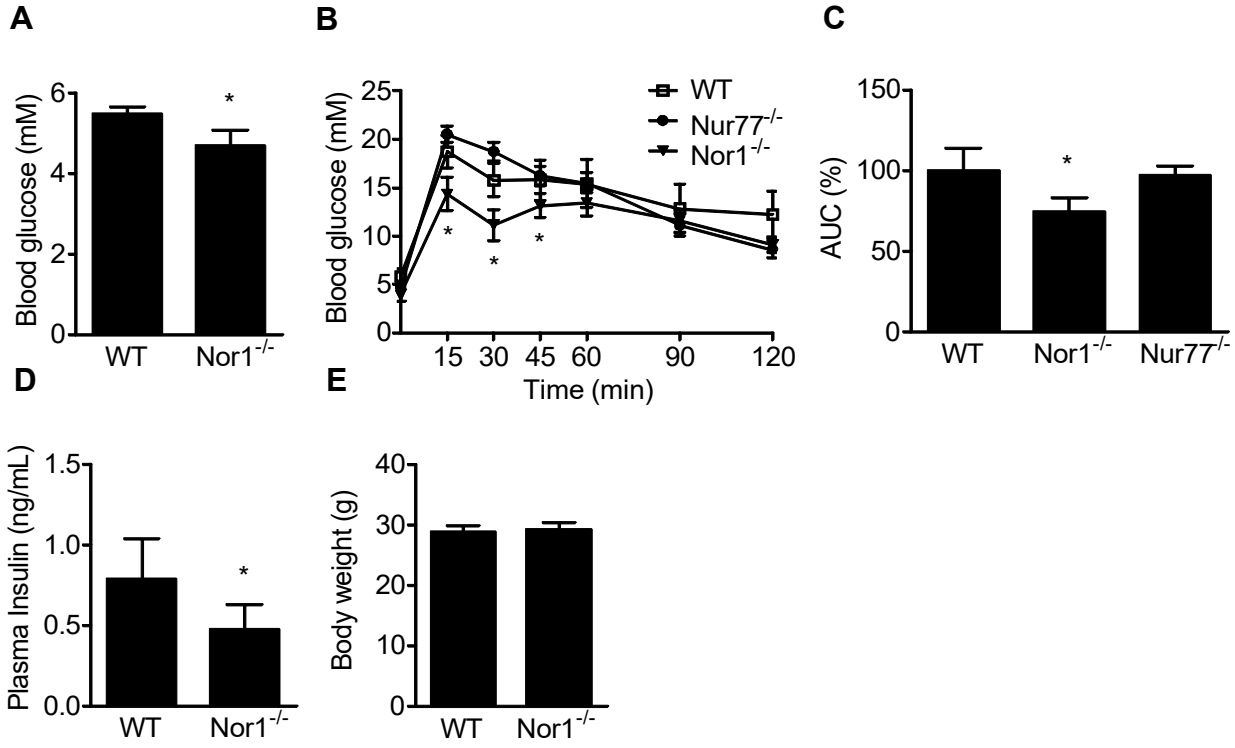


Figure 3.2: Invalidation of Nor1 improves glucose tolerance. A) Random fed blood glucose in wild type vs *Nor1*$^{-/-}$ mice. B) Glucose tolerance test in WT, *Nor1*$^{-/-}$ and *Nur77*$^{-/-}$ mice. C) Area under the curve. D) Plasma insulin levels 60 minutes after glucose injection. E) Body weight of 6-month old wild type and *Nor1*$^{-/-}$ mice. All results are represented as means +/- SEM; *, $p < 0.05$ versus wild type animals.

Nor1 expression is upregulated by pro-inflammatory cytokines

Whereas Nor1 has been extensively studied in muscle [37], its regulation and biological functions remain relatively unexplored in beta-cells. We therefore studied the expression profile of Nor1 and explored the mechanisms by which the nuclear receptor could exert its effect on β-cell mass.

We thus investigated the tissue distribution of Nor1 in the pancreas by immunofluorescence in mice (Fig 3.3). Our results indicate that in wild-type pancreata Nor1 is most strongly expressed in islets and co-localizes with both insulin (Fig 3.3A) and glucagon (Fig 3.3B). The signal for Nor1 was absent in tissue sections from *Nor1*$^{-/-}$ mice, indicating the specificity of the detected signal.

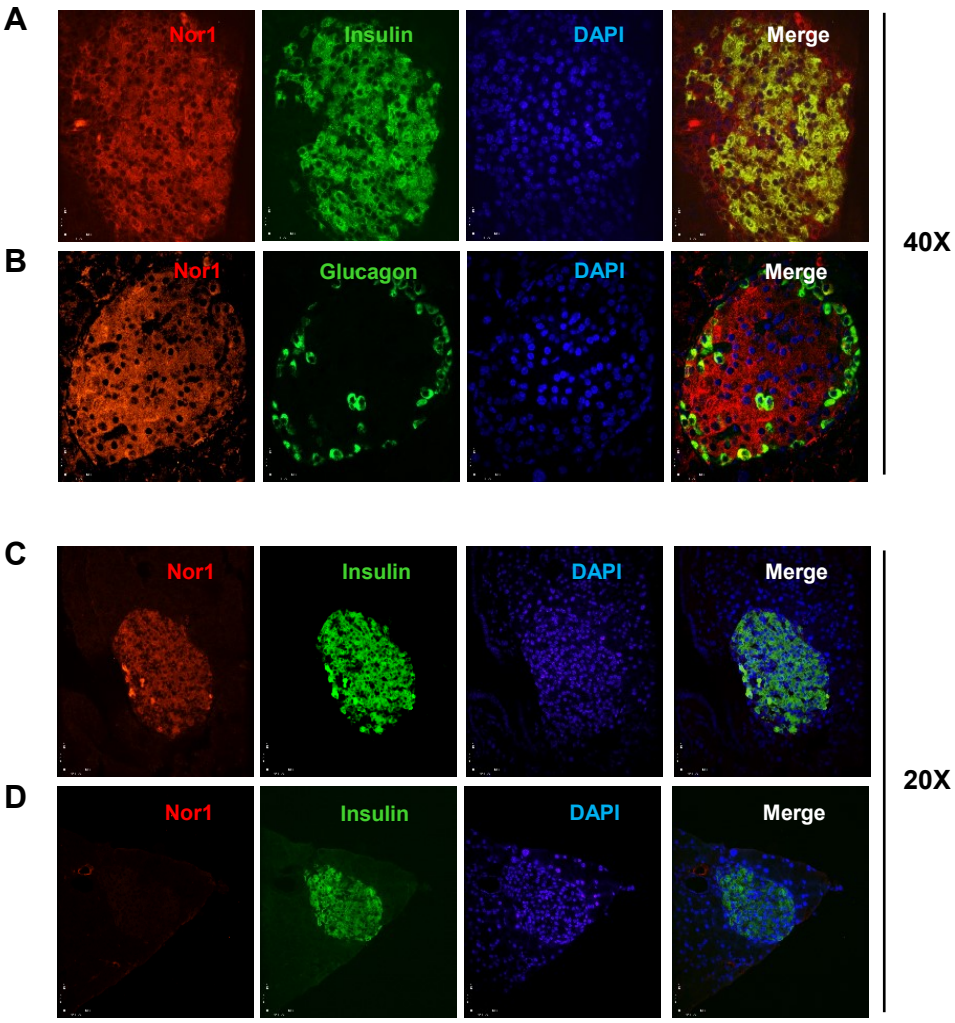


Figure 3.3: Nor1 expression in pancreatic islets. A) Double immunofluorescence staining for Nor1 (red) and insulin (green) in wild type mice. B) Double immunofluorescence staining for Nor1 (red) and glucagon (green) in wild type mice. C-D) Double immunofluorescence staining for Nor1 and insulin in WT (C) and *Nor1*$^{-/-}$ animals (D).[3]

To investigate the regulation of Nr4a gene expression, we exposed INS cells to either a cytokine cocktail comprising IL-1β and IFNγ or elevated concentrations of glucose (glucotoxicity) for various periods of time ranging from 0 to 24 hours, and measured the expression of all 3 members of the *Nr4a* family by qPCR. Cytotoxic concentrations of cytokines induced a rapid

[3] Dr. Nidheesh Dadheech provided technical guidance concerning the immunofluorescence experiments and performed the staining and image capturing on the Nor1$^{-/-}$ sections.

and transient increase in the expression of *Nor1*, with a maximum induction of ~14-fold at 4h (Fig 4.4A). Interestingly, this effect was relatively specific to *Nor1*. Indeed, *Nur77* was increased by a much lesser extent compared to *Nor1* (~2-fold at 1h) whereas *Nurr1* expression remained unaltered. Suppl Fig 3.1 expresses the results differently to illustrate the relative expression of each Nr4a member before (Panel A) and after cytokine treatment (Panel B). High glucose induced a ~5-fold increase in *Nor1* expression after 8 hours (Fig 3.4B). Once again, this effect of glucose was specific to *Nor1* since *Nur77* and *Nurr1* mRNA levels were not significantly altered. We confirmed the upregulation of Nor1 by cytokines at the protein level. Exposure of INS cells to cytokines for 6 h caused a 3-fold increase in Nor1 protein levels (Fig 3.4C).

We next sought to confirm the effects of cytokines on *Nor1* mRNA expression in human islets. Consistently with our previous results obtained in INS cells, cytokines upregulated *Nor1* expression in human islets (Fig 3.4D), albeit to a lesser extent. On the contrary, both *Nur77* and *Nurr1* mRNA levels remained unchanged (Fig 3.4E-F).

To further characterize the effects of cytokines on *Nor1* expression, we tested increasing concentrations of IL-1β and IFNγ, and evaluated the respective contribution of each cytokine singly. *Nor1* expression was increased in a dose-dependent manner in response to the cytokine cocktail in INS cells (Fig 3.4G). Also, when both cytokines are used separately the effect of IL-1β on Nor1 expression was greater than that of IFNγ alone (~6-fold versus ~1.5-fold respectively) (Fig 3.4H).

Altogether, these results identify *Nor1* as a cytokine-responsive gene in β-cells and thus raise the possibility that Nor1 could exert pro-apoptotic effects in β-cells. Indeed, such a pro-apoptotic action of Nor1 would dovetail with the increased β-cell mass observed in *Nor1*$^{-/-}$ animals.

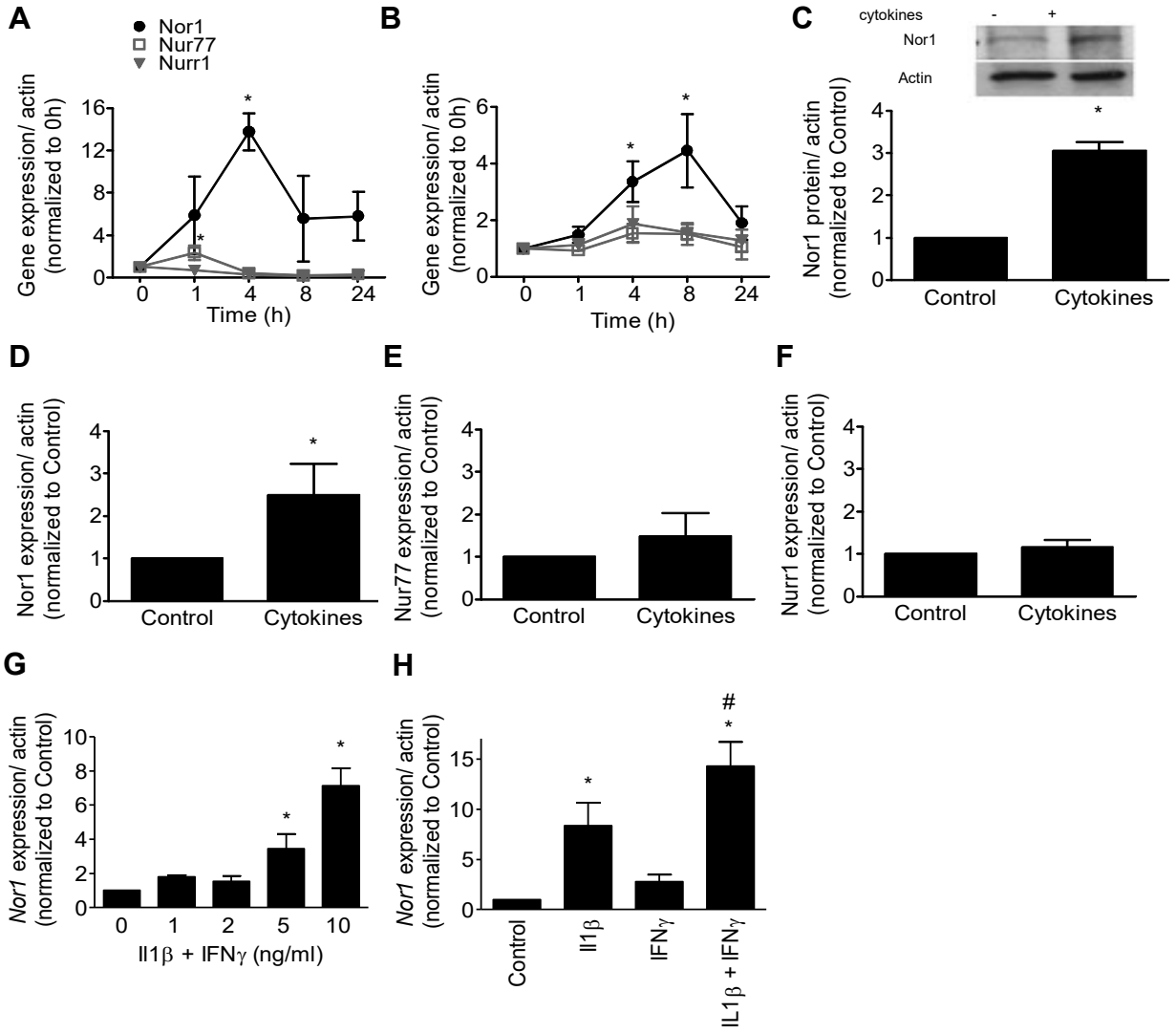


Figure 3.4: Nor1 is upregulated by pro-inflammatory cytokines in INS and human *beta*-cells. A-B) mRNA levels of *Nor1*, *Nur77* and *Nurr1* was determined by qPCR in INS832/13 cells exposed to cytokines (IL-1β 10 ng/ml and IFNγ 10 ng/ml) in A or an elevated concentration of glucose (25 mM, G25) in B for the indicated periods of time (n=5). Results were normalized to actin and are expressed as fold change over non-stimulated cells. C) Nor1 protein levels were determined in INS832/13 cells incubated for 6h in the presence or absence of cytokines (n=4). Inset: a representative image is shown. D-F) The effect of cytokines on *Nor1*, *Nur77* and *Nurr1* expression was determined by qPCR in human islets after 8 h of treatment (n=3). G) Dose-response relationship between *Nor1* gene expression and cytokine concentrations. INS832/13

cells were exposed to increasing concentrations of IL-1β and IFNγ for 4 h (n=5). H) The effects of IL-1β and IFNγ 10 ng/ml were tested separately or in combination for 4 h (n=5). All results are represented as means +/- SEM; *, $p < 0.05$ versus control; #, $p < 0.05$ versus IL-1β.

Nor1 is a novel mediator of β-cell death

To investigate the potential pro-apoptotic role of Nor1 in β-cells, we sought to perform gain-of-function and loss-of-function studies using Nor1 overexpression and siRNA-mediated knockdown, respectively. Fig 3.5A shows that Nor1 protein levels were increased by 3- to 4-fold in INS cells overexpressing Nor1-GFP or Nor1-Flag compared to control cells. We then transfected INS cells with either Nor1-Flag or a control vector before incubating them in the presence or absence of cytokines. Nor1 overexpression induced a ~3-fold increase in apoptosis (Fig 3.5B) and this effect was not additive to that of cytokines. The pro-apoptotic action of Nor1 was confirmed in human islets. Islet cells were dispersed, seeded into 24 wells plates and transfected with either Nor1-GFP or a control GFP plasmid. Then, we measured apoptosis by counting the number of TUNEL-positive (red) nuclei in the transfected cell population only (green). Ectopic expression of Nor1 increased apoptosis by 5-fold (Fig 3.5C).

To further test the hypothesis that Nor1 represents a novel mediator of cytokine-induced β-cell apoptosis, we performed siRNA-mediated *Nor1* knockdown (Fig 3.5D). Cytokine-induced β-cell apoptosis was completely abolished by two different siRNA sequences (Fig 3.5E), thereby indicating that Nor1 is necessary for the pro-apoptotic action of cytokines in β-cells.

To gain insight into the molecular mechanisms by which Nor1 causes β-cell apoptosis, we tested the effects of Nor1 on cytochrome c release in β-cells. Indeed, it was previously suggested that Nor1 could translocate to the mitochondria and activate the intrinsic (mitochondrial) pathway of apoptosis in thymocytes [38]. Fig 3.5F shows that ectopic expression of Nor1 induced cytochrome c release from the mitochondria, to the same extent as cytokines. This result suggests that Nor1-mediated β-cell apoptosis involves the intrinsic pathway as well. Future studies should delve deeper into the mechanisms by which Nor1 promotes β-cell death.

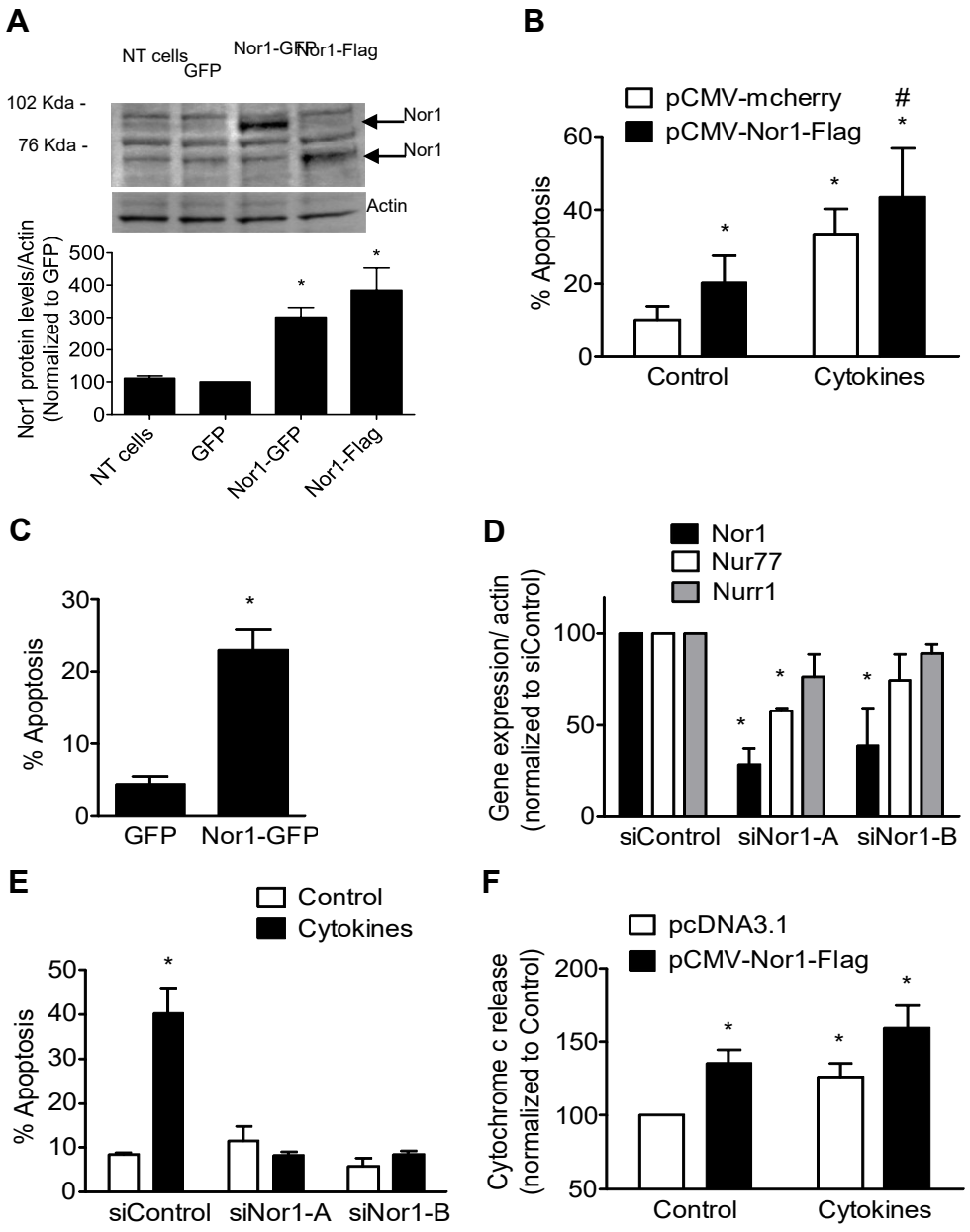


Figure 3.5: Nor1 plays a critical role in β-cell apoptosis. A) Nor1 protein levels were evaluated in non-transfected INS832/13 cells (NT) or 24 hours after transfection with a control GFP plasmid, Nor1-GFP, or Nor1-Flag (n=5). All results are represented as means +/- SEM; *, $p < 0.05$ versus control GFP. Arrows indicate Nor1-GFP (migrating at a higher molecular weight) and Nor1-Flag. B) Apoptosis was assessed by TUNEL assay in INS832/13 cells transfected with Nor1 (black bars) or a control vector (white bars) and subsequently incubated in the presence or absence of cytokines for 24 h (n=3). C) Apoptosis was measured by TUNEL assay in dispersed

human islets 24h after transfection with Nor1-GFP or GFP alone. The results are represented as the percentages of TUNEL-labelled nuclei in the population of GFP-positive cells (n=3). D) *Nor1*, *Nur77* and *Nurr1* expression was evaluated by qPCR in INS832/13 cells 48h after transfection with a control siRNA or 2 different Nor1 specific siRNA sequences (n= 3). E) siRNA-mediated knockdown of Nor1 prevents cytokine-induced apoptosis. Determination of apoptosis by TUNEL in INS832/13 cells transfected with control or Nor1 specific siRNAs and incubated in the presence (black bars) or absence (white bars) of cytokines as described above (n=3). F) Nor1 causes cytochrome c release. Cells were treated as described in (B), fractionated into cytoplasmic and mitochondrial fractions, and analyzed by ELISA (n=4). All results are represented as means +/- SEM; *, $p < 0.05$ versus control; # $p < 0.05$ versus PCMV-Nor1-Flag without cytokines.

To further characterize the biological roles of Nor1 in β-cells, we measured insulin secretion in INS cells overexpressing Nor1 (Fig 3.6). Ectopic expression of Nor1 inhibited glucose-stimulated insulin secretion but did not affect KCl-induced exocytosis.

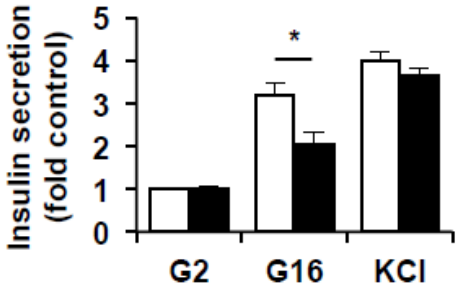


Figure 3.6: Nor1 impairs glucose-stimulated insulin secretion. Cells were transfected with either Nor1 (black bars) or a control plasmid (white bars). The next day, insulin secretion was measured in cells treated with 2 mM glucose (G2), 16 mM glucose (G16) or 35 mM KCl, and cells were lysed for determinations of insulin content. Insulin secretion data were normalized to insulin content. Results are means +/- SEM (n=4). *, $p<0.05$.[4]

[4] Barbara Villela helped immensely with both the realization and the counting of the TUNEL experiments.

Nor1 expression is increased in type 2 diabetes

Because IL-1β is upregulated in diabetic islets [39], we reasoned that Nor1 could be increased in islets of patients with type 2 diabetes. Fig 3.7 shows that *Nor1* mRNA levels are increased by ~15-fold in human islets obtained from patients with type 2 diabetes compared to healthy individuals. We thus postulate that the upregulation of *Nor1* in type 2 diabetic islets could contribute to β-cell demise and may represent a target for therapeutic interventions.

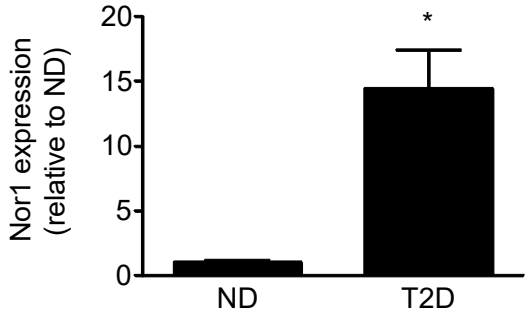


Figure 3.7: Nor1 is upregulated in type 2 diabetic islets. We compared *Nor1* expression in isolated human islets from healthy or type 2 diabetic donors (n=7). Results shown are means +/- SEM; *, $p < 0.05$.

3.4 Discussion

The biological roles of Nr4as in metabolic diseases are gathering increasing interest [16, 40]. Whereas their roles in liver and muscle have been studied extensively, their biological actions in pancreatic β-cells remain relatively unexplored.

We herein demonstrate that adult *Nor1*$^{-/-}$ mice show increased β-cell mass and that this phenotype is not reiterated by genetic deletion of either *Nur77* or *Nurr1*. The increase in β-cell mass in *Nor1*$^{-/-}$ animals was accompanied by lower blood glucose in the random fed state and improved glucose tolerance during ipGTT. We believe the effect of Nor1 on β-cell mass involves, at least in part, an increase in pancreatic β-cell replication since we detected abundant Ki67 staining in *Nor1*$^{-/-}$ mice. This distinct phenotype of *Nor1*$^{-/-}$ mice is consistent with the unique DNA binding properties of Nor1, which support distinct biological functions for Nor1 compared to its two Nr4a orthologs. Indeed, Nur77 and Nurr1 can bind the Nur responsive element (NurRE) as homodimers [41, 42] whereas Nor1 displays a much lower affinity for the NurRE resulting into a negligible transcriptional activation [41, 42]. In addition, both Nur77 and Nurr1, but not Nor1, can form heterodimers or interact with retinoid X receptors (RXRs) to target DR5 elements [8, 43]. Thus, Nor1 displays distinct binding characteristics to DNA regulatory elements and cofactors. This dovetails with a previous report which has demonstrated that Nur77 and Nor1 regulate distinct sets of genes in β-cells [26].

Because our study employed whole body *Nor1*$^{-/-}$, it is unclear whether the effects of Nor1 on pancreatic β-cell mass are direct or secondary to changes in other tissues. To our knowledge, Floxed-Nor1 mice have not been generated yet, depriving us of conducting tissue-specific deletion of Nor1 in β-cells. Thus, to specifically address this question, we resorted to investigate Nor1 regulation and potential role in apoptosis *in vitro* in INS cells and isolated human islets.

Our expression study shows that Nor1 expression is rapidly increased by cytokines. Indeed, cytokine treatments elicited a ~14-fold and ~3-fold increase in Nor1 expression in INS cells and human islets, respectively. High glucose also induced Nor1 expression but its effect was less pronounced and occurred later. Interestingly, high glucose was found to stimulate production of IL-1β in β-cells [39]. Since we herein demonstrate that IL-1β is a key inducer of *Nor1*

expression in β-cells, this raises the possibility that glucose induces *Nor1* expression via the production of pro-inflammatory cytokines. Be that as it may, the upregulation of *Nor1* upon cytokine treatments hinted towards a pro-apoptotic role for Nor1 in β-cells, an effect that over time could in part explain the increase in β-cell mass observed in *Nor1*$^{-/-}$ mice.

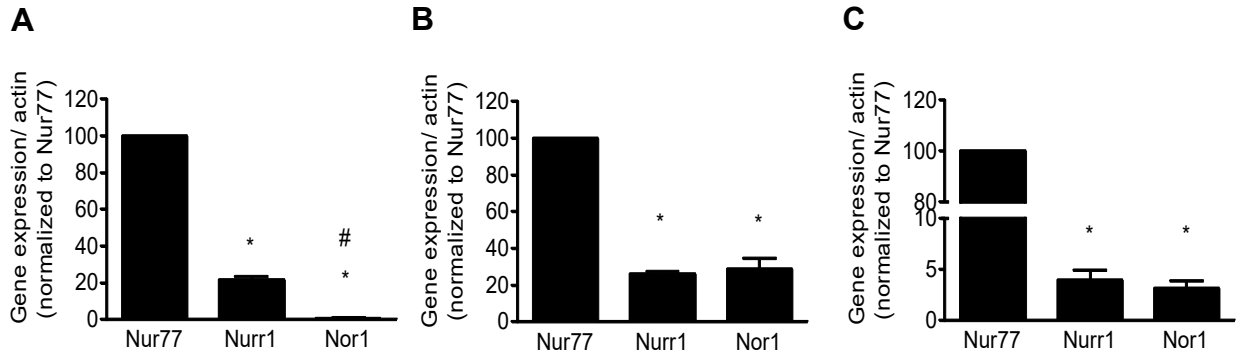
Our functional assays using gain and loss of function for Nor1 in INS cells and human islets demonstrated a critical role for Nor1 in β-cell apoptosis. This holds great clinical significance since Butler et al. showed that type 2 diabetic patients present a lower β-cell mass compared to healthy subjects and that this reduction was associated with increased rates of apoptosis [3]. Since we detected an increase in *Nor1* expression in type 2 diabetic islets, our study strongly suggests that Nor1 could participate in the β-cell demise that precedes diabetes onset. To unravel a potential mechanism by which Nor1 could promote β-cell apoptosis, we tested its potential effect on the intrinsic pathway of apoptosis. Nor1 was found to provoke cytochrome c release in INS cells, suggesting that Nor1-mediated β-cells apoptosis could involve mitochondrial stress. This is reminiscent of its role in thymocytes where Nor1 was demonstrated to relocate to the mitochondria and to associate with Bcl family members to cause apoptosis [38]. Our findings, describing Nor1 as a negative regulator of β-cell mass expansion, conflict with those presented in a recent study [33]. Indeed, Tessem and colleagues characterized Nur77 and Nor1 as transcriptional targets of Nkx6.1 in β-cells. They also showed that both Nur77 and Nor1 were necessary for Nkx6.1-mediated β-cell proliferation in rat islets via a mechanism that could implicate transcriptional repression of *p21*. The causes of this discrepancy are unknown. However, whereas the authors confirmed their results in Nur77$^{-/-}$ mice, they did not study *Nor1*$^{-/-}$ animals.

Single nucleotide polymorphisms (SNPs) located within the *Nor1* locus have been associated with increased insulin secretion and a higher insulinogenic index in diabetic as well as non-diabetic patients [29, 31]. At this point, it is unclear whether the improvement in insulin secretion in individuals bearing this mutation results from improved islet function, increased β-cell mass or both. Further, some controversies reside over the role of Nor1 in insulin secretion. In one study, Nor1 was suggested to mediate Exendin4-induced potentiation of glucose-induced insulin secretion [29]. Conversely, another study characterized Nor1 as a negative regulator of insulin

secretion [44]. Our own results align with the latter since, in our hands, Nor1 was found to impair glucose-stimulated insulin secretion. Interestingly, Nor1 did not affect KCl-evoked exocytosis suggesting a potential metabolic effect of Nor1.

In conclusion, our work shows that the nuclear receptor Nor1 is a key mediator of cytokine-induced apoptosis in β-cells, thereby characterizing Nor1 as a negative regulator of β-cell mass. Since we also show that Nor1 is increased in type 2 diabetes, we propose that Nor1 represents an interesting molecular target for therapeutic intervention.

3.5 Supplementary figure



Suppl Fig 3.1: Relative expression of Nr4a members in β-cells and human islets. (A-B) *Nur77, Nurr1* and *Nor1* mRNA levels were determined by qPCR in INS832/13 cells incubated during 4h in the absence (A) or presence (B) of cytokines (Il1β 10 ng/ml and IFNγ 10 ng/ml). (C) Nr4as expression was evaluated by qPCR in human islets. Results are presented as means +/- SEM, n=3 for each. *, $p < 0.05$ versus Nur77; #, $p < 0.05$ versus Nurr1.

3.6 References

1. Saltiel AR. New perspectives into the molecular pathogenesis and treatment of type 2 diabetes. Cell 2001; 104:517-529
2. Meier JJ, Bonadonna RC. Role of reduced beta-cell mass versus impaired beta-cell function in the pathogenesis of type 2 diabetes. Diabetes Care 2013; 36 Suppl 2:S113-119
3. Butler AE, Janson J, Bonner-Weir S, Ritzel R, Rizza RA, Butler PC. Beta-cell deficit and increased beta-cell apoptosis in humans with type 2 diabetes. Diabetes 2003; 52:102-110
4. Meier JJ, Breuer TG, Bonadonna RC, Tannapfel A, Uhl W, Schmidt WE, Schrader H, Menge BA. Pancreatic diabetes manifests when beta cell area declines by approximately 65% in humans. Diabetologia 2012; 55:1346-1354
5. Rhodes CJ. Type 2 diabetes-a matter of beta-cell life and death? Science 2005; 307:380-384
6. Prentki M, Nolan CJ. Islet beta cell failure in type 2 diabetes. J Clin Invest 2006; 116:1802-1812
7. Committee NRN. A unified nomenclature system for the nuclear receptor superfamily. Cell 1999; 97:161-163
8. Kurakula K, Koenis DS, van Tiel CM, de Vries CJ. NR4A nuclear receptors are orphans but not lonesome. Biochim Biophys Acta 2014; 1843:2543-2555
9. Lammi J, Aarnisalo P. FGF-8 stimulates the expression of NR4A orphan nuclear receptors in osteoblasts. Mol Cell Endocrinol 2008; 295:87-93
10. Arredondo C, Orellana M, Vecchiola A, Pereira LA, Galdames L, Andres ME. PIASgamma enhanced SUMO-2 modification of Nurr1 activation-function-1 domain limits Nurr1 transcriptional synergy. PLoS One 2013; 8:e55035
11. Galleguillos D, Vecchiola A, Fuentealba JA, Ojeda V, Alvarez K, Gomez A, Andres ME. PIASgamma represses the transcriptional activation induced by the nuclear receptor Nurr1. The Journal of biological chemistry 2004; 279:2005-2011
12. Han YH, Cao X, Lin B, Lin F, Kolluri SK, Stebbins J, Reed JC, Dawson MI, Zhang XK. Regulation of Nur77 nuclear export by c-Jun N-terminal kinase and Akt. Oncogene 2006; 25:2974-2986
13. Kovalovsky D, Refojo D, Liberman AC, Hochbaum D, Pereda MP, Coso OA, Stalla GK, Holsboer F, Arzt E. Activation and induction of NUR77/NURR1 in corticotrophs by CRH/cAMP: involvement of calcium, protein kinase A, and MAPK pathways. Mol Endocrinol 2002; 16:1638-1651
14. Volakakis N, Malewicz M, Kadkhodai B, Perlmann T, Benoit G. Characterization of the Nurr1 ligand-binding domain co-activator interaction surface. J Mol Endocrinol 2006; 37:317-326
15. Wingate AD, Campbell DG, Peggie M, Arthur JS. Nur77 is phosphorylated in cells by RSK in response to mitogenic stimulation. Biochem J 2006; 393:715-724

16. Close AF, Rouillard C, Buteau J. NR4A orphan nuclear receptors in glucose homeostasis: A minireview. Diabetes Metab 2013; 39:478-484

17. Lévesque D RC. Role of Members of the Nur (NR4A) Transcription Factors in Dopamine-Related Neurodegenerative and Neuropsychiatric Disorders. In: Ritsner M e, ed. The Handbook of Neuropsychiatric Biomarkers, Endophenotypes and Genes Netherlands: Springer; 2009:195-210.

18. Fu Y, Luo L, Luo N, Zhu X, Garvey WT. NR4A orphan nuclear receptors modulate insulin action and the glucose transport system: potential role in insulin resistance. The Journal of biological chemistry 2007; 282:31525-31533

19. Kanzleiter T, Wilks D, Preston E, Ye J, Frangioudakis G, Cooney GJ. Regulation of the nuclear hormone receptor nur77 in muscle: influence of exercise-activated pathways in vitro and obesity in vivo. Biochim Biophys Acta 2009; 1792:777-782

20. Pei L, Waki H, Vaitheesvaran B, Wilpitz DC, Kurland IJ, Tontonoz P. NR4A orphan nuclear receptors are transcriptional regulators of hepatic glucose metabolism. Nat Med 2006; 12:1048-1055

21. Veum VL, Dankel SN, Gjerde J, Nielsen HJ, Solsvik MH, Haugen C, Christensen BJ, Hoang T, Fadnes DJ, Busch C, Vage V, Sagen JV, Mellgren G. The nuclear receptors NUR77, NURR1 and NOR1 in obesity and during fat loss. Int J Obes (Lond) 2012; 36:1195-1202

22. Catoire M, Mensink M, Boekschoten MV, Hangelbroek R, Muller M, Schrauwen P, Kersten S. Pronounced effects of acute endurance exercise on gene expression in resting and exercising human skeletal muscle. PLoS One 2012; 7:e51066

23. Kawasaki E, Hokari F, Sasaki M, Sakai A, Koshinaka K, Kawanaka K. Role of local muscle contractile activity in the exercise-induced increase in NR4A receptor mRNA expression. J Appl Physiol (1985) 2009; 106:1826-1831

24. Mahoney DJ, Parise G, Melov S, Safdar A, Tarnopolsky MA. Analysis of global mRNA expression in human skeletal muscle during recovery from endurance exercise. FASEB J 2005; 19:1498-1500

25. Berriel Diaz M, Lemke U, Herzig S. Discovering orphans' sweet secret: NR4A receptors and hepatic glucose production. Cell Metab 2006; 4:339-340

26. Briand O, Helleboid-Chapman A, Ploton M, Hennuyer N, Carpentier R, Pattou F, Vandewalle B, Moerman E, Gmyr V, Kerr-Conte J, Eeckhoute J, Staels B, Lefebvre P. The nuclear orphan receptor Nur77 is a lipotoxicity sensor regulating glucose-induced insulin secretion in pancreatic beta-cells. Mol Endocrinol 2012; 26:399-413

27. Chao LC, Wroblewski K, Ilkayeva OR, Stevens RD, Bain J, Meyer GA, Schenk S, Martinez L, Vergnes L, Narkar VA, Drew BG, Hong C, Boyadjian R, Hevener AL, Evans RM, Reue K, Spencer MJ, Newgard CB, Tontonoz P. Skeletal muscle Nur77 expression enhances oxidative metabolism and substrate utilization. J Lipid Res 2012; 53:2610-2619

28. Chao LC, Zhang Z, Pei L, Saito T, Tontonoz P, Pilch PF. Nur77 coordinately regulates expression of genes linked to glucose metabolism in skeletal muscle. Mol Endocrinol 2007; 21:2152-2163

29. Ordelheide AM, Gerst F, Rothfuss O, Heni M, Haas C, Thielker I, Herzberg-Schafer S, Bohm A, Machicao F, Ullrich S, Stefan N, Fritsche A, Haring HU, Staiger H. Nor-1, a novel incretin-responsive regulator of insulin genes and insulin secretion. Mol Metab 2013; 2:243-255

30. Pearen MA, Eriksson NA, Fitzsimmons RL, Goode JM, Martel N, Andrikopoulos S, Muscat GE. The nuclear receptor, Nor-1, markedly increases type II oxidative muscle fibers and resistance to fatigue. Mol Endocrinol 2012; 26:372-384

31. Weyrich P, Staiger H, Stancakova A, Schafer SA, Kirchhoff K, Ullrich S, Ranta F, Gallwitz B, Stefan N, Machicao F, Kuusisto J, Laakso M, Fritsche A, Haring HU. Common polymorphisms within the NR4A3 locus, encoding the orphan nuclear receptor Nor-1, are associated with enhanced beta-cell function in non-diabetic subjects. BMC Med Genet 2009; 10:77

32. Nonogaki K, Kaji T, Ohba Y, Sumii M, Wakameda M, Tamari T. Serotonin 5-HT2C receptor-independent expression of hypothalamic NOR1, a novel modulator of food intake and energy balance, in mice. Biochemical and biophysical research communications 2009; 386:311-315

33. Tessem JS, Moss LG, Chao LC, Arlotto M, Lu D, Jensen MV, Stephens SB, Tontonoz P, Hohmeier HE, Newgard CB. Nkx6.1 regulates islet beta-cell proliferation via Nr4a1 and Nr4a3 nuclear receptors. Proc Natl Acad Sci U S A 2014; 111:5242-5247

34. Lee SL, Wesselschmidt RL, Linette GP, Kanagawa O, Russell JH, Milbrandt J. Unimpaired thymic and peripheral T cell death in mice lacking the nuclear receptor NGFI-B (Nur77). Science 1995; 269:532-535

35. Rojas P, Joodmardi E, Hong Y, Perlmann T, Ogren SO. Adult mice with reduced Nurr1 expression: an animal model for schizophrenia. Mol Psychiatry 2007; 12:756-766

36. Hohmeier HE, Mulder H, Chen G, Henkel-Rieger R, Prentki M, Newgard CB. Isolation of INS-1-derived cell lines with robust ATP-sensitive K+ channel-dependent and -independent glucose-stimulated insulin secretion. Diabetes 2000; 49:424-430

37. Pearen MA, Goode JM, Fitzsimmons RL, Eriksson NA, Thomas GP, Cowin GJ, Wang SC, Tuong ZK, Muscat GE. Transgenic muscle-specific Nor-1 expression regulates multiple pathways that effect adiposity, metabolism, and endurance. Mol Endocrinol 2013; 27:1897-1917

38. Thompson J, Burger ML, Whang H, Winoto A. Protein kinase C regulates mitochondrial targeting of Nur77 and its family member Nor-1 in thymocytes undergoing apoptosis. Eur J Immunol 2010; 40:2041-2049

39. Maedler K, Sergeev P, Ris F, Oberholzer J, Joller-Jemelka HI, Spinas GA, Kaiser N, Halban PA, Donath MY. Glucose-induced beta cell production of IL-1beta contributes to glucotoxicity in human pancreatic islets. J Clin Invest 2002; 110:851-860

40. Pearen MA, Muscat GE. Minireview: Nuclear hormone receptor 4A signaling: implications for metabolic disease. Mol Endocrinol 2010; 24:1891-1903

41. Wilson TE, Fahrner TJ, Milbrandt J. The orphan receptors NGFI-B and steroidogenic factor 1 establish monomer binding as a third paradigm of nuclear receptor-DNA interaction. Mol Cell Biol 1993; 13:5794-5804

42. Maira M, Martens C, Philips A, Drouin J. Heterodimerization between members of the Nur subfamily of orphan nuclear receptors as a novel mechanism for gene activation. Mol Cell Biol 1999; 19:7549-7557

43. Gissendanner CR, Kelley K, Nguyen TQ, Hoener MC, Sluder AE, Maina CV. The Caenorhabditis elegans NR4A nuclear receptor is required for spermatheca morphogenesis. Dev Biol 2008; 313:767-786

44. Gao W, Fu Y, Yu C, Wang S, Zhang Y, Zong C, Xu T, Liu Y, Li X, Wang X. Elevation of NR4A3 Expression and Its Possible Role in Modulating Insulin Expression in the Pancreatic Beta Cell. PLoS One 2014; 9

3.7 Additional results

In the interest of clarity, a group of results have been left out of the article. However, these results provide interesting information concerning the expression and role of Nor1 in β-cells. They are therefore presented here.

3.7.1 Nor1 promoter activity

Cytokines significantly increase Nor1 expression in β-cells. The effect of cytokines on Nor1 expression rises to reach a peak after 4 hours of exposition before to progressively decrease. We sought to investigate how cytokines modulate Nor1 expression. More precisely, we aimed to determine on which DNA sequence in the *Nor1* gene cytokines exert their effect to regulate Nor1 expression.

Methods

To do so, we used a Dual-Luciferase® Reporter Assay (E1910, Promega, Madison, WI, USA). INS832/13 cells were co-transfected with a control reporter vector (pGL4-hRLuc for *Renilla* Luciferase) and one of the following Firefly Luciferase-conjugated vector containing a 500-1000 base pair DNA sequence of the Nor1 gene:

- pGL3Nor1_Luc1 (base pair 0 to 1000)
- pGL3Nor1_Luc2 (base pair -1000 to 0)
- pGL3Nor1_Luc3 (base pair 1000 to 1500)

These vectors were a kind gift from Dr. Nick Barlev (University of Leicester, UK). Transfected cells were plated in a clear-bottom, white walled, 96 wells plate, 9 wells per plasmid pair and 70000 cells/well. Cells were then left overnight in a 37ºC and 5% CO_2 atmosphere to allow adherence to the wells before they were treated with cytokines (Il1β 10 ng/mL and IFNγ 10 ng/mL) during 0 (control), 1 or 4 hours. Samples were then lysed in the plate using passive lysis buffer provided with the kit and a freeze/thaw cycle. Luciferin, the substrate of the Firefly Luciferase, was then added and Firefly Luciferase bioluminescent reaction was measured. The reaction was quenched and the luminescent reaction of *Renilla* Luciferase was activated and

subsequently measured. All measurements were done in triplicate. The activity of the experimental reporter (Firefly Luciferase) was normalized to the activity of the control reporter.

Results

Cytokines significantly increased the activity of the reporter conjugated to the Nor1 DNA sequence located between base pair 0 and 1000 (Fig 3.8). This effect was observed after both 1h and 4h of exposure to cytokines with no difference between the 2 times. Cytokines also significantly decreased the promoter activity of Nor1 after 4h.

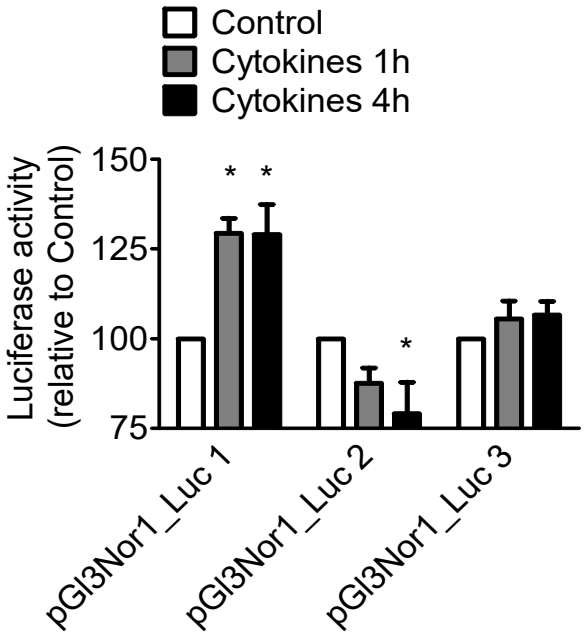


Figure 3.8: Cytokines modulate Nor1 expression via an element located within the first 1000 base pairs of the Nor1 gene. INS832/13 cells were transfected with different regions of the Nor1 gene linked to Firefly Luciferase (pGL3Nor1_Luc1, 2 or 3) and then exposed to cytokines during 0 to 4 hours. Firefly Luciferase activity was measured and normalized on the activity of the co-transfected pGL4-linked *Renilla* Luciferase. Results are expressed relative to control for each vector pair. N=3. All results are represented as means +/- SEM; *, $p < 0.05$ versus control.

We expected cytokines to positively affect Nor1 expression by an action on the promoter. The fact that cytokines exert their stimulating effect on Nor1 expression via a transcribed region of the gene was unexpected. More detailed evaluation of the molecular mechanism of Nor1

regulation by cytokines is needed. However, these preliminary results suggest that further analysis would not be straight forward. Therefore, in the interest of time, we decided not to pursue this line of investigation.

3.7.2 Nor1 expression in mice models of diabetes

Nor1 expression is increased by cytokines *in vitro*. Pro-inflammatory cytokines (Il1β, IFNγ and TNFα) are elevated in the blood stream in obesity and are driving β-cell death in type 1 diabetes [69, 215]. We therefore aimed to evaluate if Nor1 is increased in the β-cells of animal models of obesity and type 1 diabetes.

For type 1 diabetes, we used the best available mouse model: the NOD/SchiLtJ mouse [216]. At the time of weaning, NOD mice start presenting a mononuclear infiltrate around their islets (peri-insulitis). That infiltrate is mostly constituted of T cells (CD4+ and CD8+) but also contains NK cells, B lymphocytes, dendritic cells and macrophages [217, 218]. This peri-insulitis evolves into invasive insulitis at 10-15 weeks of age, affecting 100% of mice by 20 weeks [216, 218]. The consequence of insulitis is the specific immune destruction of β-cells and the subsequent spontaneous development of diabetes in 60-80% of female mice and 20-30% of males [217].

For obesity, we used the high fat diet model. It has been shown that obesity, accompanied by low grade systemic inflammation, insulin resistance and impaired fasting glucose develop when certain mouse strains (AKR/J, C57L/J, A/J, C3H/HeJ, DBA/2J, and C57BL/6J) are fed a diet containing 40-60% of calories from fat during 8-16 weeks [219-221]. The degree of hyperglycemia depends on the % of calories from fat in the diet and the duration of the diet.

Methods

Female 8 weeks old NOD mice were received from the Jackson Laboratory (Bar Harbor, ME, USA). The mice were housed three per cage on soft bedding in a temperature-controlled environment (held at a constant temperature of 20°C), on a 12h-light/dark cycle (lights on at 6:15 A.M.) with *ad libitum* access to food and water. Blood glucose was measured bi-weekly (Figure 3) with FreeStyle Lite test strips (Abbott Diabetes Care, Alameda, CA, USA). The pancreas of 5 mice were removed upon diagnosis of diabetes (non-fasting glycaemia ≥ 11.1 mmol/L on 2 consecutive days) as well as the pancreas of 4 non-diabetic controls. Pancreas were then

analyzed by immunofluorescence as described in 3.2. Images were analyzed using Perkin Elmer Spinning Disk ERS confocal microscope with Hamamatsu C9100-50 EMCCD camera and the Volocity imaging software.

6 C57BL/6J mice were fed a high-fat (60 kcal% fat) or control diet (10 kcal% fat) for 8 weeks (The Jackson Laboratory, Bar Harbor, ME). Mice were weighed and then sacrificed immediately upon reception. Pancreas were removed and analyzed by immunofluorescence as described in 3.2.

Results

Except for mouse 33, the NOD mice that became diabetic (mice 21, 22, 23 and 32), developed the disease between 15 and 19 weeks of age (Fig 3.9). The first non-diabetic control mouse was sacrificed at 9 weeks old and presented a mononuclear infiltrate around its islets. All the other NOD mice also presented signs of peri-insulitis or insulitis as revealed by DAPI staining (Fig 3.11). We also observed an apparent reduction of the intensity of the insulin signal (green) in the islets of the diabetic animals. Cytokines increase Nor1 expression in INS1 cells. We therefore expected it to be increased as well in the β-cells of the NOD mice because the islets are surrounded by mononuclear cells that secrete cytokines. Surprisingly, the red signal seems to be rather decreased in the β-cells of NOD mice compared to wild type animals with no apparent difference between ages and glycaemia.

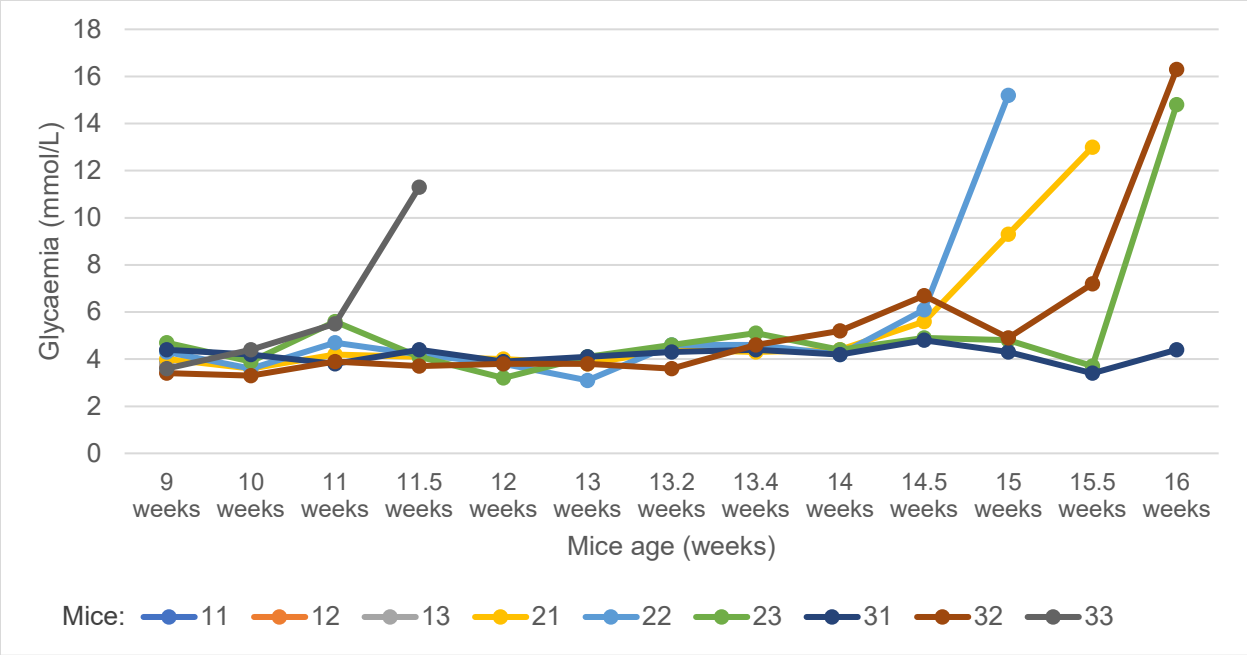


Figure 3.9: Evolution of the NOD mice glycaemia in mmol/L. 9 female NOD mice were received at 8 weeks old. Blood glucose was measured bi-weekly with FreeStyle Lite test strips.

The weights of the mice after 8 weeks of a high fat *versus* control diet is reported in figure 3.10. The mice fed a high fat diet have a significantly higher weight then the control animals. Mice fed a high fat diet develop low grade systemic inflammation as well as insulitis [221]. Il1β can also be detected in their islets. We therefore expected to observe an increase of Nor1 expression in the islets of the high fat fed animals. However, such an observation could not be made (Fig 3.11).[5]

[5] Dr. Nidheesh dadheech kindly helped with the monitoring of the mice glycaemia.

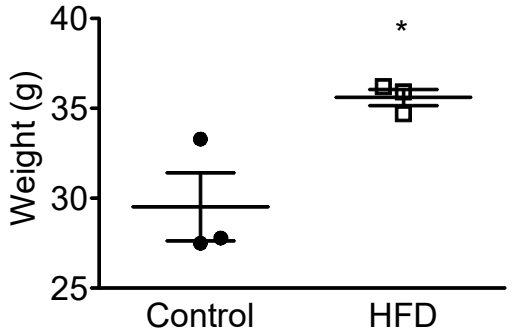


Figure 3.10: Weights in g of C57BL/6J mice after 8 weeks of high fat diet (HFD, 60% kcal fat) *versus* a control diet (10% kcal fat). Results are represented as means +/- SEM; *, $p < 0.05$ versus Control.

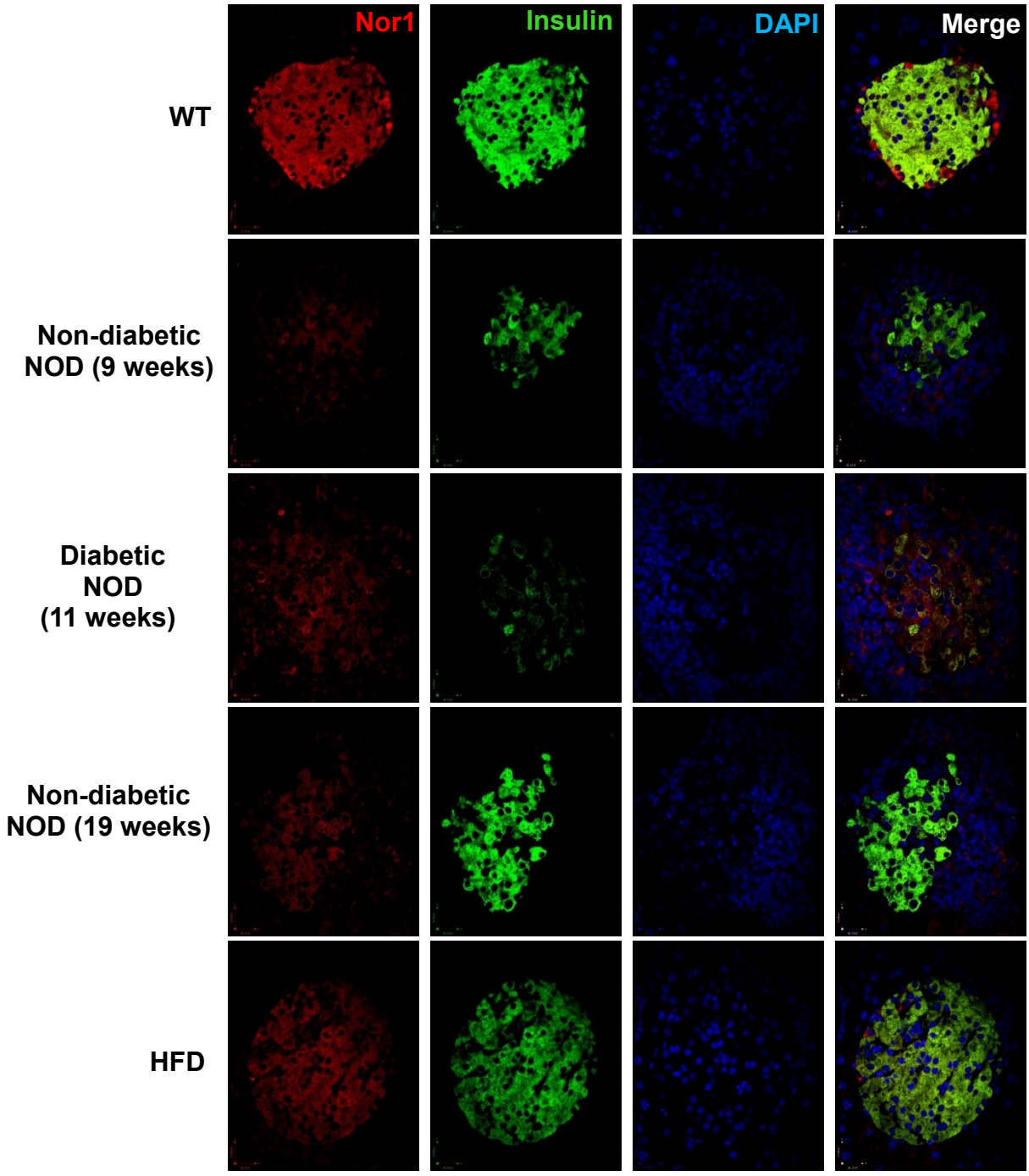


Figure 3.11: Nor1 expression in the β-cells of obese and diabetic mice. Representative images of double immunostaining of pancreatic sections from wild type mice, 2 non-diabetic NOD mice at different ages, a diabetic NOD mouse and a C57BL/6J mouse fed a high fat diet containing 60% of calories from fat (HFD) for 8 weeks. Nor1 appears in red and insulin in green.

It is difficult to draw conclusions relative to expression based on immunofluorescence alone. Indeed, immunofluorescence is not a quantitative method. Moreover, in this case, the insulin signal (green) varies between mice. It is strongly lower in the NOD mice and slightly lower in the HFD mice compared to the wild types. If there is a component of leaking from the green channel to the red, variations of the insulin signal could affect the Nor1 signal and make the images uninterpretable.

A quantitative approach of Nor1 expression in animal models of type 1 diabetes and obesity would be the evaluation of Nor1 RNA levels (qPCR) and protein levels (Western blot) in the isolated islets of these animals.

3.7.3 Insulin content

There is no consensus regarding the effect of Nor1 on insulin gene expression and insulin secretion. Gao and colleagues suggested that Nor1 negatively regulates relative insulin secretion as well as *Ins1* and *Ins2* mRNA levels [181]. However, it has also been suggested that Nor1 exerts a beneficial effect on insulin secretion and expression [178, 179]. Moreover, there is only one published report of the effect of Nor1 on insulin protein content. Indeed, Reynolds et al showed that silencing Nor1 in INS1 cells did not affect insulin content [179].

We therefore sought to investigate the effect of Nor1 on insulin gene expression and insulin content in our model.

Methods

INS832/13 cells were transfected with Nor1Flag, Nor1GFP or control vectors by nucleofection. *Ins1* mRNA levels were measured by RT-qPCR as described before.

For insulin content and protein content, transfected cells were plated in 24 wells plates (400000 cells/well). Cells were lysed 30h after transfection. For insulin content, cells were submitted to an overnight freeze/thaw cycle in 1.5% acidic ethanol (70% ethanol and 1.5% pure HCl). The lysates were then centrifuged and insulin measured in the supernatant using EZRMI-13K rat/mouse insulin Elisa kit (EMD Millipore, Etobicoke, ON). For protein content, cells were lysed in a buffer containing 50 mM Tris (pH 8.0), 150 mM NaCl and 1% v/v Triton X-100 and

supplemented with 1 tablet/10 ml of protease inhibitors (Complete Mini, Roche Diagnostics, Mannheim, Germany). Protein concentrations were determined by BCA protein assay.

Results

Insulin mRNA levels were not modified by Nor1 in INS1 cells (Fig 3.12A). Nor1 overexpression significantly decreased insulin content but that effect was lost when insulin content was normalized on protein content (Fig 3.12B). The negative effect that Nor1 exerts on insulin and protein content might be attributed to the pro-apoptotic effect of Nor1 in β-cells.

We concluded that, in accordance with the results obtained by Reynolds et al, Nor1 does not affect insulin expression in INS1 cells [179].

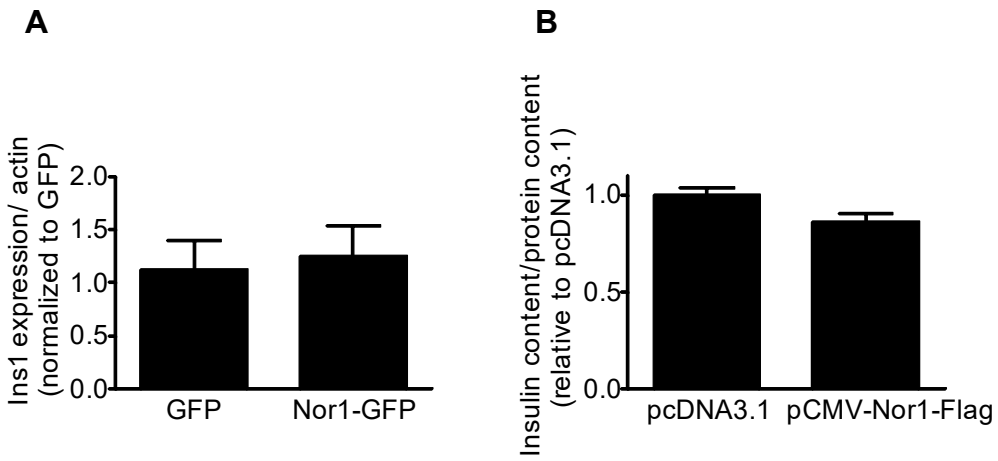


Figure 3.12: Insulin expression is not modulated by Nor1. INS832/13 cells were transfected with Nor1Flag, Nor1GFP or control vectors. A) 16h after transfection, Ins1 mRNA levels were measured by RT-qPCR. N=3. B) 30h after transfection, cells were lysed and insulin content as well as protein content were evaluated. N=3. All results are represented as means +/- SEM; *, $p < 0.05$ versus control (GFP or pcDNA3.1).

Chapter 4: Disruption of β-cell mitochondrial networks by the orphan nuclear receptor Nor1/Nr4a3

A version of this chapter has been submitted in April 2017 to Molecular Metabolism: Anne-Françoise Close, Claude Rouillard and Jean Buteau. Title: Disruption of beta-cell mitochondrial networks by the orphan nuclear receptor Nor1/Nr4a3.

4.1 Introduction

Type 2 diabetes is characterized by a progressive deterioration of both beta-cell mass and function, resulting in a relative deficit in insulin secretion [1, 2]. Indeed, significant reductions in beta-cell mass have been reported in individuals with type 2 diabetes [3, 4] an effect that implicates beta-cell apoptosis [3, 5]. However, the specific pathways and mediators that regulate beta-cell death have not yet been entirely identified.

The Nr4a family of orphan nuclear receptors comprises 3 members in mammalian cells: Nur77/Nr4a1, Nurr1/Nr4a2 and Nor1/Nr4a3 [6]. Our most recent study characterized Nor1 as a novel mediator of cytokine-induced beta-cell death. Indeed, Nor1-KO animals displayed increased beta-cell mass and improved glucose tolerance. Genetic manipulations of Nor1 in INS and human beta-cells demonstrated the pro-apoptotic actions of Nor1. Importantly, Nor1 expression was robustly elevated in islets from type 2 diabetic donors, suggesting that Nor1 could participate in the beta-cell demise that underpins the disease. However, the precise molecular mechanisms by which Nor1 could regulate beta-cell mass and function remain unclear. Previous studies have suggested that Nor1, in a manner similar but to a lesser extent than Nur77, could exert its transcriptional activity to regulate the expression of genes involved in glucose-induced insulin secretion and beta-cell proliferation [7, 8]. However, the sub-cellular localization of Nor1 has, to our knowledge, never been extensively investigated in beta-cells. Also, its biological actions are not fully understood.

Because, Nr4as do not have any identified ligands [9], their regulation is thought to be dependent on post-transcriptional modifications that dictate their sub-cellular localization. For instance, in cancer cells, Nur77 has been shown to promote cell growth when it is restrained to the nucleus [10]. In contrast, when Nur77 is translocated to the mitochondria, it has been shown to physically interact with Bcl2. Nur77 binding exposes the BH3 death domain of Bcl2, resulting in cytochrome c release and apoptosis [10-15]. Nor1 has been suggested to trigger the same pathway in thymocytes [16].

We herein aimed to address these knowledge gaps by (i) studying Nor1 sub-cellular localization in beta-cells, and (ii) use this information to derive important cues about the molecular mechanisms by which Nor1 could affect beta-cell death and function.

4.2 Material and Methods

Reagents ─RPMI 1640, fetal bovine serum, L-glutamine and all other cell culture reagents were purchased from Life Technologies (Burlington, ON).

Cell Culture ─ INS (832/13) [222] cells (passage 57-80) were grown in RPMI 1640 medium containing 11 mM glucose and supplemented with 10 mM HEPES, 10% heat-inactivated fetal bovine serum, 2 mM L-glutamine, 1 mM sodium pyruvate, and 50 μM β-mercaptoethanol at 37ºC in a humidified 5% CO_2 atmosphere. Our cytokine treatment consisted in a combination of IL1β (10 ng/ml) and IFNγ (10 ng/ml).

Human Islets ─Human islets were purchased from the Alberta Diabetes Institute Islet Core at the University of Alberta with the assistance of the Human Organ Procurement and Exchange Program and the Trillium Gift of Life Network who provide donor pancreata for research. For mitophagy quantification, islets from 4 different healthy lean donors between 50 and 65 years of age were used. Islets were cultured in 11 mM glucose RPMI medium supplemented with 10 mM HEPES, 10% heat-inactivated fetal bovine serum, 2 mM L-glutamine, 1 mM sodium pyruvate, 1% penicillin-streptomycin and 50 μM β-mercaptoethanol at 37ºC in a humidified 5% CO_2 atmosphere. Our study was approved by the Human Research Ethics Board at the University of Alberta.

Plasmid transfection ─pCMV-Nor1-GFP, pCMV-Nor1-FLAG, peGFP-LC3 and pcDNA3.1 were obtained from Origene (Rockville, MD). For INS832/13 cells: plasmids were introduced at a concentration of 5 μg of DNA for $6x10^6$ cells via nucleofection (Lonza, Allendale, NJ). Transfection efficiency was >90%. For human islets: cells were dispersed using 0.05% trypsin-EDTA and transfected the following day using Lipofectamine 2000 (Life Technologies, Burlington, ON) according to manufacturer's protocol. We obtained ~50% transfection efficiency. Cells were assayed 30h post-transfection.

Mitochondria staining ─Transfected cells were seeded onto 18mm poly-L-lysine (Sigma-Aldrich, Saint-Louis, MO) coated coverslips ($2x10^6$ cells per coverslip). 30h post-transfection, they were incubated in the presence or absence of cytokines for 1h. Media was then changed to RPMI 1640 containing 25 nM Mitotracker® Red CMXRos (Molecular Probes, Eugene, OR) and 10 μg/mL Hoechst33342 (BD Bioscience, San Jose, CA) with or without 20 μM FCCP (Cayman chemical, Ann Arbor, MI) for 15 minutes. The cells were then washed once with PBS. Live cell

images were acquired on a WaveFX spinning disk confocal microscope with a Hamamatsu C9100-50 EMCCD camera and analyzed using the Volocity imaging software.

Glucose oxidation ─Glucose oxidation was measured as described before [223] in non-ventilated 25 cm^2 cell culture flasks. Briefly, cells were pre-incubated in 2 mM glucose KRB medium for 30 minutes before being treated with 2, 7 or 11 mM glucose. 5µCi of [14C(U)]-D-glucose (Moravek Biochemicals, Brea, CA) was added to each flask. All metabolic reactions were stopped after 45 min by adding H_2SO_4 to the media. 14C-CO_2 was captured using fiberglass fibre filters and counted using a scintillation counter.

Glucose uptake ─Glucose uptake was assessed using a commercial kit (Glucose Uptake-Glo™Assay, Promega, Madison, WI). Cells were treated as described above in section 2.5 for glucose oxidation assays, except that 5% of the total glucose of each condition was 2-deoxyglucose.

ATP production ─Cells were washed with cold PBS and lysed in a buffer containing 10 mM Tris pH 7.5, 100 mM NaCl, 1 mM EDTA and 0.01% Triton X100. ATP was quantified using the ATP Determination Kit (Molecular Probes, Eugene, OR).

Metabolomic analysis ─INS832/13 cells were collected in cold HPLC grade methanol before to be snap frozen in liquid nitrogen and sent to The Metabolomics Innovation Center (University of Victoria, Victoria, BC). There, cells were lysed by vibrating homogenization. The metabolites were further extracted with ultra-sonication of the sample tubes. The samples were clarified by centrifugation. The supernatants were taken out for the following UPLC-MRM/MS analyses and the protein pellets were used for protein quantitation using a standard BCA procedure. All the quantitative analyses were conducted by ultrahigh-performance liquid chromatography–tandem mass spectrometry (UPLC-MS/MS) with multiple-reaction monitoring (MRM) scans. The LC-MS system was a Dionex Ultimate 3400RS UPLC instrument or a Waters Acquity UPLC system coupled to a Sciex 4000 QTRAP mass spectrometer. The concentrations were calculated from linearly regressed calibration curves of individual acids which were prepared with the use of their standard substances and 9 stable isotope-labeled carboxylic acids as internal calibration (IS) for quantitation.

Mitochondrial membrane potential ─Cells were treated in the presence or the absence of cytokines during 4h before the assay was performed. 20 µM FCCP was used as a negative control and 25 mM glucose as a positive control. Mitochondrial membrane potential was then

evaluated using JC-1 Mitochondrial Membrane Potential Assay Kit (Cayman chemicals, Ann Arbor, MI).

Mitochondrial ultrastructure ─Cells were pre-fixed in 4% glutaraldehyde in 0.2M sodium cacodylate buffer, post-fixed in 1% osmium tetroxide (OsO4) in 0.1M sodium cacodylate buffer, dehydrated in an ethyl alcohol series, embedded with epoxy resin, and thermally polymerized as previously described [224, 225]. Ultra thin-sections (70 nm) were cut by an ultramicrotome (Leica Microsystems, ON, Canada) and then stained with 4% uranyl acetate and Reinold's lead citrate. The contrasted sections were imaged using a Hitachi H-7650 transmission electron microscope at 80 kV equipped with a 16 megapixel EMCCD camera (XR111, Advanced Microscopy Technique, MA, USA).[6]

Quantification of mitophagy ─Fluorescence microscopy: Cells were transfected with peGFP-LC3 and with either Nor1-Flag or a control empty vector. 30h after transfection, cells were imaged as described in section 2.4. The colocalization of peGFP-LC3 positive vacuoles and mitochondria was determined using the Imaris software (Bitplane, Switzerland). Electron microscopy: The number of autophagic vacuoles targeting the mitochondria was determined as described before [226]. Briefly, we examined 3 grid squares per condition. In each square, images were taken at 400x to assess whole cell areas and at 12000x to count the number of autophagic vacuoles.

Quantification of mitochondrial DNA ─Mitochondria were isolated using a Cell Fractionation Kit (Abcam, Toronto, ON) and DNA was purified using a commercial kit (Qiagen, Germantown, MD). For detection of mtDNA, PCR was carried out using the following primers: mtCOX1 forward, 5'-GGAGCAGTATTCGCCATCAT-3' and reverse, 5'-ACGACGAGGTATCCCTGCTA-3'. The results were normalized to genomic DNA using: TBP forward, 5'-CTCAGTTACAGGTGGCAGCA-3' and reverse, 5'-CAGCACAGAGCAAGCAACTC-3'.

Western blot ─Cells were lysed in a buffer containing 50 mM Tris (pH 8.0), 150 mM NaCl and 1% v/v Triton X-100 and supplemented with protease inhibitors (Complete Mini, Roche Diagnostics, Mannheim, Germany). Cytosolic and mitochondrial protein extracts were obtained using a Cell Fractionation Kit (Abcam, Toronto, ON). Protein concentrations were determined by BCA protein assay. Equal amounts of heat-denaturated proteins from each treatment group

[6] I am extremely grateful to Dr. Woo Jung Cho for preparing the TEM samples and helping with data interpretation.

were run on Novex 10% Tris-Glycine gels (Life Technologies, Burlington, ON) and electrically transferred to nitrocellulose membranes. After blocking for 1h at room temperature with 1% BSA, membranes were incubated overnight at 4ºC with primary antibodies. The next day, membranes were incubated with Horseradish-Peroxidase linked secondary antibodies followed by exposition to Amersham ECL Western Blotting Detection Reagents (GE Healthcare, Mississauga, ON) and film development. The primary antibodies used in our studies were: rabbit anti-VDAC antibody (Cell Signaling, Danvers, MA) (1:1000) and rabbit anti-Tubulin antibody (Cell Signaling, Danvers, MA) (1:1000).

Citrate Synthase Activity ─Cells were harvested in a solution containing methylsulfonylmethane (MSM)/EDTA supplemented with 1% Sodium Cholate Hydrate. Citrate synthase activity was then evaluated by measuring the conversion of 5,5' dithiobis 2 nitrobenzoic acid (DTNB, 0.1 mM) into 2-nitro-5-benzoic acid (TNB), which absorbs specifically at 412 nm. The reaction was carried out in a buffer containing 0.25% triton X100, 0.5 mM oxaloacetate and 0.31 mM acetyl-CoA.

Statistical analysis ─Data are presented as means ± SEM. Statistical analyses were performed with GraphPad Prism® using ANOVA followed by Bonferroni post-test.

4.3 Results

Nor1 translocates to the mitochondria in response to pro-inflammatory cytokines.
Our previous study identified a critical role of the nuclear receptor Nor1 in cytokine-induced β-cell apoptosis (submitted). However, the mode of action of Nor1 remained elusive and, to our knowledge, the transcriptional targets of Nor1 have not been the subject of extensive investigation. We thus sought to define the expression profile of β-cells overexpressing Nor1, reasoning that this would provide important cues about its biological function. We analyzed the transcriptome of INS832/13 cells transfected with Nor1 *versus* a control vector by RNA sequencing (Suppl Fig 4.1). Surprisingly, relatively few genes were significantly altered in Nor1-expressing cells. Only 18 genes were differentially expressed using a cut-off false discovery rate of 0.05. Moreover, the vast majority of those genes were encoded by the mitochondrial genome.

We then examined the sub-cellular localization of Nor1-GFP in β-cells treated with a cytokine cocktail comprising IL1β (10 ng/ml) and IFNγ (10 ng/ml). Mitochondrial and nuclei were stained with Mitotracker Red and Hoescht, respectively. In untreated cells, Nor1-GFP showed a diffuse cytosolic signal, which did not colocalize with the nucleus or the mitochondria (Fig 4.1). However, upon cytokine treatment Nor1 was found to translocate to the mitochondria, as detected by the perfect overlap of the green and red signals. These observations cast some doubts on the possibility that Nor1 acts as a transcription factor in β-cells and prompted us to investigate a potential effect of Nor1 on mitochondrial function.

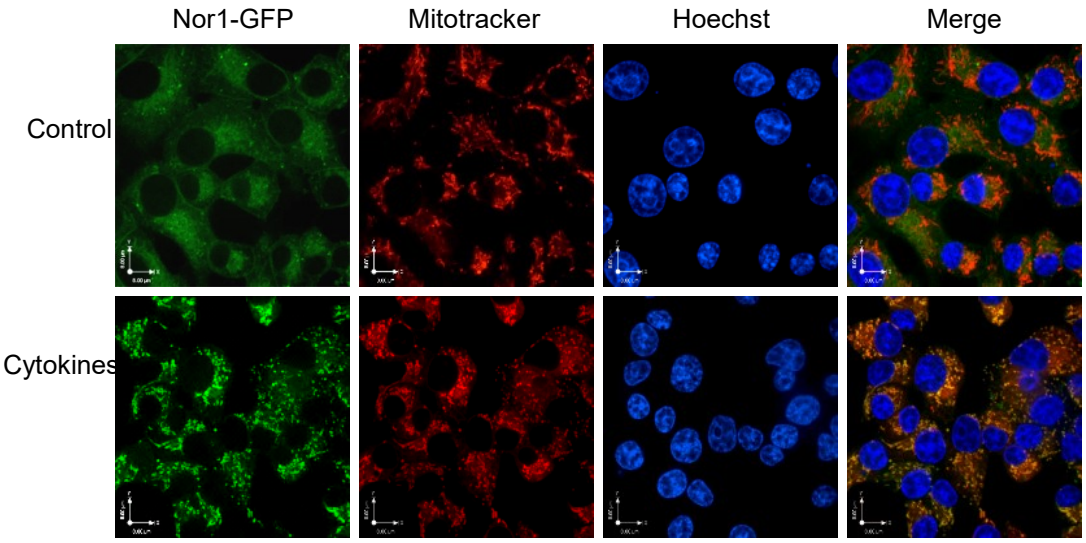


Figure 4.1: Nor1 translocates to the mitochondria in response to cytokines in INS832/13-cells**.** Images show representative INS832/13 cells transfected with either Nor1-GFP or a control GFP plasmid and subsequently incubated in the presence or in the absence of cytokines (IL-1β 10 ng/ml and IFNγ 10 ng/ml) for 1 hour. Mitochondria are stained in red (Mitotracker) and nuclei in blue (Hoechst).

Nor1 affects mitochondrial function.

Nor1 has been shown to interact with Bcl-2 in immune cells [206]. We thus investigated whether Nor1 could bind Bcl-2 in β-cells by conducting co-immunoprecipitation experiments. Our results suggest that Nor1 does not bind Bcl-2, whether be it in the absence or presence of cytokines (Suppl Fig 4.2).

We then sought to measure the effect of Nor1 on glucose metabolism and ATP production in INS832/13 cells, and to use a metabolomics approach to define the metabolic perturbations linked to Nor1 overexpression. Nor1 significantly reduced glucose oxidation in cells exposed to intermediate (7 mM) or high (11 mM) glucose concentrations (Fig 4.2A) compared to control cells. This effect was not due to a reduction in glucose uptake (Fig 4.2B), suggesting that glucose metabolism is impaired. Consistently, Nor1 decreased the production of ATP by ~30% in cells exposed to 11 mM glucose, an effect that was not additive to the effect of cytokines (Fig 4.2C). We reasoned that the decrease in ATP production could be the result of increased anaerobic glycolysis in Nor1-expressing cells and measured lactate production. Lactate levels were

increased by ~50% in Nor1-transfected cells (Fig 4.2D). We also used targeted metabolomic profiling to interrogate the glycolytic and TCA pathways and further characterize Nor1 action. The results are presented in supplementary figure 4.3. We detected significant changes in several glycolytic intermediates and TCA metabolites, revealing a metabolic thread by which Nor1 could affect cellular energy homeostasis.

Nor1 significantly increased mitochondrial membrane potential by ~30% (Fig 4.2D). This is reminiscent of a model [22-24] in which hyperpolarization of the inner mitochondrial membrane in pro-apoptotic conditions has been proposed to provoke mitochondrial matrix swelling and ruptures in the outer membrane. We thus examined mitochondria morphology by TEM in INS cells transfected with Nor1 or a control vector (Fig 4.2E-G). Nor1-expressing cells displayed mitochondrial matrix swelling and subsequent herniation through breaks in the outer membrane. These features were absent in control cells. Outer mitochondrial membrane ruptures allow for the release of cytochrome c from the intermembrane space into the cytosol. We have shown that Nor1 does indeed significantly increase cytochrome c release by ~35% in β-cells (submitted). Altogether, these results unveil a mechanism by which Nor1 could activate the intrinsic pathway of apoptosis in β-cells.

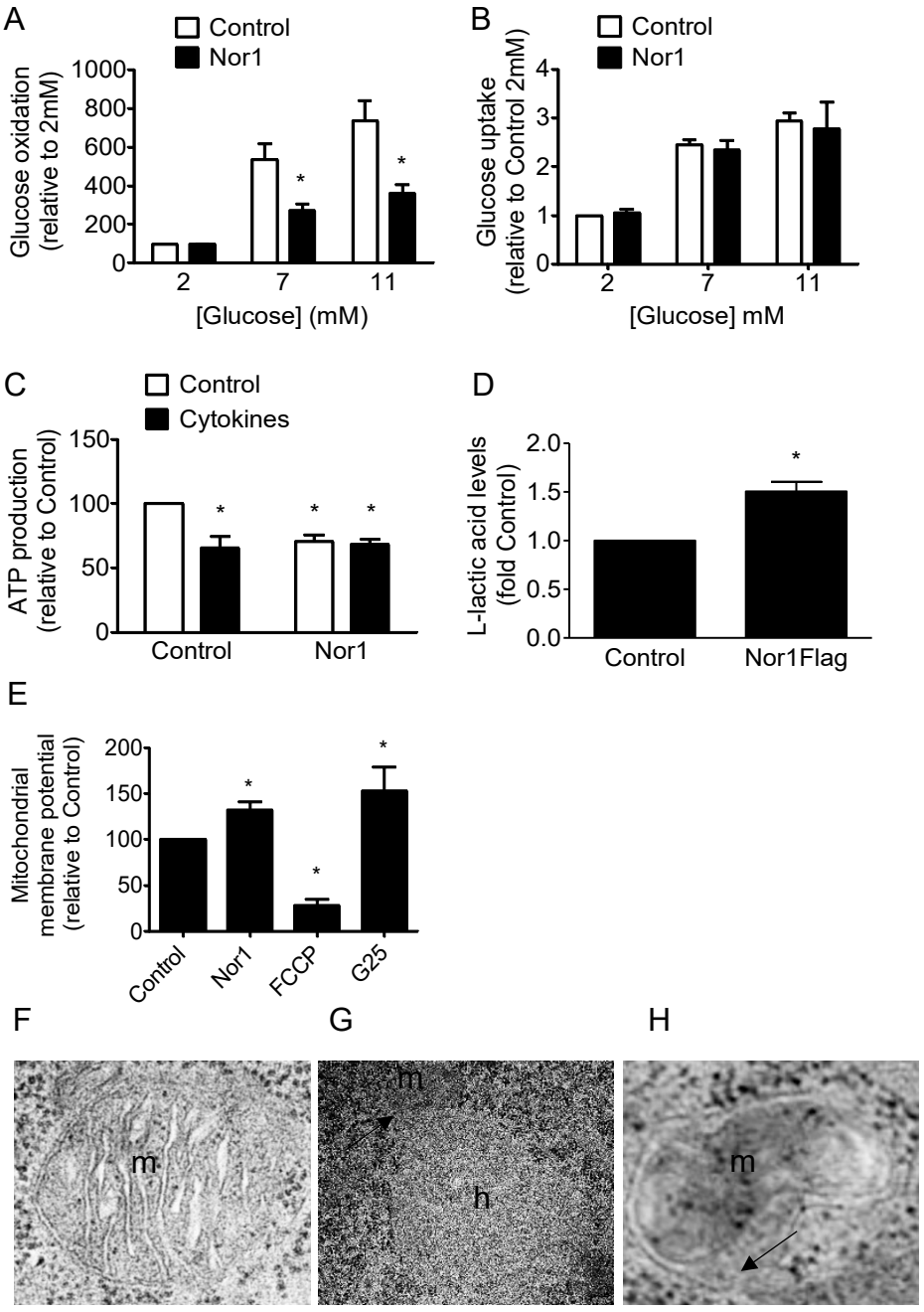


Figure 4.2: Nor1 impairs mitochondrial function in β-cells. Glucose oxidation (A) and glucose uptake (B) were measured in INS832/13 cells transfected with Nor1 (black bars) or a control vector (white bars) and exposed to 2, 7 or 11 mM glucose during 45 minutes. C) ATP production in INS832/13 cells transfected with Nor1 or a control vector in the presence (black bars) or in the absence (white bars) of cytokines. (D) L-Lactic acid levels in INS832/13 cells transfected with Nor1 or a control vector. (E) Mitochondrial membrane potential was determined by evaluating

JC-1 fluorescence in INS832/13 cells transfected with Nor1 *versus* control. Cells were exposed to 20 μM FCCP (15 minutes) and 25 mM glucose (4 hours) as negative and positive controls, respectively. F-G-H) Representative TEM images of mitochondria from INS832/13 cells transfected with Nor1 (G-H) *versus* control (F). m, mitochondria; h, hernia; arrows identify breaks in the outer mitochondrial membrane. Magnification 12000-50000x. All results are represented as means +/- SEM of 3 separate experiments. *, $p < 0.05$ *versus* control.

Nor1 induces mitochondrial fragmentation in β-cells.

We then studied a potential disrupting action of Nor1 on the mitochondrial networks by fluorescence microscopy (Fig 4.3). Our images show a strong disruption of the networks in Nor1-expressing cells. Indeed, mitochondria displayed a more punctuated aspect in INS cells and dispersed human islet cells transfected with Nor1 compared to controls (Fig 4.3A-B). Moreover, mitochondrial fragmentation was also observed in TEM images (Fig 4.3C). Measurements of mitochondrial lengths revealed that Nor1 induces a shift in the distribution of mitochondria towards smaller sizes (Fig 4.3D). Indeed, there was an accumulation of smaller mitochondria (< 499 nm in size) in cells transfected with Nor1 compared to control. On the contrary, large mitochondria of >1500 nm were virtually absent in Nor1-expressing cells. We next evaluated if the malfunctioning, fragmented mitochondria were subjected to mitophagy.

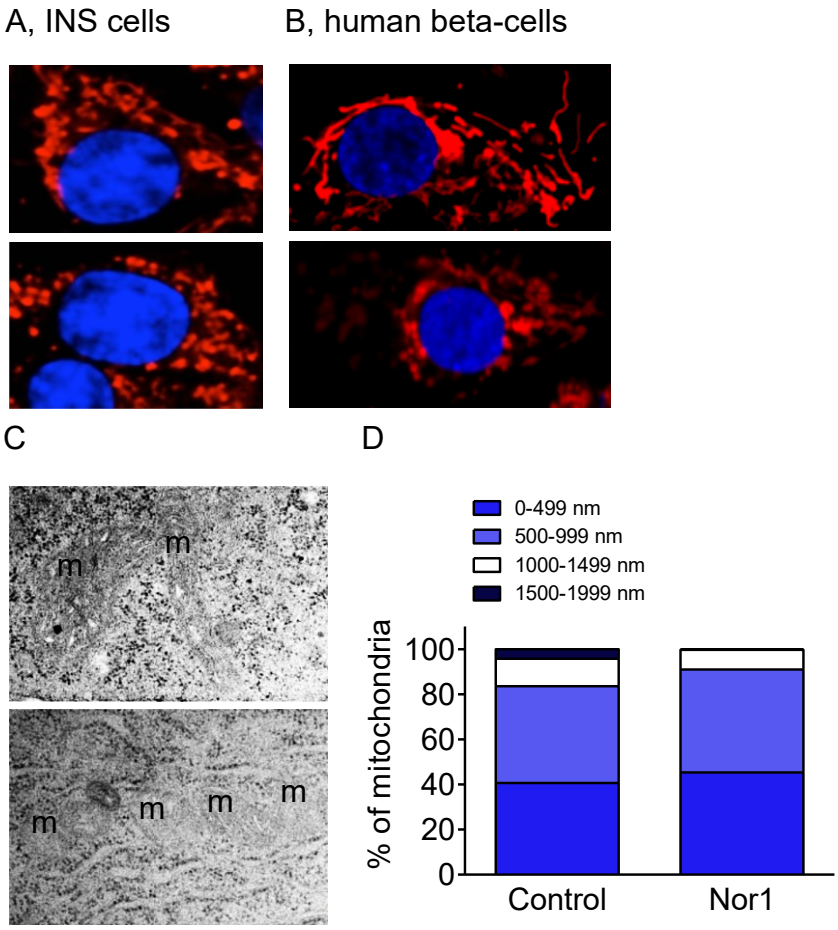


Figure 4.3: Nor1 induces mitochondrial fragmentation in β-cells. Representative images of INS832/13 cells (A) or dispersed human islet cells (B) transfected with Nor1 (bottom) or a control vector (top). Mitochondria were stained using Mitotracker Red, and the nuclei using Hoechst. Magnification 60x. C) TEM images showing mitochondrial fragmentation in INS cells transfected with Nor1 (bottom) compared to a control vector (top). m denotes mitochondria. Magnification was 30000x. D) Relative size distribution of mitochondria in TEM images. For each condition, the mitochondria of 4 grid squares, each containing 4 cells were measured (Control: 261 mitochondria. Nor1: 287 mitochondria).

Nor1 increases mitophagy in β-cells.

We first assessed mitophagy by quantifying the colocalization of mitochondria (in red) with the established autophagosome marker LC3-GFP (green) in INS832/13 cells (Fig 4.4A-C) and dispersed human islet cells transfected with Nor1 *versus* a control vector. We detected a

significant increase in the colocalization of the green and red signals in Nor1-expressing cells (Fig 4.4E-F). Indeed, the number of mitochondria co-localizing with LC3-GFP increased by 2-3 fold in Nor1-expressing INS cells (Fig 4.4E) and dispersed human islet cells (Fig 4.4F), strongly suggesting that Nor1 could increase mitophagy in β-cells. We then quantified the number of autophagosomes targeting the mitochondria in our TEM images (Fig 4.4F-G) and detected a significant increase (~3.4 fold) in cells transfected with Nor1 (Fig 4.4H).

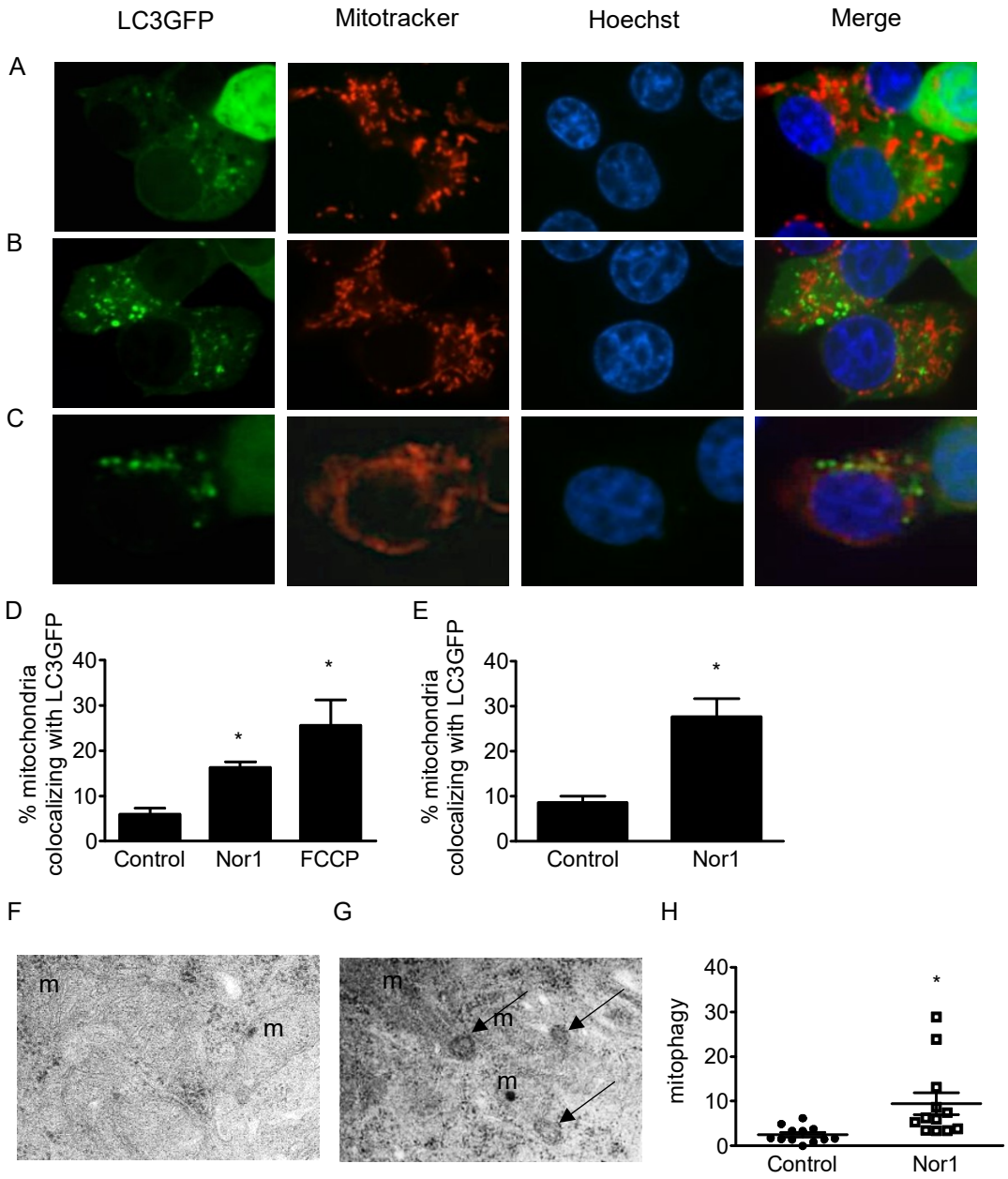


Figure 4.4: Nor1 increases mitophagy in β-cells. A-C) Immunofluorescence images of INS832/13 cells transfected with LC3-GFP along with Nor1 (B) or a control vector (A and C). FCCP (20 μM, 15 minutes) was used as a positive control in (C). Magnification 60x. D) Quantification of the colocalization between LC3-GFP and mitochondria in INS832/13 cells (n=3). Results are expressed as the percentage of mitochondria (red signal) colocalizing with LC3-GFP in each group. E) Quantification of the colocalization between LC3GFP and mitochondria in β-cells dispersed from human islets (n=4). Results are expressed as fold change over untreated control cells. F-G) TEM images of mitochondria in control INS832/13 cells (G) and cells transfected with Nor1 (F). Arrows indicate autophagosomes. Magnification 30000x. H) Quantification of the number of autophagosomes targetting mitochondria per cell area in TEM images. All results are represented as means +/- SEM; *, $p < 0.05$ *versus* control.

Nor1 does not affect mitochondrial content in β-cells.

Next, we sought to determine if the increase in mitophagy could cause a decrease in mitochondrial content. The mitochondrial area relative to total cell area assessed on TEM images was not modified by Nor1 (Fig 4.5A). We then assessed mitochondrial content by various methods, namely (i) quantification of mitochondrial DNA content, (ii) measurements of citrate synthase activity, and (iii) VDAC levels. The quantification of mitochondrial DNA over genomic DNA did not show any differences between Nor1-expressing cells and control cells (Fig 4.5B). Similarly, Nor1 did not affect citrate synthase activity in our system (Fig 4.5C). Finally, VDAC protein levels were not changed by Nor1 (Fig 4.5D). These results suggest that Nor1 does not reduce mitochondrial content in β-cells.

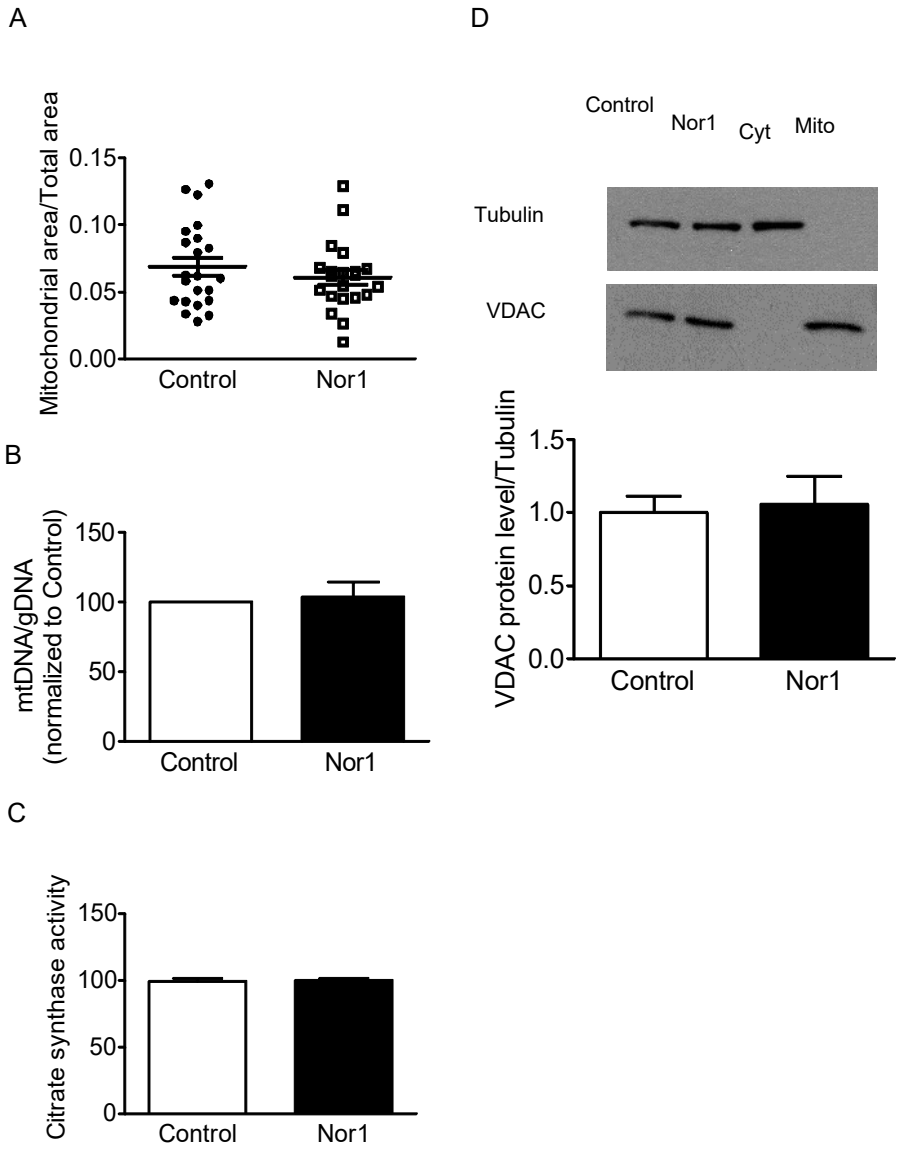


Figure 4.5: Nor1 does not alter mitochondrial content in β-cells. A) Mitochondrial area was determined from TEM images of INS832/13 cells transfected with Nor1 or a control vector, and normalized to total cell area. B) Quantification of mitochondrial DNA in control cells (white bar) and cells overexpressing Nor1 (black bar). The results were normalized to genomic DNA contents (n=5). C) Citrate synthase activity was measured in control cells (white bar) or cells overexpressing Nor1 (black bar) (n=4). D) VDAC protein levels were measured by western blot in INS cells transfected with Nor1 or a control vector. Cytoplasmic (Cyt) and mitochondrial (Mito) fractions were used as negative and positive controls, respectively. Results were normalized to tubulin (n=5). All results are represented as means +/- SEM.

4.4 Discussion

Recently, Nr4as have garnered considerable interest for their potential implication in metabolic diseases [9, 25]. In particular, their biological actions in pancreatic β-cells remain relatively unexplored. Our recent unpublished work characterized Nor1 as a mediator of cytokine-induced β-cell death. Indeed, Nor1 gain-of-function elicited β-cell apoptosis whereas Nor1 loss-of-function prevented cytokine-induced apoptosis. Moreover, Nor1-knockout animals displayed higher β-cell mass and improved glucose tolerance. Finally, we went on to show that Nor1 was increased in type 2 diabetic islets, suggesting that Nor1 could play a role in the etiology of diabetes.

We herein documented the sub-cellular localization of Nor1 in untreated and cytokine-treated cells. Surprisingly, we showed that in β-cells exposed to cytokines Nor1 undergoes mitochondrial translocation. In all tested conditions, we could not detect nuclear Nor1, suggesting that the "nuclear receptor" may not act primarily as a transcription factor in β-cells. This would confer a distinct biological role for Nor1 as compared to the other members of the Nr4a family. Indeed, Nur77 and Nurr1 have been shown previously to translocate to the nucleus and regulate transcription in response to various stressors in β-cells [26, 27]. A similar honing of Nor1 to the mitochondria has been described in immune cells [10-16]. However, the biological outcome appears to be different since, in these cell models, Nor1 was found to interact with Bcl2 to expose its BH3 domain and trigger the intrinsic pathway of apoptosis. In our study, we could not unveil a Nor1/Bcl2 interaction in β-cells using similar approaches.

The canonical pathway of intrinsic apoptosis involves the dissipation of the mitochondrial membrane potential [28]. However, our data indicated that Nor1 rather increases mitochondrial membrane potential. An alternative model exists and proposes that pro-apoptotic stimuli could induce mitochondrial hyperpolarization, resulting in an osmotic imbalance capable of provoking outer mitochondrial membrane rupture, and the release of intermembrane space proteins such as cytochrome c to induce cell death [22-24]. Our results dovetail with this alternative model and thereby unveil a possible novel mode of action for Nor1 in β-cells.

Our results also show that mitochondrial Nor1 impairs mitochondrial function and energy production in β-cells. This was concomitant with increased mitochondrial fragmentation and, possibly, the induction of mitophagy. These observations are relevant since mitochondrial dynamics govern the maintenance of optimal mitochondrial function [29] and it has been previously demonstrated that disruption of the mitochondrial network leads to reduced glucose oxidation and ATP production [30]. Moreover, mitochondrial fragmentation also actively participates in the triggering of apoptosis [31]. Thus, our results linking Nor1 to mitochondrial fragmentation provides a molecular mechanism by which Nor1 could both stimulate β-cell apoptosis and impair β-cell function, two defects that participate in β-cell demise in diabetes.

Mitochondrial dynamics also dictate the removal of these organelles by autophagy [32]. Small, rounded, fragmented mitochondria are indeed targeted by autophagosomes. We thus quantified mitophagy and detected an accumulation of double-membrane vacuoles targeting the mitochondria in Nor1-expressing cells. Interestingly, the number of autophagosomes is increased in the β-cells of diabetic individuals compared to non-diabetic subjects [33]. To our knowledge, the role of Nr4a members in autophagy remains unexplored. A single publication reported that Nur77 could induce autophagic cell death following its translocation to the mitochondria [14, 34]

In conclusion, our study demonstrates for the first time that the nuclear receptor Nor1 translocates to the mitochondria in β-cells to disrupt mitochondrial networks. As a result, Nor1 could play a critical role in both β-cell function and viability. These results are important considering that we previously detected an up-regulation of Nor1 in type 2 diabetic islets. We thus believe that Nor1 constitutes a potential molecular target to improve or maintain functional β-cell mass.

4.5 References

1. Saltiel, A.R., *New perspectives into the molecular pathogenesis and treatment of type 2 diabetes.* Cell, 2001. **104**(4): p. 517-29.
2. Meier, J.J. and R.C. Bonadonna, *Role of reduced beta-cell mass versus impaired beta-cell function in the pathogenesis of type 2 diabetes.* Diabetes Care, 2013. **36 Suppl 2**: p. S113-9.
3. Butler, A.E., et al., *Beta-cell deficit and increased beta-cell apoptosis in humans with type 2 diabetes.* Diabetes, 2003. **52**(1): p. 102-10.
4. Meier, J.J., et al., *Pancreatic diabetes manifests when beta cell area declines by approximately 65% in humans.* Diabetologia, 2012. **55**(5): p. 1346-54.
5. Rhodes, C.J., *Type 2 diabetes-a matter of beta-cell life and death?* Science, 2005. **307**(5708): p. 380-4.
6. *A unified nomenclature system for the nuclear receptor superfamily.* Cell, 1999. **97**(2): p. 161-3.
7. Ordelheide, A.M., et al., *Nor-1, a novel incretin-responsive regulator of insulin genes and insulin secretion.* Mol Metab, 2013. **2**(3): p. 243-55.
8. Tessem, J.S., et al., *Nkx6.1 regulates islet beta-cell proliferation via Nr4a1 and Nr4a3 nuclear receptors.* Proc Natl Acad Sci U S A, 2014. **111**(14): p. 5242-7.
9. Close, A.F., C. Rouillard, and J. Buteau, *NR4A orphan nuclear receptors in glucose homeostasis: a minireview.* Diabetes Metab, 2013. **39**(6): p. 478-84.
10. Zhang, X.K., *Targeting Nur77 translocation.* Expert Opin Ther Targets, 2007. **11**(1): p. 69-79.
11. Kiss, B., et al., *Retinoids induce Nur77-dependent apoptosis in mouse thymocytes.* Biochim Biophys Acta, 2015. **1853**(3): p. 660-70.
12. Chen, H.Z., et al., *Akt phosphorylates the TR3 orphan receptor and blocks its targeting to the mitochondria.* Carcinogenesis, 2008. **29**(11): p. 2078-88.
13. Rapak, A., et al., *Apoptosis of lymphoma cells is abolished due to blockade of cytochrome c release despite Nur77 mitochondrial targeting.* Apoptosis, 2007. **12**(10): p. 1873-8.
14. Wang, A., et al., *Phosphorylation of Nur77 by the MEK-ERK-RSK cascade induces mitochondrial translocation and apoptosis in T cells.* J Immunol, 2009. **183**(5): p. 3268-77.
15. Yang, H., et al., *Induction and intracellular localization of Nur77 dictate fenretinide-induced apoptosis of human liver cancer cells.* Biochem Pharmacol, 2010. **79**(7): p. 948-54.
16. Thompson, J., et al., *Protein kinase C regulates mitochondrial targeting of Nur77 and its family member Nor-1 in thymocytes undergoing apoptosis.* Eur J Immunol, 2010. **40**(7): p. 2041-9.
17. Hohmeier, H.E., et al., *Isolation of INS-1-derived cell lines with robust ATP-sensitive K+ channel-dependent and -independent glucose-stimulated insulin secretion.* Diabetes, 2000. **49**(3): p. 424-30.

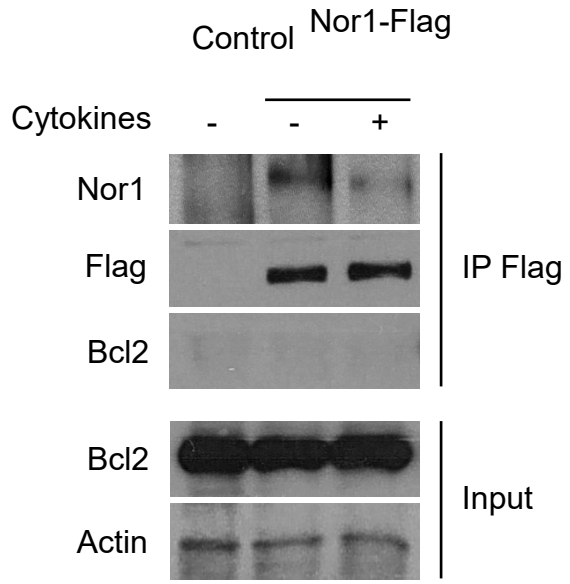
18. Buteau, J., et al., *Metabolic diapause in pancreatic beta-cells expressing a gain-of-function mutant of the forkhead protein Foxo1.* J Biol Chem, 2007. **282**(1): p. 287-93.
19. Cho, W.J., et al., *Matrix metalloproteinase-2, caveolins, focal adhesion kinase and c-Kit in cells of the mouse myocardium.* J Cell Mol Med, 2007. **11**(5): p. 1069-86.
20. Katragadda, D., et al., *Epoxyeicosatrienoic acids limit damage to mitochondrial function following stress in cardiac cells.* J Mol Cell Cardiol, 2009. **46**(6): p. 867-75.
21. Eskelinen, E.L., *Fine structure of the autophagosome.* Methods Mol Biol, 2008. **445**: p. 11-28.
22. Giovannini, C., et al., *Mitochondria hyperpolarization is an early event in oxidized low-density lipoprotein-induced apoptosis in Caco-2 intestinal cells.* FEBS Lett, 2002. **523**(1-3): p. 200-6.
23. Leal, A.M., et al., *Violacein induces cell death by triggering mitochondrial membrane hyperpolarization in vitro.* BMC Microbiol, 2015. **15**: p. 115.
24. Vander Heiden, M.G., et al., *Outer mitochondrial membrane permeability can regulate coupled respiration and cell survival.* Proc Natl Acad Sci U S A, 2000. **97**(9): p. 4666-71.
25. Pearen, M.A. and G.E. Muscat, *Minireview: Nuclear hormone receptor 4A signaling: implications for metabolic disease.* Mol Endocrinol, 2010. **24**(10): p. 1891-903.
26. Briand, O., et al., *The nuclear orphan receptor Nur77 is a lipotoxicity sensor regulating glucose-induced insulin secretion in pancreatic beta-cells.* Mol Endocrinol, 2012. **26**(3): p. 399-413.
27. Yu, C., et al., *The Orphan Nuclear Receptor NR4A1 Protects Pancreatic beta-Cells from Endoplasmic Reticulum (ER) Stress-mediated Apoptosis.* J Biol Chem, 2015. **290**(34): p. 20687-99.
28. Lemasters, J.J., et al., *The mitochondrial permeability transition in cell death: a common mechanism in necrosis, apoptosis and autophagy.* Biochim Biophys Acta, 1998. **1366**(1-2): p. 177-96.
29. Morciano, G., et al., *Intersection of mitochondrial fission and fusion machinery with apoptotic pathways: Role of Mcl-1.* Biol Cell, 2016. **108**(10): p. 279-293.
30. Bach, D., et al., *Mitofusin-2 determines mitochondrial network architecture and mitochondrial metabolism. A novel regulatory mechanism altered in obesity.* J Biol Chem, 2003. **278**(19): p. 17190-7.
31. Suen, D.F., K.L. Norris, and R.J. Youle, *Mitochondrial dynamics and apoptosis.* Genes Dev, 2008. **22**(12): p. 1577-90.
32. Twig, G., et al., *Fission and selective fusion govern mitochondrial segregation and elimination by autophagy.* EMBO J, 2008. **27**(2): p. 433-46.
33. Masini, M., et al., *Autophagy in human type 2 diabetes pancreatic beta cells.* Diabetologia, 2009. **52**(6): p. 1083-6.
34. Bouzas-Rodriguez, J., et al., *The nuclear receptor NR4A1 induces a form of cell death dependent on autophagy in mammalian cells.* PLoS One, 2012. **7**(10): p. e46422.

4.6 Supplementary material

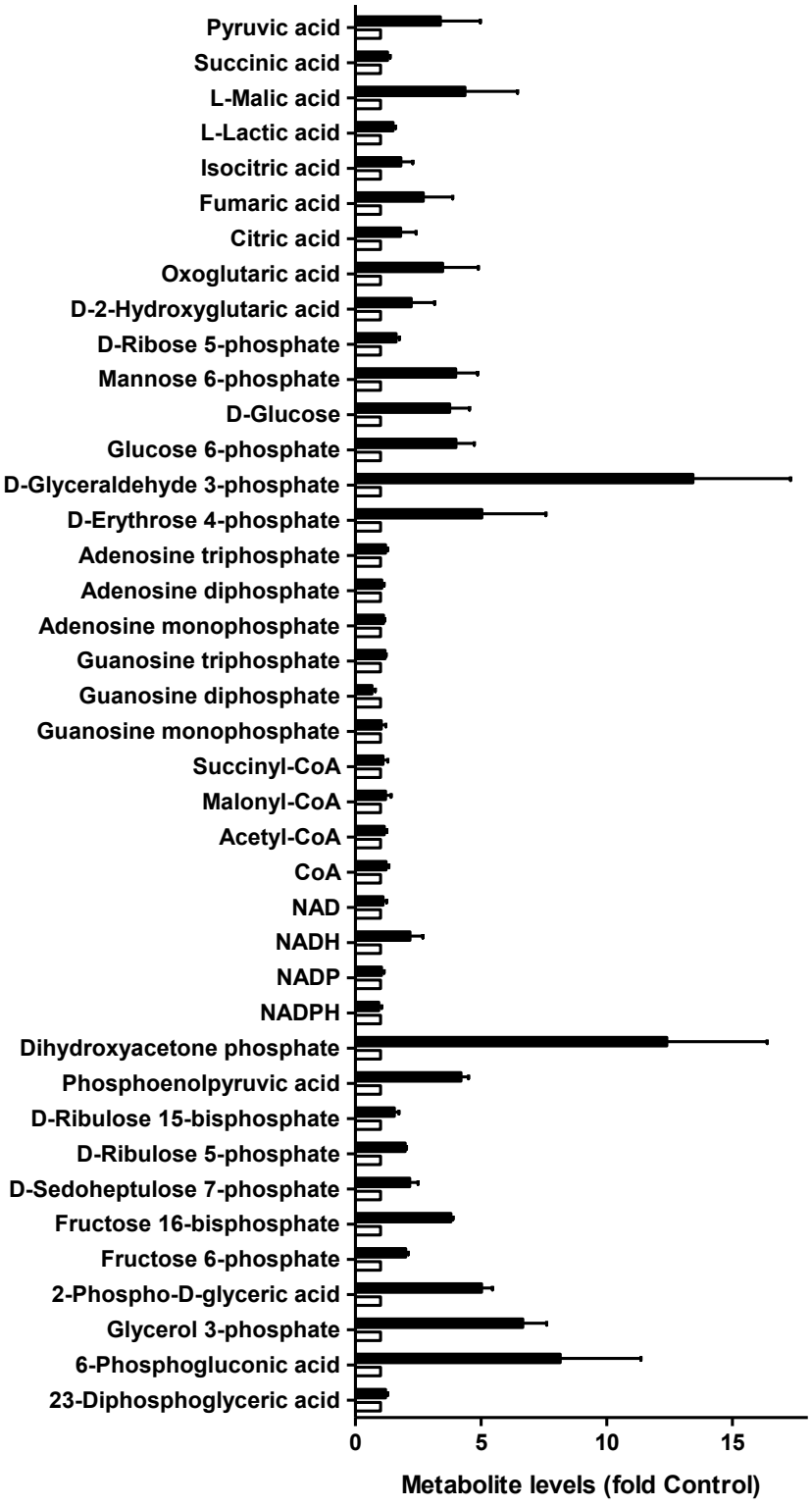
4.6.1 Supplementary figures

Ensembl Ger	logFC	logCPM	PValue	FDR	EntrezGene I	RefSeq mRN/	RGD Symbol	Description	Transcript St	Strand	Associated G	Associated G	Ensembl Tra	Gene End (bj	Gene Start (t	Ensembl Family Descriptio
pNor1	10.6338232	1.65685657	3.31E-113	3.98E-109	0											Human Nor1
ENSRNOG00(	-0.5443062	9.24084346	3.54E-08	0.00021271	7066256		0	0 NADH dehyd	10160	1	MT-ND4	UniProtKB Ge	ENSRNOT000	11537	10160	NADH UBIQUINONE OXIDO
ENSRNOG00(	-0.5064031	6.45389524	8.14E-08	0.00031357	7066255		0	0 NADH dehyd	9870	1	MT-ND4L	UniProtKB Ge	ENSRNOT000	10166	9870	NADH UBIQUINONE OXIDO
ENSRNOG00(	-0.6204325	8.20499162	1.04E-07	0.00031357	7066257		0	0 NADH dehyd	11736	1	MT-ND5	UniProtKB Ge	ENSRNOT000	13565	11736	NADH UBIQUINONE OXIDO
ENSRNOG00(	-0.2309044	7.33722306	2.30E-06	0.00506381	306451	NM_0011691	Tenm3	odz, odd Oz/	46462637	1	Tenm3	RGD Symbol	ENSRNOT000	46657495	46462637	TENEURIN TEN ODD OZ/TE
ENSRNOG00(	-0.4820625	10.0869311	2.53E-06	0.00506381	7066259		0	0 cytochrome I	14136	1	CYTB	UniProtKB Ge	ENSRNOT000	15278	14136	CYTOCHROME B COMPLEX
ENSRNOG00(	-0.4830264	9.1402972	4.1366E-06	0.0070996	7066247		0	0 NADH dehyd	2740	1	ND1	EntrezGene	ENSRNOT000	3694	2740	NADH UBIQUINONE OXIDO
ENSRNOG00(	-0.5916894	5.78569638	7.89E-06	0.0118527	7066258		0	0 NADH dehyd	13543	-1	MT-ND6	UniProtKB Ge	ENSRNOT000	14061	13543	NADH UBIQUINONE OXIDO
ENSRNOG00(	0.36051267	3.23587062	1.4199E-05	0.01553672	100912007	NM_001270	Tceb1	transcription	2052950	1	Tceb1	RGD Symbol	ENSRNOT000	2058835	2052950	TRANSCRIPTION ELONGAT
ENSRNOG00(	0.27803743	4.41766447	1.5519E-05	0.01553672	0		0	0 Uncharacteri	197560288	-1	0	0	ENSRNOT000	197560952	197560288	60S ACIDIC RIBOSOMAL P0
ENSRNOG00(	0.24024198	5.68923829	1.4704E-05	0.01553672	100360522		0	LOC1003605260S acidic rib	11392283	-1	RPLP1	UniProtKB Ge	ENSRNOT000	11393688	11392283	60S ACIDIC RIBOSOMAL P1
ENSRNOG00(	-0.2009795	7.71814968	1.2241E-05	0.01553672	25398		0	0 voltage-depe	36502533	1	Cacna1a	EntrezGene	ENSRNOT000	36725231	36502533	VOLTAGE DEPENDENT TYP
ENSRNOG00(	0.34834004	4.87086538	1.9738E-05	0.01693791	100361193		0	LOC1003611	Protein LOC1	142294230	1	Tbca	EntrezGene	ENSRNOT000	142294553	142294230 TUBULIN SPECIFIC CHAPER
ENSRNOG00(	-0.9533985	0.16330493	1.8708E-05	0.01693791	690467		0	LOC689635	Protein LOC6	40084947	1	0	0	ENSRNOT000	40087313	40084947 UNCHARACTERIZED
ENSRNOG00(	0.33922043	3.23862082	2.3209E-05	0.01858872	691532	NM_0010055	Rps25	ribosomal pr	47346312	1	Rps25	RGD Symbol	ENSRNOT000	47348692	47346312	40S RIBOSOMAL S25
ENSRNOG00(	0.39318754	2.78036036	4.1577E-05	0.03121904	0		0	0	86620412	-1	0	0	ENSRNOT000	86620871	86620412	0
ENSRNOG00(	0.36916867	3.69564611	5.1434E-05	0.03570672	0		0	0 Ac2-067; Unc	255457574	1	EEF1B2L	UniProtKB Ge	ENSRNOT000	255461596	255457574	ELONGATION FACTOR 1 BE
ENSRNOG00(	0.22727652	5.73541193	5.3498E-05	0.03570672	64041	NM_022274	Birc5	baculoviral IA	106494775	1	Birc5	RGD Symbol	ENSRNOT000	106503091	106494775	BACULOVIRAL IAP REPEAT
ENSRNOG00(	-0.1982324	7.08596302	8.5689E-05	0.05204218	360652	NM_001108	Sdk2	sidekick cell a	101978490	-1	Sdk2	RGD Symbol	ENSRNOT000	102096741	101978490	SIDEKICK PRECURSOR
ENSRNOG00(	-0.2790666	4.75583699	8.6636E-05	0.05204218	366949		0	Mafa	Killer cell lect	116671892	-1	Mafa	RGD Symbol	ENSRNOT000	116672977	116671892 TRANSCRIPTION FACTOR M
ENSRNOG00(	-0.4018683	11.3504327	9.9834E-05	0.05711451	7066249		0	0 cytochrome c	5323	1	COI	UniProtKB Ge	ENSRNOT000	6867	5323	CYTOCHROME C OXIDASE S
ENSRNOG00(	0.3917117	2.38016264	0.00014278	0.07797292	680964		0	LOC680964	Protein LOC6	36606405	-1	0	0	ENSRNOT000	36607617	36606405 UNKNOWN
ENSRNOG00(	-0.2301013	4.4652683	0.00015891	0.07954771	310640	NM_001107	Fam189b	family with s	207911887	1	Fam189b	RGD Symbol	ENSRNOT000	207917765	207911887	UNKNOWN
ENSRNOG00(	-0.2730055	4.06070434	0.00015877	0.07954771	362931		0	0 brain-specific	115675214	1	Bai1	EntrezGene	ENSRNOT000	115733670	115675214	BRAIN SPECIFIC ANGIOGEN
ENSRNOG00(	0.46716736	5.55825778	0.0001736	0.08109854	100911683		0	Snrpgl2	Protein LOC1	92715996	-1	Snrpgl2	RGD Symbol	ENSRNOT000	92716226	92715996 UNKNOWN
ENSRNOG00(	0.30704769	3.70623413	0.00017551	0.08109854	499157	NM_001271(	Svip	small VCP/p9	108412833	-1	Svip	RGD Symbol	ENSRNOT000	108415318	108412833	UNKNOWN
ENSRNOG00(	0.40779692	3.21174487	0.00018312	0.08148056	689134	NM_001135(	Sec61g	SEC61, gamm	99067709	1	Sec61g	RGD Symbol	ENSRNOT000	99074084	99067709	UNKNOWN
ENSRNOG00(	0.24996531	5.4400465	0.00021855	0.09377527	65136	NM_031706	Rps8	ribosomal pr	139813899	-1	Rps8	RGD Symbol	ENSRNOT000	139816616	139813899	40S RIBOSOMAL S8
ENSRNOG00(	0.22220324	6.26727658	0.00023712	0.09823388	64001		0	0 cytochrome I	80739205	1	Cyb5a	RGD Symbol	ENSRNOT000	80770883	80739205	CYTOCHROME B5
ENSRNOG00(	0.21616245	5.14826574	0.0002506	0.10035814	689399	NM_001246	Cenpw	centromere p	31347827	1	Cenpw	RGD Symbol	ENSRNOT000	31352722	31347827	CENTROMERE W CENP W (
ENSRNOG00(	0.29344329	6.08365117	0.00028894	0.11197732	25045	NM_031853	Dbi	diazepam bir	41256375	-1	Dbi	RGD Symbol	ENSRNOT000	41283626	41225979	ACYL COA BINDING ACBP D
ENSRNOG00(	0.20849949	5.50128648	0.00032416	0.11801536	360461	NM_001108	Fopnl	FGFR1OP N-t	11412943	1	Fopnl	RGD Symbol	ENSRNOT000	11433757	11412943	LISH DOMAIN CONTAINING
ENSRNOG00(	-0.4839119	4.72390382	0.00031493	0.11801536	0		0	0	46834107	1	0	0	ENSRNOT000	46834200	46834107	0
ENSRNOG00(	1.64897634	-0.4039137	0.00041312	0.14597747	100909544	NM_212495	Brd2	bromodomai	7323527	1	Brd2	RGD Symbol	ENSRNOT000	7330397	7323527	BROMODOMAIN
ENSRNOG00(	0.29441623	4.8685085	0.00043902	0.1465124	0		0	0 Cyclin-depen	27496741	1	CDKN3	UniProtKB Ge	ENSRNOT000	27508022	27496741	CYCLIN DEPENDENT KINAS
ENSRNOG00(	0.20005128	6.23634081	0.00043661	0.1465124	81778	NM_031114	S100a10	S100 calcium	210966310	-1	S100a10	RGD Symbol	ENSRNOT000	210975004	210966310	S100 A10 CALPACTIN I LIGH
ENSRNOG00(	0.15412796	6.0267653	0.00047041	0.15274328	498600	NM_001128(	Fam32a	family with s	19164840	-1	Fam32a	RGD Symbol	ENSRNOT000	19169329	19164840	FAM32A OVARIAN TUMOR
ENSRNOG00(	-0.2428699	4.79635647	0.00050549	0.15981493	29433	NM_019210	Pak3	p21 protein (	113372070	1	Pak3	RGD Symbol	ENSRNOT000	113489867	113372070	SERINE/THREONINE KINAS
ENSRNOG00(	0.25879492	3.40193483	0.00051882	0.15982223	310537	NM_001107(	RGD1560010	RGD1560010	197725440	1	RGD1560010	RGD Symbol	ENSRNOT000	197728572	197725440	UNCHARACTERIZED
ENSRNOG00(	0.21571045	5.50827017	0.00054317	0.16314082	66028	NM_023972	Arl6ip5	ADP-ribosyla	194077679	1	Arl6ip5	RGD Symbol	ENSRNOT000	194100520	194077679	PRA1 FAMILY 3 ADP RIBOS
ENSRNOG00(	-0.1820632	6.87271591	0.00056274	0.1648964	688540	NM_0011347	Mast3	microtubule	20257645	1	Mast3	RGD Symbol	ENSRNOT000	20271835	20257645	MICROTUBULE ASSOCIATE
ENSRNOG00(	0.27064704	3.20600542	0.00058345	0.16689546	688776		0	LOC688776	Protein LOC6	74146654	-1	0	0	ENSRNOT000	74147124	74146654 60S RIBOSOMAL
ENSRNOG00(	0.23703314	7.16515622	0.00061932	0.16910143	100188937	NM_0011347	Cox7c	cytochrome c	14553406	-1	Cox7c	RGD Symbol	ENSRNOT000	14555427	14553406	UNKNOWN
ENSRNOG00(	0.20753368	5.16782201	0.00061717	0.16910143	100912122	NM_017187	RGD1564519	high mobility	35886764	-1	Hmgb2	RGD Symbol	ENSRNOT000	35889338	35886764	HIGH MOBILITY GROUP B1
ENSRNOG00(	0.21815046	4.3204324	0.00068668	0.17552661	287302	NM_0011057	Mrpl22	mitochondria	43395542	1	Mrpl22	RGD Symbol	ENSRNOT000	43405875	43395542	39S RIBOSOMAL L22 MITO
ENSRNOG00(	0.20271039	4.70754273	0.00067946	0.17552661	361956	NM_0010141	Rsrc1	arginine/seri	183491965	1	Rsrc1	RGD Symbol	ENSRNOT000	183881122	183491965	SERINE/ARGININE RELATED
ENSRNOG00(	-1.3687462	0.92578041	0.00066985	0.17552661	100911238	NM_0011005	Pomp	proteasome	11094518	1	Pomp	RGD Symbol	ENSRNOT000	11107003	11094518	PROTEASOME MATURATIC
ENSRNOG00(	0.46664526	1.47027411	0.00088184	0.18608777	287726	NM_0011058	Rdm1	RAD52 motif	89337811	1	Rdm1	RGD Symbol	ENSRNOT000	89348066	89337811	RAD52 MOTIF CONTAINING
ENSRNOG00(	0.33401294	4.49261361	0.00082802	0.18608777	100361574		0	0 polymerase (	75138464	1	Polr2k	EntrezGene	ENSRNOT000	75141444	75138464	POLR2K FRAGMENT
ENSRNOG00(	0.29532647	2.74152794	0.00084608	0.18608777	298062	NM_201271	Lppr1	lipid phospha	72309081	-1	Lppr1	RGD Symbol	ENSRNOT000	72594906	72309081	LIPID PHOSPHATE PHOSPHA
ENSRNOG00(	0.29484899	3.87621709	0.00088289	0.18608777	690940		0	LOC685520	Protein LOC6	250371993	-1	0	0	ENSRNOT000	250373468	250371993 HIGH MOBILITY GROUP B1
ENSRNOG00(	0.24003422	4.46694999	0.00086608	0.18608777	500651	NM_0010477	Sptssa	serine palmit	85080146	-1	Sptssa	RGD Symbol	ENSRNOT000	85091608	85080146	UNKNOWN
ENSRNOG00(	0.17722168	4.83575178	0.00076105	0.18608777	311903	NM_0010083	Mrrf	mitochondria	20773165	1	Mrrf	RGD Symbol	ENSRNOT000	20829830	20773165	RIBOSOME RECYCLING FAC
ENSRNOG00(	0.16908961	5.65704208	0.00079729	0.18608777	100125373	NM_0011033	Pnrc2	proline-rich r	157985391	-1	Pnrc2	RGD Symbol	ENSRNOT000	157988374	157985391	PROLINE RICH NUCLEAR RE
ENSRNOG00(	-0.1900368	5.65362183	0.00086383	0.18608777	0		0	0 unc-13 homo	19917585	-1	Unc13a	MGI Symbol	ENSRNOT000	19958035	19917585	UNC 13 HOMOLOG MUNC1
ENSRNOG00(	-0.2094065	4.91525569	0.00081114	0.18608777	24684		0	0 prolactin rec	84378168	-1	Prlr	EntrezGene	ENSRNOT000	84554681	84361344	PROLACTIN RECEPTOR PRE
ENSRNOG00(	-0.2307601	4.59271366	0.00074784	0.18608777	140433	NM_001191(	Lama5	laminin, alph	181517512	1	Lama5	RGD Symbol	ENSRNOT000	181565641	181517512	LAMININ SUBUNIT ALPHA I
ENSRNOG00(	0.17001777	6.09413983	0.00090951	0.1869078	286899	NM_173119	Hsbp1	heat shock fa	62733736	1	Hsbp1	RGD Symbol	ENSRNOT000	62738068	62733736	UNKNOWN
ENSRNOG00(	-0.1897579	4.29467999	0.00091789	0.1869078	361710	NM_001004(	Sipa1	signal-induce	227941671	-1	Sipa1	RGD Symbol	ENSRNOT000	227953682	227941671	SIGNAL INDUCED PROLIFER
ENSRNOG00(	0.30659758	2.86144833	0.00094111	0.18844064	300278		0	RGD1566136	Protein LOC1	111679710	-1	RGD1566136	RGD Symbol	ENSRNOT000	111680497	111679710 40S RIBOSOMAL S9
ENSRNOG00(	0.45830178	1.37259678	0.00100983	0.19207799	29168	NM_053299	Ubd	ubiquitin D (I	3917783	-1	Ubd	RGD Symbol	ENSRNOT000	3919728	3917783	UBIQUITIN D DIUBIQUITIN
ENSRNOG00(	0.26850312	4.42122335	0.0009987	0.19207799	680122		0	0 sin3 associat	35925240	1	Sap30	MGI Symbol	ENSRNOT000	35930358	35925240	HISTONE DEACETYLASE CO
ENSRNOG00(	0.14596975	6.96207198	0.00097703	0.19207799	25203	NM_171991	Ccnb1	cyclin B1 (Ccr	49941586	-1	Ccnb1	RGD Symbol	ENSRNOT000	49948770	49941586	G2/MITOTIC SPECIFIC CYCL
ENSRNOG00(	-0.3935772	10.5110314	0.00103921	0.19207799	7066253		0	0 Cytochrome c	8599	1	MT-CO3	UniProtKB Ge	ENSRNOT000	9382	8599	CYTOCHROME C OXIDASE S
ENSRNOG00(	-0.4071208	2.21963957	0.00102835	0.19207799	309150		0	0 Protein RGD1	226285566	-1	RGD1308234	RGD Symbol	ENSRNOT000	226292919	226285566	CARNOSINE SYNTHASE 1 E
ENSRNOG00(	0.14984835	6.35812589	0.00106655	0.19414388	25541	NM_138508	Scp2	sterol carrier	131495400	-1	Scp2	RGD Symbol	ENSRNOT000	131584319	131495400	NON SPECIFIC LIPID TRANS

Suppl Fig 4.1: RNA sequencing. A) Quantification of RNA extracted and sequenced from INS832/13 cells transfected with Nor1 or a control vector (n=4 for each). All results are represented as means +/- SEM. Darker blue indicates a cutoff FDR of 0.05. Lighter blue indicates a cutoff FDR of 0.1.



Suppl Fig 4.2: Nor1 does not colocalize with Bcl2 in beta-cells. INS cells were transfected with Nor1-Flag or left untransfected (NT), and treated with of without cytokines for 1h. We immunoprecipitated Nor1-Flag and blotted for Bcl2, Flag and Nor1. Actin was used as control. Representative images of 3 experiments are shown.



Suppl Fig 4.3: Nor1 affects metabolic pathways in beta-cells. INS832/13 cells were transfected with Nor1 (black) or a control vector (white) (n=3 for each). Central Carbon Metabolism

Metabolites were quantified by ultrahigh-performance liquid chromatography–tandem mass spectrometry. All results are represented as means +/- SEM.

4.6.2 Supplementary methods

RNA sequencing

In brief, polyA-selected mRNA was purified from total RNA isolated with the RNeasy Mini Kit (Qiagen). mRNAs were reverse-transcribed to cDNA, paired-end repaired, 3′-monoadenylated, and adaptor-ligated. cDNA products (200 bp) were amplified and libraries submitted to quality control with the Agilent 2100 Bioanalyzer (Agilent Technologies, Wokingham, U.K.). The RNA integrity number (RIN) values for all samples were >7.5.

Immunoprecipitation and Western Blot

Cells were lysed in a buffer containing 50 mM Tris (pH 8.0), 150 mM NaCl and 1% v/v Triton X-100 and supplemented with 1 tablet/10 ml of protease inhibitors (Complete Mini, Roche Diagnostics, Mannheim, Germany). Protein concentrations were determined by BCA protein assay and Flag was immunoprecipitated by adding 1 µg mouse anti-DDK antibody (Origene, Rockville, MD) to 1 mg of proteins per condition. Antibodies: rabbit anti-bcl2 (Cell Signaling 2876S 1:1000); anti-Nor1 (R&D systems H7833 1:500); anti-DDK 1:1000 and mouse anti-actin (Santa Cruz Biotechnology sc-8432 1:1000).

4.7 Additional results: Respiratory flux

Upon observation of the translocation of Nor1 to the mitochondria in response to cytokines, we aimed to evaluate the effect of Nor1 on mitochondrial function in β-cells. The dominant role of mitochondria is the production of energy in the form of ATP. It does so through a process called respiration that requires high oxygen consumption (Fig 1) [227]. Our first approach to study mitochondrial function was therefore to measure oxygen consumption by respirometry in INS832/13 cells transfected with Nor1Flag *versus* a control vector.

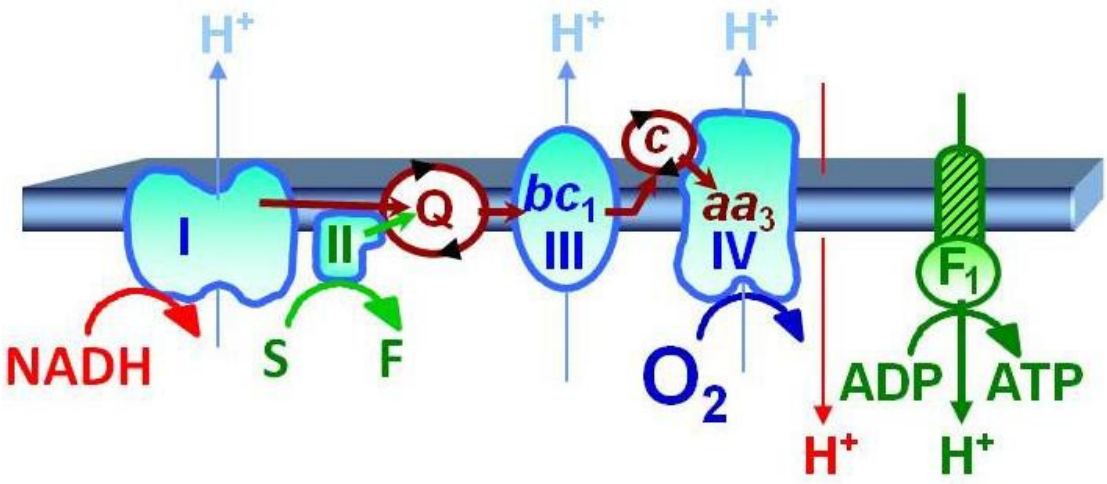


Figure 4.6: Electron Transfer System (ETS) and ATP synthase (green). The inner mitochondrial membrane complexes (C) I, II, III and IV are represented in blue. NADH-linked substrates feed electrons into CI. Succinate (S) supports electron flux through CII via $FADH_2$. Electrons can be transferred to Coenzyme Q from CI or CII. Electrons are then transferred to CIII (cytochrome b) and CIV via cytochrome c. Oxygen is the recipient of the last electron. This oxidation chain is accompanied by the building of a high concentration of protons in the inter-membrane space creating the proton-motive force. The release of the protons to the mitochondrial matrix through ATP synthase provides the energy necessary for ADP phosphorylation into ATP [228]. Used with permission.

4.7.1 Methods

The measurements were made using oxygraph-2k[7] (Oroboros Instruments, Innsbruck, Austria). 24h after transfection, equal numbers of INS832/13 cells transfected with Nor1Flag or a control vector suspended in complete RPMI media containing 11 mM of glucose were transferred into the oxygraph chambers (2 chambers per condition) (Fig 2). All calibrations and experiments were performed at 37°C with magnetic stirring set at 750 rpm.

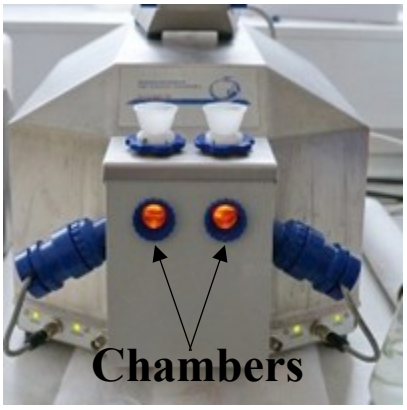


Figure 4.7: Oroboros oxygraph-2k. Adapted from [228]. Used with permission.

In a closed oxygraph chamber, the oxygen concentration declines overtime as a result of respiratory processes (Fig 3) [228]. Oxygen concentrations (nmol/mL) in the samples were measured and analysed by the Datlab software (OROBOROS INSTRUMENTS). Oxygen flux (pmol O_2/s/10^6 cells) or respiration was calculated as the negative time derivative of measured oxygen concentration.

[7] Dr. Hélène Lemieux kindly allowed us to use her oxygraphs and provided training as well as technical guidance.

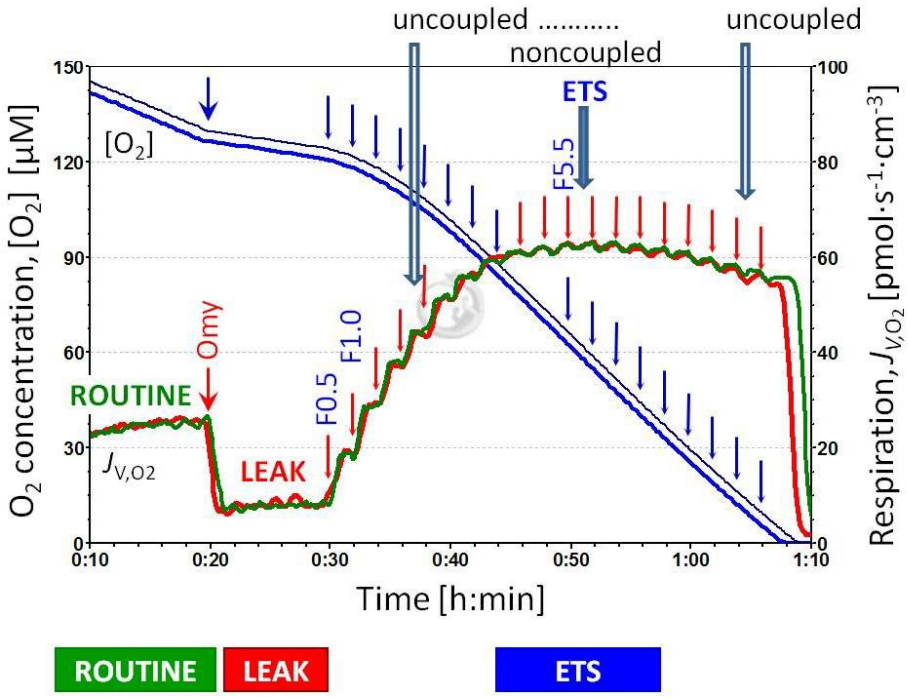


Figure 4.8: Graphic representation of oxygen concentration (blue line) and oxygen consumption (green and red) over time in hematopoietic 32D cells in response to oligomycin (2 µg/mL) and successive steps of FCCP injections (0.1 µL of 10 mM FCCP per step). Routine and Leak state as well as maximum capacity of the Electron Transfer System (ETS) are shown. Adapted from [228]. Used with permission.

The basal oxygen consumption or flux is called Routine respiration. The Leak state is then induced by adding the ATP synthase inhibitor oligomycin to the system. The proton gradient through the inner mitochondrial membrane is thus increased because the protons can no more escape through the ATP synthase. This creates a negative feedback on the electron transfer chain and reduces the oxygen flux (Fig 3). If subsequently, oxidation is uncoupled by adding FCCP, oxygen flux is not controlled by phosphorylation anymore and progressively reaches its maximum which corresponds to the maximal capacity of the ETS.

The cells' Routine and Leak oxygen flux as well as the ETS can be very informative regarding mitochondrial function. However, it is also useful to measure the contribution of each of the ETS complexes to electron transfer and therefore oxygen consumption. This can be done by injecting inhibitors and substrates specific of each complex successively into the chambers (Fig 4). First

cells were permeabilized with optimum digitonin concentration. Leak respiration (no phosphorylation in the absence of ADP) was measured following the injection of malate+pyruvate+glutamate (M+P+G). These 3 NADH-linked substrates feed electrons into CI. Oxidative phosphorylation capacity was then measured after addition of ADP. Next, Cytochrome c was added to test the integrity of the outer mitochondrial membrane. The addition of succinate, an $FADH_2$-linked substrate allowed the supply of electrons to CII and the measurement of respiration with convergent electron flow through CI and CII. The ETS was evaluated by uncoupling with optimum FCCP concentration. The inhibition of CI by addition of rotenone (Rot) allowed the measurement of CII ETS capacity. Later, the inhibition of CIII by antimycin A (Ama) provided an estimation of ROX, the Residual Oxygen Flux due to non-respiratory side reactions. Finally, N,N,N',N'-Tetramethyl-*p*-Phenylenediamine (TMPD) and ascorbate were added. TMPD is an artificial substrate used to measure complex IV activity and ascorbate helps maintain TMPD in the reduced form. Sodium azide, an inhibitor of CIV was the last reagent injected into the chambers.

Here are the final concentrations of the products used during the experiment:

- Digitonin (0.2 µL of 10 mg/mL digitonin)
- Malate (2 mM)
- Pyruvate (5 mM)
- Glutamate (10 mM)
- ADP (2.5 mM)
- Cytochrome c (10 µM)
- Succinate (10 mM)
- FCCP (0.05 µM steps)
- Rotenone (1 µM)
- Antimycin A (2.5 µM)
- Ascorbate (2 mM)
- TMPD (0.5 mM)
- Sodium Azide (100 mM)

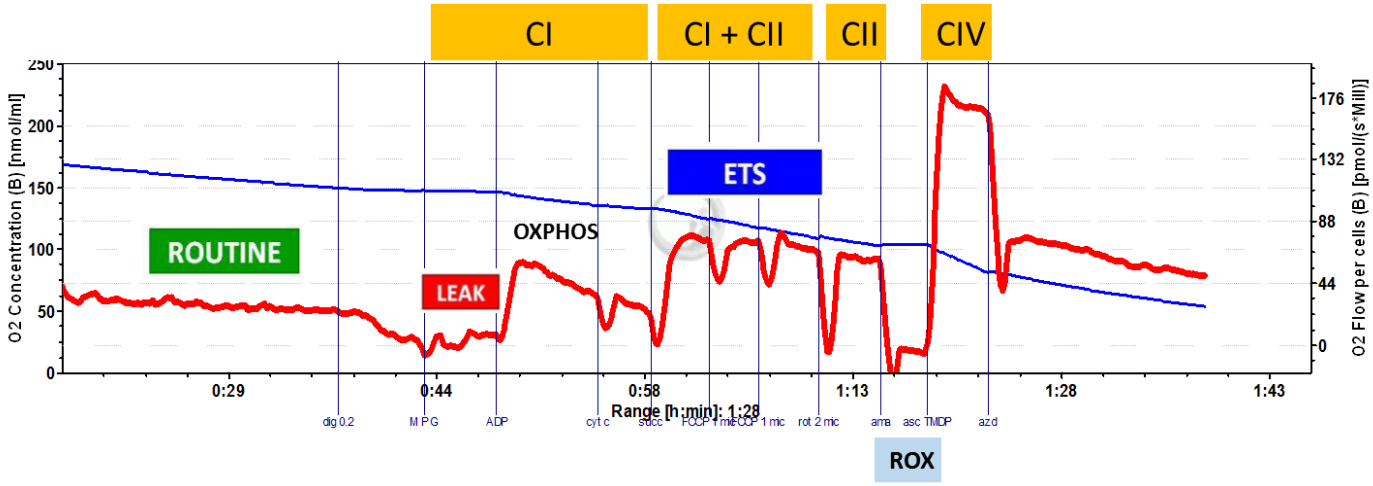


Figure 4.9: Example of an output from an oxygraphy chamber containing permeabilized INS832/13 cells (transfected with a control vector). The blue line represents the oxygen concentration (nmol O_2/mL) in the chamber. The red line represents oxygen flux (pmol O_2/s/10^6 cells). Dig= Digitonin, MPG= Malate, Pyruvate, Glutamate. Rot= Rotenone. Ama= Antimycin A. Asc= ascorbate. Azd= azide. [AF Close, unpublished data].

Average oxygen flux was measured at each stage and non-respiratory side reactions were corrected for by subtracting the oxygen flux measured in the ROX state. Oxygen flux after azide injection was substracted from the measurement of CIV activity.

4.7.2 Results

Overexpressing Nor1 did not modify routine or leak respiration in intact β-cells (Fig 5A). The maximum capacity of the Electron Transfer System was not affected either. We then evaluated the contribution of each mitochondrial respiratory complex to electron transfer in permeabilized INS1 cells (Fig 5B). We could not detect any difference between the cells transfected with Nor1 and the controls.

We have observed (chapter 4) that Nor1 reduces glucose oxidation (measured by CO_2 production) as well as ATP production in β-cells. Our data also suggest that Nor1 diverts the glycolysis-derived pyruvate to lactate production. This might result in a reduction of the production of Tricarboxylic Acid Cycle intermediates and NADH. These intermediates are the

substrates of the ETS. The increase of lactate production by Nor1 could therefore explain the reduction of CO2 and ATP production without any alteration of the ETS itself.

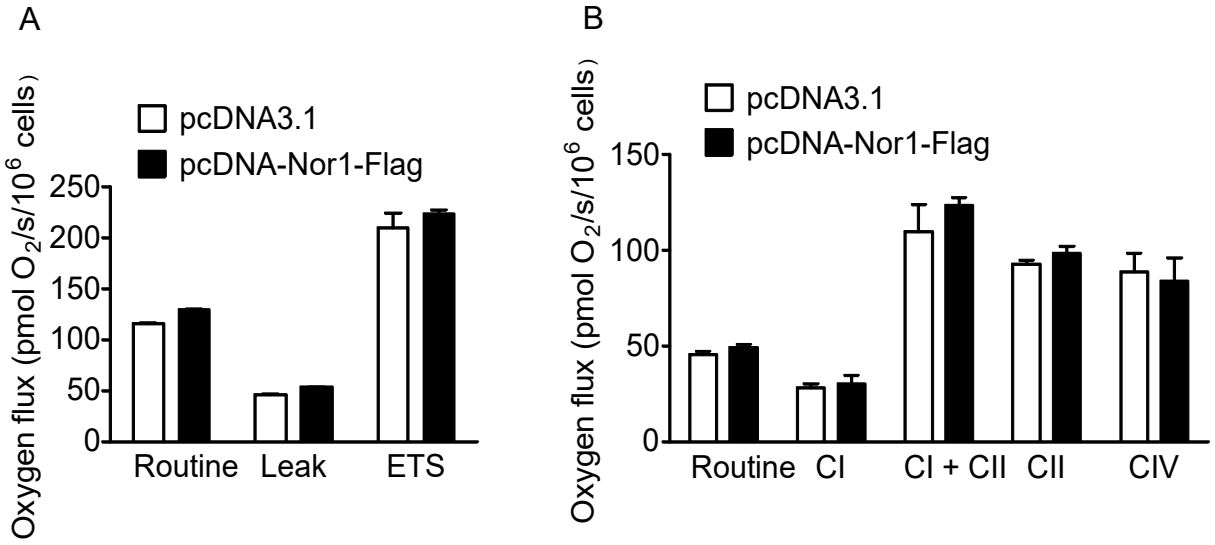


Figure 4.10: Sequential respiratory states in INS832/13 cells 24h after transfection with Nor1Flag or a control vector. (A) Intact cells. N=2. (B) Permeabilized cells. N=4. Routine: basal respiratory state. Leak: after addition of oligomycin (2 µg/mL). ETS: following successive steps of FCCP injections (0.1 µL of 10 mM FCCP per step). CI: oxygen flux after addition of MPG (malate+pyruvate+glutamate). CI+CII: after addition of succinate. CII: after inhibition of CI by Rotenone. CIV: after addition of TMPD and ascorbate. All results are represented as means +/- SEM.

Chapter 5: A critical role for the neural zinc factor ST18 in pancreatic β-cell apoptosis

This research was originally published in J Biol Chem. Cyndi Henry, Anne-Françoise Close and Jean Buteau (2014) A critical role for the neural zinc factor ST18 in pancreatic β-cell apoptosis. *J Biol Chem* 289(12):8413-9.

5.1 Introduction

Zinc fingers are small structural motifs and one of the most abundant DNA binding structures. They are classified according to the number and position of the cysteine and histidine residues, which are responsible for zinc coordination. The Neural Zinc Finger/Myelin Transcription Factor family comprises three members, namely NZF1 (also known as MyT1L), MyT1 (also known as NZF2) and Suppression of Tumorigenicity 18 (ST18, also known as NZF3/MyT3). ST18 was characterized based on structural homology with the two other NZF family members [1]. Thus, they each harbor six Cys-X_5-Cys-X_{12}-His-X_4-Cys (C2HC) zinc finger motifs arranged in two main clusters and recognize the core consensus element AAAGTTT [1]. In contrast to its two paralogs, which have been shown to act both as transcriptional activators and repressors, ST18 has been shown to solely repress transcription of genes containing the NZF binding motif in their promoter [1]. This was attributed to the absence of specific acidic and serine/threonine-rich domains that are found in NZF1 and MyT1.

NZF1 and MyT1 have been suggested to play a role in neuronal [2] and endocrine pancreatic [3, 4] development, respectively. Nevertheless, the biological functions of the NZF transcription factors remain elusive. Very little is known about the regulation of ST18 expression, its biological role or its transcriptional targets. Loss of ST18 expression was reported in breast tumors and various breast cancer cell lines [5]. Also, ectopic expression of ST18 reduced the tumorigenicity of MCF-7 cells [5], thereby suggesting a role for ST18 in tumor suppression. Furthermore, manipulation of ST18 expression in fibroblasts unravelled its potential proapoptotic and proinflammatory roles [6]. ST18 expression was reported in the developing pancreas but its potential role in mature β-cells has never been investigated [4].

In this study, we identify the transcriptional repressor ST18 as a novel regulator of β-cell mass and function. We show that ST18 expression is induced by cytotoxic concentrations of free fatty acids or cytokines. ST18 gain-of-function causes β-cell apoptosis, curtails proliferation and impairs insulin secretion. Conversely, ST18 knockdown prevents palmitate- and cytokine-induced β-cell apoptosis. These data suggest that ST18 could represent a novel molecular target in diabetes.

5.2 Experimental procedures

Material - RPMI 1640, fetal calf serum and glutamine were purchased from Life Technologies (Burlington, ON). All other cell culture reagents were from Sigma (St-Louis, MO).

Commercial PCR array - ST18 tissue distribution was investigated using a rat TissueScan array from Origene (Rockville, MD), according to the manufacturer's protocol. Each qPCR array contains high quality cDNAs from 46 different tissues. Two pairs of arrays were provided in the kit and they were probed for actin and ST18 in duplicates.

Animals – Animal care and handling were performed in accordance with the Canadian Guide for the Care and Use of Laboratory Animals. All experimental procedures received prior approval of the Laval University animal care committee. C57BL/6J mice were fed a high-fat (60 kcal% fat) or control diet (10 kcal% fat) for 8 weeks (Jackson, Bar Harbor, ME). Pancreas were removed and analyzed by immunofluorescence as described below.

Isolation of pancreatic islets - Islets were isolated from male (250 g) Wistar rats. Animals were anesthetized and the pancreatic duct was perfused with a solution containing 0.7 mg/ml type V collagenase. The pancreas was removed and digested in a 37°C water bath. Islets were subsequently purified on a Histopaque gradient, hand-picked under a microscope and cultured in complete RPMI medium.

Cell culture - INS (832/13) cells (passage 36-60) were grown in RPMI-1640 medium supplemented with 10 mM HEPES, 10% heat-inactivated fetal calf serum, 2 mM L-glutamine, 1 mM sodium pyruvate, and 50 µM ß-mercaptoethanol at 37°C in a humidified 5% CO_2 atmosphere. Cells at 70% confluence were washed with PBS and pre-incubated in serum-free RPMI supplemented with 3 mM glucose and 0.1% bovine serum albumin (BSA).

Immunohistochemistry - Cells were seeded onto poly-lysine-coated coverslips, and cultured as described above. Cells were then washed, fixed in paraformaldehyde and incubated with an anti-ST18 antibody. Pancreas were fixed in paraformaldehyde and embedded in paraffin. We mounted 10 µm sections on slides and performed immunohistochemistry with a cocktail of antibodies comprising anti-ST18 and anti-insulin or anti-glucagon antibodies. Primary antibodies were from Sigma (St-Louis, MO). Images were acquired on a fully automated fluorescence microscope (Olympus, Markham, ON) equipped with Suveyor and ImagePro Plus software. Densitometry results are reported as mean signal intensity per surface area in the islets.

Electrophoretic mobility shift assay - EMSA was performed as described in [8] with slight modifications. Briefly, 5 µg of protein extracts were incubated with a biotin-labelled probe for 20 min at room temperature. Samples were analyzed on 4% non-denaturing polyacrylamide gels and revealed with streptavidin-HRP. A 50-fold excess of un-labelled oligonucleotide was added to assess the specificity of the binding.

Western blot - Proteins were extracted and quantified by BCA assay (Roche, Rockford, IL) prior to fractionation on 4-12% polyacrylamide gels (Life Technologies, Burlington, ON).

Transfection - DNA vectors were introduced into INS832/13 cells by nucleofection (Lonza, Mississauga, ON) at a concentration of 5 µg of DNA for 6×10^6 cells. Cells were assayed the following day.

siRNA-mediated knockdown - ST18-specific stealth siRNAs were purchased from Life Technologies (Burlington, ON) and were transfected with Lipofectamine RNAiMax following the manufacturer's protocol. We have used two siRNAs targeting different ST18 sequences (s141450 and s141448). Briefly, cells were plated in 24 wells plates and incubated 24h to obtain adherence. For each well, 100 pmol of siRNA was mixed with 4 µL of Lipofectamine RNAiMax in RPMI 1640 medium containing no additive. Experiments were conducted 48h after siRNA transfection.

Human islets - Human islets were purchased from Prodo Laboratories (Irvine, CA). Islets from three different lean donors, between 45 and 61 years of age and without any history of diabetes or metabolic disorder, were used. Our study was approved by the Human Research Ethics Board at the University of Alberta.

Apoptosis - Apoptosis was assessed by TUNEL assay (DNA fragmentation) using a fluorescein *in situ* cell death detection kit (Roche, Indianapolis, IN). In brief, INS or human islet cells were seeded onto poly-lysine-coated coverslips, fixed in paraformaldehyde, permeabilized with 0.1% Triton X-100 in PBS, blocked with 1% BSA and incubated for 1 h with a TUNEL reaction mix. The coverslips were then washed in PBS and mounted with antifade containing Hoechst. The fluorescence was visualized under a fluorescence microscope at 400X magnification. At least 600 cells were analyzed for each experimental condition.

Proliferation - Proliferation was evaluated using an ELISA-based BrdU incorporation kit (Roche, Indianapolis, IN). In brief, INS832/13 cells were transfected with the indicated DNA vectors, seeded in 96-well plates at 70% confluence and incubated overnight in serum-free RPMI

medium supplemented with 3 mM glucose and 0.1% bovine serum albumin (BSA). BrdU was added to the culture medium for the last 1 h of the incubation period. Cells were then fixed, incubated with a peroxidase-conjugated anti-BrdU antibody and the immune complexes were quantified using a spectrophotometer to measure absorbance (Bio-Rad, Hercules, CA).

Insulin secretion and total insulin content assays - Cells were cultured in 24-well plates, washed and incubated for 30 min in 2.8 mM glucose KRBH buffer before incubation for 30 min at different glucose concentrations (2.8 mM and 16 mM) or 35 mM KCl to induce cell depolarization. At the end of the incubation, culture medium was collected, centrifuged to remove cells and assayed for insulin content by radioimmunoassay (Linco, St. Charles, MO). Total insulin content was measured after acid ethanol extraction. Insulin secretion results are presented as % of insulin content to normalize for the loss of β-cells by apoptosis. Insulin contents were normalized to total protein content.

Calculations and Statistics - Data are presented as means ± SEM. Statistical analyses were performed with SPSS using ANOVA.

5.3 Results

Tissue distribution of the neural zinc finger transcription factor ST18

We first established the expression pattern of ST18 using a commercial PCR array (Fig 5.1). Our data show that ST18 is broadly expressed with the most abundant expression observed in the thyroid, the spinal cord, the aorta and the brain. Along the gastrointestinal tract, ST18 was moderately expressed in the stomach, the liver, and the pancreas. It was virtually absent in the intestine.

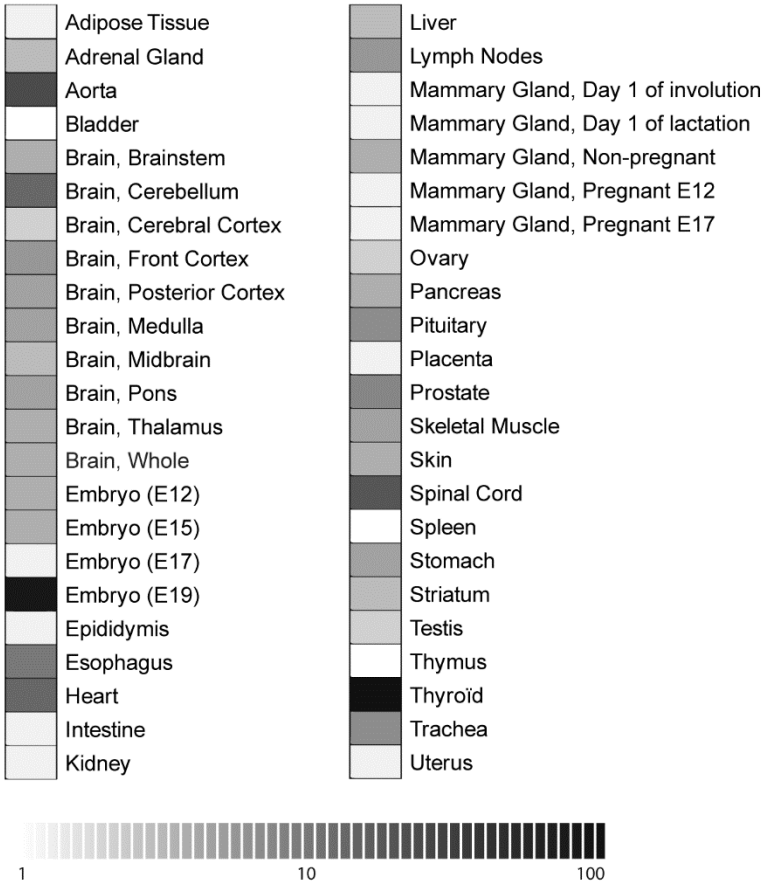


Figure 5.1: Tissue distribution of ST18. Relative ST18 expression levels were determined by qPCR using a commercial array. Results are presented on a logarithmic scale.

We next examined the precise tissue distribution of ST18 within the pancreas by immunofluorescence and PCR. Immunostaining of rat pancreas sections indicated that ST18

expression was restricted to the endocrine pancreas (Fig 5.2A). Indeed, ST18 staining co-localized with both insulin (top panels) and glucagon (lower panels) but remained undetected in acinar and ductal cells. To further confirm the pancreatic distribution of ST18, we analyzed its expression by PCR in isolated rat islets and acinar tissues. Consistent with our immunostaining analysis, ST18 expression was restricted to the islet fraction, with no expression detected in exocrine cells (Fig 5.2B). Insulin and amylase were used as controls. Quantification of ST18 expression by real-time qPCR indicated that its expression was ~10 fold higher in isolated islets than in whole pancreas and reached 50% of that in the brain (Fig 5.2C). ST18 was not detected in the spleen, which was used as a negative control.

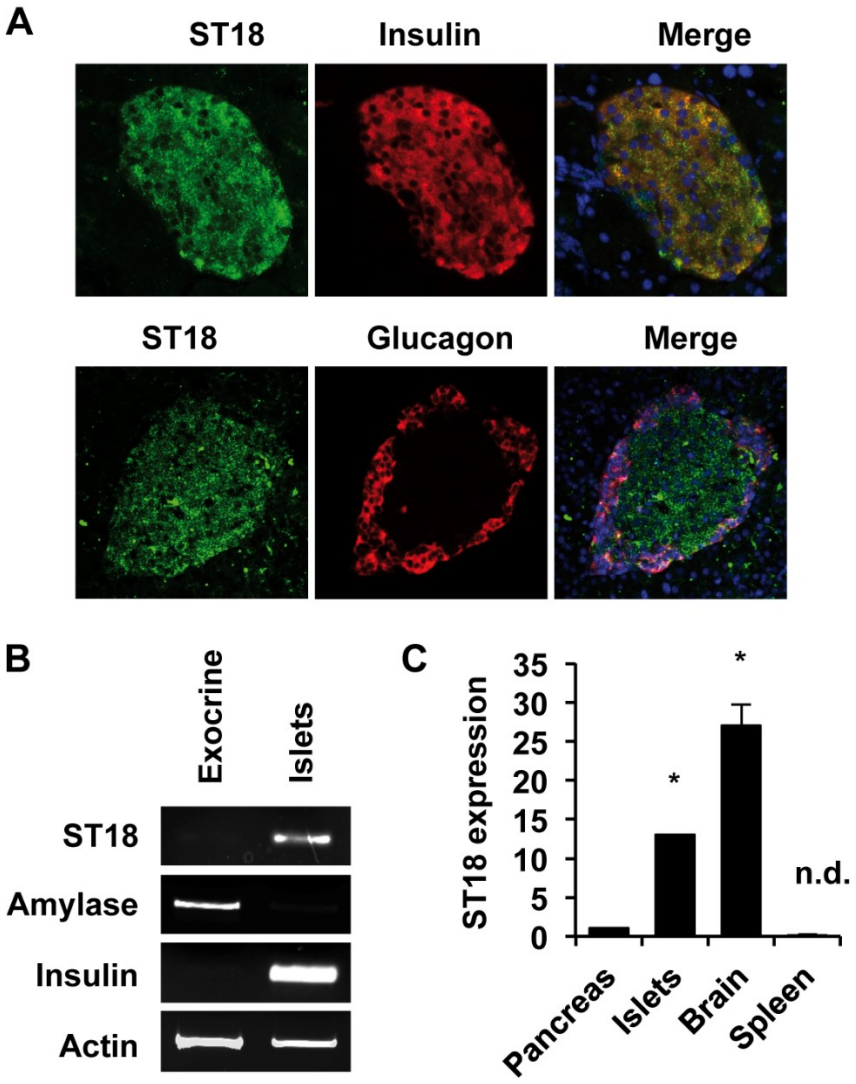


Figure 5.2: ST18 expression is restricted to endocrine cells in the pancreas. A) Double-immunostaining for ST18 (green) and insulin or glucagon (red). B) ST18 expression was investigated by PCR in rat exocrine and islet fractions. Amylase, insulin and actin were used as controls. Representative images of at least 3 separate experiments are shown. C) Quantitative PCR results demonstrating the relative ST18 expression in rat whole pancreas, islets, brain and spleen. Means ± SE of 5 animals. *, $p<0.05$ vs whole pancreas; n.d., not detected.

Regulation of ST18 expression and activity

We next sought to investigate ST18 expression/activity in response to various environmental cues, reasoning that it could provide valuable clues to help grasp its role in β-cells.

We first measured ST18 expression in INS832/13 cells following exposure to 10% serum, elevated glucose, free fatty acids (palmitate and oleate), a cytokine cocktail comprising IL1β and IFNγ, or the glucoincretin hormone GLP-1. ST18 expression was significantly increased in the presence of palmitate, oleate as well as cytokines (Fig 5.3A). Conversely, it was decreased by elevated glucose concentrations. ST18 expression remained unaltered by the glucoincretin hormone GLP-1. In Fig 5.3A, expression of ST18 was greater in cells incubated in the absence of serum overnight as compared to those incubated with serum. Fig 5.3B demonstrates that this increase in ST18 expression in serum-deprived cells occurred in a time-dependent manner. Altogether, these observations suggest that conditions deleterious to β-cell function induce ST18 expression.

We next tested whether the rise in ST18 expression translates into increased ST18 protein levels (Fig 5.3C). Our data show that overnight serum deprivation increased ST18 protein levels in INS cells. Furthermore, the rise in ST18 protein levels was accentuated by cytotoxic concentrations of palmitate (0.4 mM). We next confirmed the effects of palmitate on ST18 expression in normal islet tissue. Thus, isolated human islets exposed to 0.4 mM palmitate overnight displayed a 2-fold increase in ST18 expression as compared to control untreated islets (Fig 5.3D).

In order to assess ST18 DNA binding activity, we performed electrophoretic mobility shift assays using a biotin-labelled DNA probe containing an ST18 consensus sequence [1]. Our data show that ST18 DNA binding activity was increased by palmitate and cytokines (Fig 5.3E). The observed shift could be competed with an excess of cold probe. Unfortunately, currently available anti-ST18 antibodies are not suitable for supershift experiments.

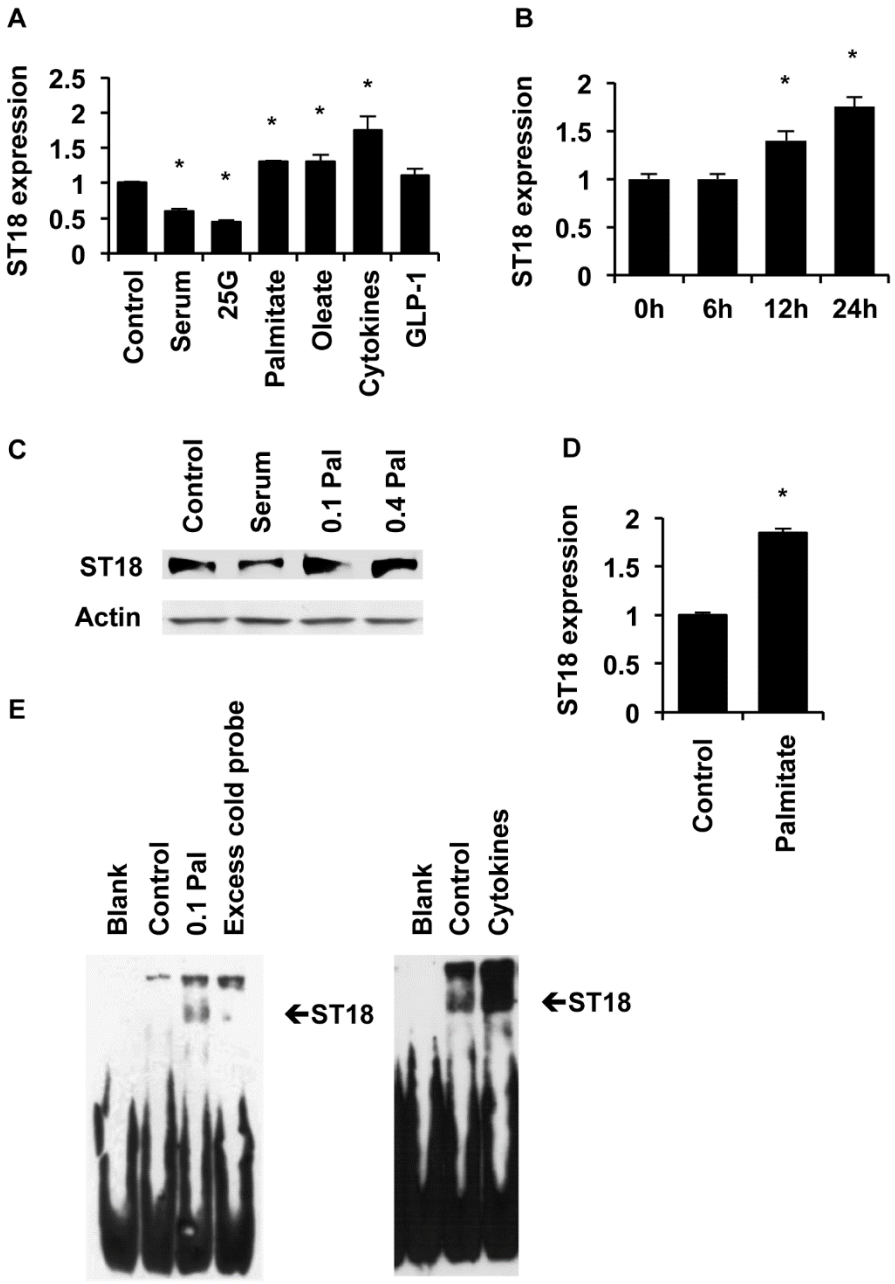


Figure 5.3: ST18 expression and activity are up-regulated by palmitate or cytokines in INS832/13 cells. A) ST18 expression was investigated by qPCR in INS832/13 cells incubated for 24h in the absence or presence of serum, 25 mM glucose (25G), 0.1 mM palmitate, a cytokine cocktail (10 ng/ml IFN-γ and 10 ng/ml IL1β), and 10 nM GLP-1. B) qPCR results demonstrating the time-dependent increase in ST18 expression following serum deprivation. C) ST18 protein levels were determined by western blot after overnight incubation in the absence or presence of serum and palmitate (0.1 mM and 0.4 mM bound to BSA). D) ST18 DNA binding

activity was evaluated by EMSA. Nuclear extracts were prepared from cells incubated in the presence or absence or palmitate (left panel) and cytokines (right panel) for 1h. Means ± SE of 3 experiments, each comprising duplicates. *, $p<0.05$ vs control. Representative images of at least 3 separate experiments are shown.

We next sought to test whether fatty acids could also induce ST18 expression in islets *in vivo*. ST18 levels were evaluated by immunofluorescence in pancreas sections from diet-induced obese animals. Mice fed a high fat diet (60 kcal% fat) for 12 weeks displayed a significant increase in nuclear ST18 signals compared to control animals fed a 10% fat diet (Fig 5.4A and B, respectively).

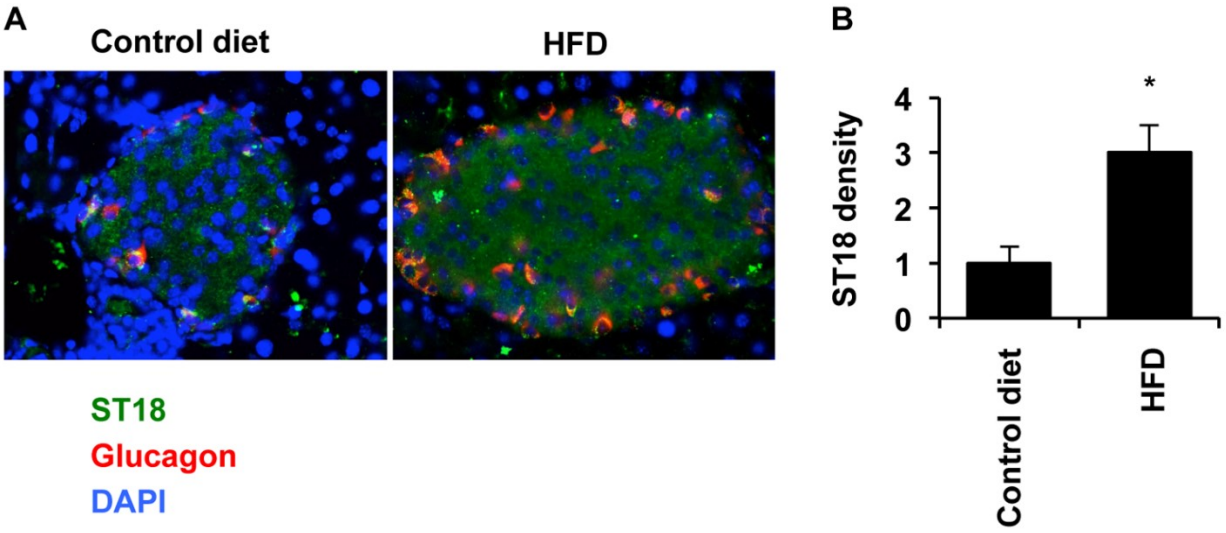


Figure 5.4: Nuclear localization of ST18 is increased in islets of diet-induced obese animals.
ST18 subcellular localization was investigated by immunofluorescence in pancreas sections from 16-week old mice fed a control or high fat diet. A) Typical immunofluorescence images are shown. ST18 was stained in green and glucagon in red. Nuclei were counterstained in blue. B) Quantification of ST18 signal intensity in islet cells. Means ± SE of 4 animals for each category; *, $p<0.05$ vs chow diet.

The increase in ST18 expression/activity in response to free fatty acids and cytokines raised the possibility that ST18 could play a role in apoptosis. This prompted us to examine the role of ST18 in lipotoxicity and cytokine-stimulated β-cell death.

The potential role of ST18 in β-cell apoptosis and proliferation

We evaluated apoptosis by TUNEL assay in INS832/13 cells with either siRNA-mediated ST18 knockdown or ST18 overexpression. In non-stimulated conditions, gene silencing efficiency was ~70% as measured by qPCR (Fig 5.5A). A 50% reduction was achieved in the presence of palmitate or cytokines, which completely abolished the rise in ST18 expression and returned its levels to control values (Fig 5.5A). ST18 protein levels were assessed by western blot. In the presence of palmitate, siRNA-mediated ST18 knockdown reduced protein levels by ~70% after 48h (Fig 5.5B). Conversely, cells with ST18 gain-of-function displayed a 5- to 10-fold increase in ST18 protein levels (Fig 5.5C), similarly to what was obtained with elevated concentrations of palmitate as shown in Fig 5.3.

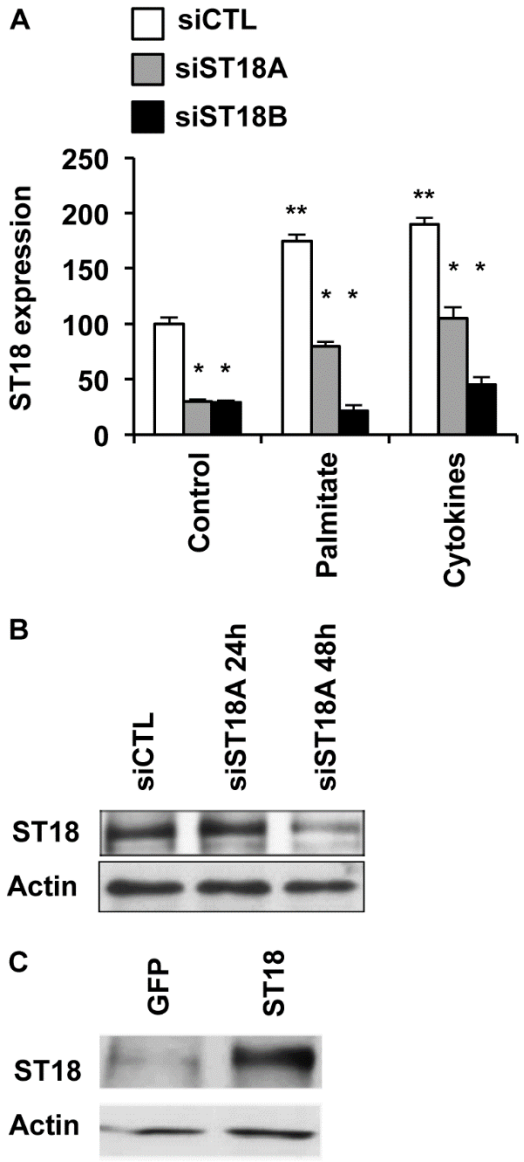


Figure 5.5: Manipulation of ST18 expression in INS cells. A) INS832/13 cells were transfected with control siRNA (siCtrl) or two different ST18-specific siRNAs (siST18A and B) and, the following day, were exposed to palmitate (0.1 mM) and cytokines for an additional 24h. ST18 expression was measured by qPCR. *, $p<0.05$ vs corresponding siCtrl; **, $p<0.05$ vs untreated cells. B) ST18 protein levels were determined by western blot in cells treated as described above in the presence of palmitate. C) ST18 protein levels were determined by western blot in cells transfected with either control (GFP) or pCMV6-ST18 (ST18) vectors for 24h. Representative images of at least 3 experiments are shown.

Knockdown of ST18 expression significantly decreased β-cell apoptosis caused by serum deprivation, cytotoxic concentrations of palmitate or cytokines (Fig 5.6A). Conversely, ST18 overexpression caused a marked 4-fold increase in apoptosis and this effect was not additive to the effect of palmitate (Fig 5.6B). These data identify ST18 as a novel mediator of lipotoxicity and cytokine-toxicity in β-cells.

We next tested whether the cytotoxic effect of ST18 could be complemented by an inhibitory action on β-cell proliferation. The effect of ST18 on INS832/13 cell proliferation was investigated by BrdU incorporation. ST18 loss-of-function increased proliferation by 30% and prevented the deleterious effect of palmitate (Fig 5.6C). However, the reduction in proliferation caused by cytokines was unchanged by ST18 knockdown. Consistently with its effect on apoptosis, forced expression of ST18 blunted proliferation by 50% and this effect was non-additive to that of palmitate (Fig 5.6D).

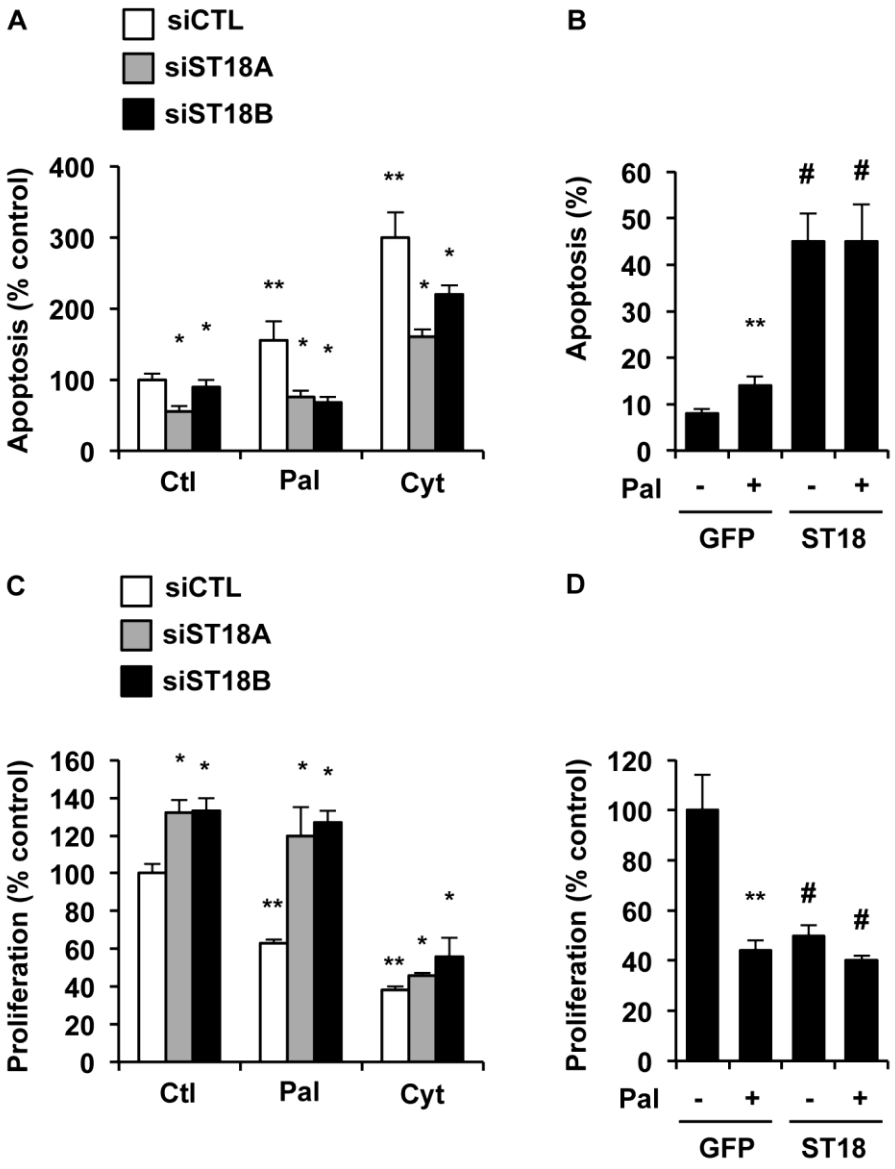


Figure 5.6: ST18 induces β-cell apoptosis and suppresses proliferation in INS832/13 cells. A) Apoptosis was measured by TUNEL assay after overnight incubation in the presence or absence of 0.1 mM palmitate and cytokines, in INS832/13 cells transfected with either control siRNAs or ST18-specific siRNAs. B) Apoptosis was evaluated in the absence or presence of palmitate in INS832/13 cells transfected with control GFP or ST18 expression vectors. C-D) The effects of palmitate and cytokines on proliferation were examined by BrdU incorporation assay in INS832/13 cells with either ST18 loss-of-function (C) or gain-of-function (D), as described above. All results represent means ± SE of at least 3 experiments, each performed in duplicates. *, $p<0.05$ vs control siRNA; **, $p<0.05$ vs untreated cells; #, $p<0.05$ vs control vector.

We sought to confirm the pro-apoptotic action of ST18 in isolated human islet cells. We thus transfected human islet cells with GFP or ST18-GFP and determined the percentage of dead cells in transfected (green) cells by Tunel staining. Fig 5.7 shows that ST18 gain-of-function induced apoptosis by 2.5 fold in human islet cells. Altogether, our data identify ST18 as a negative regulator of β-cell mass, via its combined effect on proliferation and apoptosis.

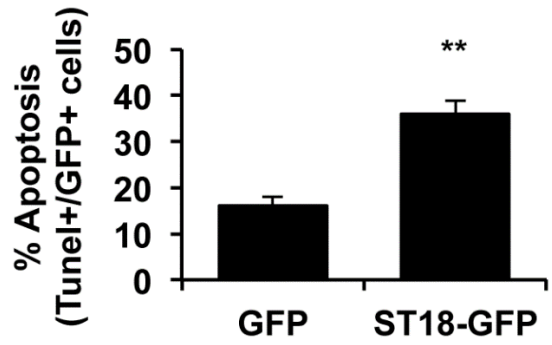


Figure 5.7: Pro-apoptotic action of ST18 in isolated human islet cells. Dispersed human islet cells were transfected with either GFP or ST18-GFP. The following day, apoptotic cells were detected by Tunel assay. Results show the percentages of Tunel-positive cells in transfected (green) cells. Means ± SE of 3 experiments, each comprising duplicates. *, $p<0.05$ vs GFP control.

We next examined the subcellular localization of ST18 by immunofluorescence in the absence or presence of palmitate in INS832/13 cells (Fig 5.8). In the control situation, ST18 was predominantly localized in the cytoplasm of INS832/13 cells (Fig 5.8A, left panel). Upon palmitate treatment, the nuclear localization of ST18 increased (Fig 5.8A, middle panel). Forced expression of ST18 also promoted the nuclear translocation of ST18, concomitantly with apoptosis (Fig 5.8A, right panel). We next investigated the subcellular localization of ST18 in dispersed human islet cells using ST18-GFP (Fig 5.8B). ST18 was found almost exclusively in the cytoplasm of healthy human islet cells (Fig 5.8B, asterisks). However, we detected nuclear ST18 in islet cells undergoing apoptosis (Fig 5.8B, white arrow). Thus, ST18 is predominantly cytoplasmic in unchallenged INS832/13 cells and translocates to the nucleus upon palmitate treatment. ST18 nuclear localization in human islet cells coincides with apoptosis.

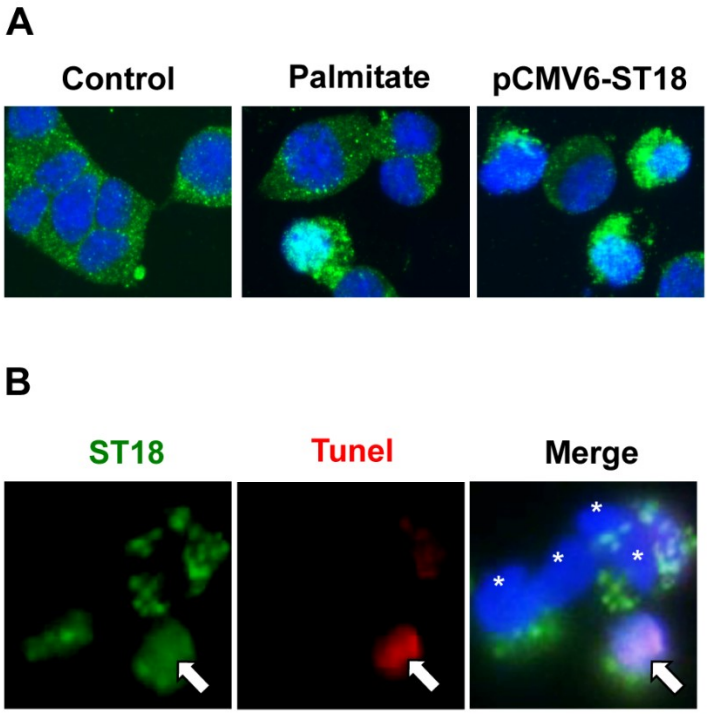


Figure 5.8: Nuclear localization of ST18 in β-cell apoptosis. A) The subcellular localization of ST18 was investigated by immunofluorescence (green) in INS832/13 cells cultured in the absence or presence of palmitate for 24h, as well as in INS832/13 cells overexpressing ST18. Nuclei are counter-stained in blue. Representative images of 4 separate experiments are shown. B) Dispersed islet cells were transfected with ST18-GFP and, the following day, apoptotic cells were detected by Tunel assay (red). Healthy cells (asterisks) show cytoplasmic localization of ST18 whereas apoptotic cells (arrow) display abundant nuclear ST18.

ST18 affects insulin secretion by a distal mechanism

Finally, we sought to investigate the effect of ST18 on β-cell function by measuring insulin secretion in INS832/13 cells with ST18 overexpression (Fig 5.9A). ST18 slightly increased basal insulin release and completely abolished both glucose- and KCl-stimulated insulin secretion. The fact that ST18 inhibited the effects of KCl suggests that ST18 could act (at least in part) via a mechanism distal to calcium mobilization. Forced expression of ST18 did not significantly alter insulin content (Fig 5.9B).

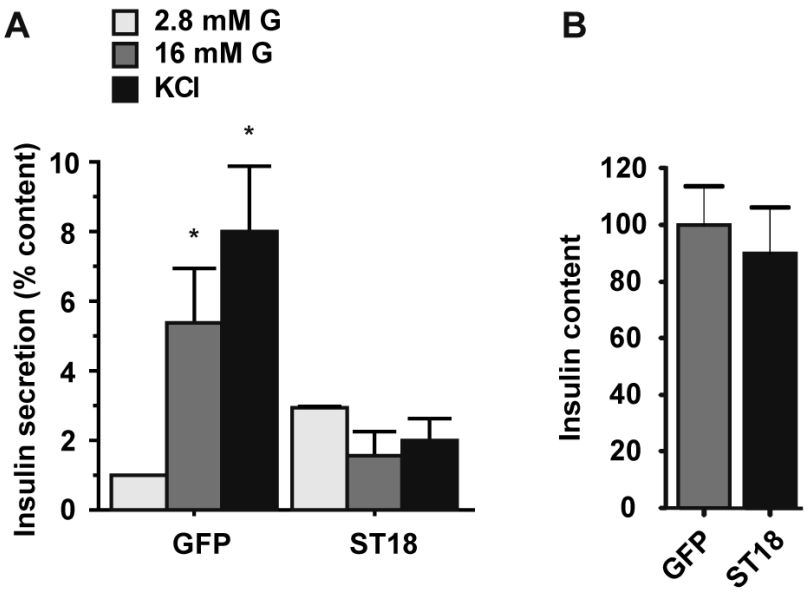


Figure 5.9: ST18 impairs insulin secretion. A) Insulin secretion was determined in INS832/13 cells transfected with either control GFP or ST18 vectors. Cells were incubated at low (2.8 mM) or high (16 mM) glucose concentrations, or in the presence of 35 mM KCl to induce cell depolarization. B) Insulin content was normalized to total protein content and expressed as percent of control. Means ± SE of at least 3 experiments, each performed in duplicates. *, $p<0.05$.

5.4 Discussion

ST18 was originally identified in rat brain tissue based on sequence homology with previous members of the NZF family [1]. However, very little is known about its biological function as it remains relatively unexplored. Our study is the first to investigate ST18 expression in the pancreas and to functionally characterize ST18 in β-cells.

We demonstrate that, in the pancreas, ST18 expression is restricted to endocrine cells. We also provide evidence that both ST18 expression and activity are increased by prolonged exposure to free fatty acids and cytokines. This observation is potentially important because hyperlipidemia and cytokine production are common features of both type 1 as well as type 2 diabetes [11] and, collectively, contribute to β-cell demise. Indeed, local production of cytokines within islets induces β-cell apoptosis in type 1 as well as in type 2 diabetes [12] whereas chronic exposure to elevated levels of free fatty acids causes β-cell apoptosis and impairs glucose-stimulated insulin secretion, a phenomenon often referred to as "lipotoxicity" [13, 14]. Using siRNA-mediated ST18 knockdown, we herein demonstrate that the pro-apoptotic action of free fatty acids and cytokines are mediated by ST18 in β-cells.

The pro-apoptotic action of ST18 is consistent with its role in tumor suppression. ST18 was reported to be down-regulated in breast cancer cell lines and in a majority of breast tumors [1]. Also, ectopic ST18 expression in the breast cancer cell line MCF-7 blunted colony formation in soft agar and tumor formation in a xenograft mouse model [1]. However, the authors did not specifically measure apoptosis in their system. Another publication demonstrated a proinflammatory role for ST18 in fibroblasts [6]. In this study, siRNA-mediated knockdown of ST18 reduced TNFα-induced apoptosis and proinflammatory gene expression, whereas its overexpression significantly enhanced apoptosis. These data, along with ours, support an overarching role of ST18 in cell death. However, the biological role of ST18 is likely to be more complex. A more recent publication reported that ST18 expression was up-regulated during neuronal differentiation *in vitro* and *in vivo* [15]. The authors went on to show that forced expression of ST18 in P19 cells (a model of neuronal progenitor cells) caused spontaneous differentiation. The action of ST18 on neuronal differentiation was synergistic to that of the bHLH protein Neurog1. Thus, the biological role of ST18 may be context-dependent.

The role of ST18 in neuronal differentiation is reminiscent of the role of its paralog MyT1 in endocrine cell differentiation. The group of Dr. Gu has shown that MyT1 is required for proper endocrine cell differentiation [4] and that Myt1 and Ngn3 form a feed-forward loop in pancreatic progenitor cells [16]. This raises questions about a possible redundancy between all three NZF paralogs. However, the bulk of evidence suggests that NZF members could each exert specific actions: *i)* NZF members display different binding affinities for closely related sequences [1]; *ii)* ST18 acts as a transcriptional repressor on reporter constructs containing a consensus binding element whereas MyT1 and NZF2 act as transcriptional activators [1]; *iii)* ST18 lacks acidic and serine/threonine-rich regulatory domains that are present in its two paralogs [1]; *iv)* and finally, the three NZF members are not equipotent in inducing neuronal differentiation [15].

Unfortunately, the pathways that govern ST18 expression have never been explored. Likewise, its transcriptional targets remain elusive, thereby depriving us from very valuable clues about its mode of action. As more molecular tools and genetically engineered animals are made available, we expect future studies to fill this critical gap in knowledge.

In summary, we characterized ST18 as a transcriptional mediator of lipotoxicity and cytokine-induced β-cell apoptosis. ST18 could therefore represent a common effector of fatty acid- and cytokine-induced β-cell death. We suggest that ST18 could be a potential target for the preservation/regeneration of β-cell mass and function.

5.5 References

1. Yee, K. S., and Yu, V. C. (1998) Isolation and characterization of a novel member of the neural zinc finger factor/myelin transcription factor family with transcriptional repression activity. *J Biol Chem* **273**, 5366-5374
2. Kim, J. G., Armstrong, R. C., v Agoston, D., Robinsky, A., Wiese, C., Nagle, J., and Hudson, L. D. (1997) Myelin transcription factor 1 (Myt1) of the oligodendrocyte lineage, along with a closely related CCHC zinc finger, is expressed in developing neurons in the mammalian central nervous system. *J Neurosci Res* **50**, 272-290
3. Gu, G., Wells, J. M., Dombkowski, D., Preffer, F., Aronow, B., and Melton, D. A. (2004) Global expression analysis of gene regulatory pathways during endocrine pancreatic development. *Development* **131**, 165-179
4. Wang, S., Zhang, J., Zhao, A., Hipkens, S., Magnuson, M. A., and Gu, G. (2007) Loss of Myt1 function partially compromises endocrine islet cell differentiation and pancreatic physiological function in the mouse. *Mech Dev* **124**, 898-910
5. Jandrig, B., Seitz, S., Hinzmann, B., Arnold, W., Micheel, B., Koelble, K., Siebert, R., Schwartz, A., Ruecker, K., Schlag, P. M., Scherneck, S., and Rosenthal, A. (2004) ST18 is a breast cancer tumor suppressor gene at human chromosome 8q11.2. *Oncogene* **23**, 9295-9302
6. Yang, J., Siqueira, M. F., Behl, Y., Alikhani, M., and Graves, D. T. (2008) The transcription factor ST18 regulates proapoptotic and proinflammatory gene expression in fibroblasts. *Faseb J* **22**, 3956-3967
7. Hohmeier, H. E., Mulder, H., Chen, G., Henkel-Rieger, R., Prentki, M., and Newgard, C. B. (2000) Isolation of INS-1-derived cell lines with robust ATP-sensitive K+ channel-dependent and -independent glucose-stimulated insulin secretion. *Diabetes* **49**, 424-430
8. Buteau, J., Roduit, R., Susini, S., and Prentki, M. (1999) Glucagon-like peptide-1 promotes DNA synthesis, activates phosphatidylinositol 3-kinase and increases transcription factor pancreatic and duodenal homeobox gene 1 (PDX-1) DNA binding activity in beta (INS-1)-cells. *Diabetologia* **42**, 856-864
9. Buteau, J., El-Assaad, W., Rhodes, C. J., Rosenberg, L., Joly, E., and Prentki, M. (2004) Glucagon-like peptide-1 prevents beta cell glucolipotoxicity. *Diabetologia* **47**, 806-815

10. Buteau, J., Spatz, M. L., and Accili, D. (2006) Transcription factor FoxO1 mediates glucagon-like peptide-1 effects on pancreatic beta-cell mass. *Diabetes* **55**, 1190-1196
11. Rhodes, C. J. (2005) Type 2 diabetes-a matter of beta-cell life and death? *Science* **307**, 380-384
12. Donath, M. Y., Storling, J., Maedler, K., and Mandrup-Poulsen, T. (2003) Inflammatory mediators and islet beta-cell failure: a link between type 1 and type 2 diabetes. *J Mol Med* **81**, 455-470
13. El-Assaad, W., Buteau, J., Peyot, M. L., Nolan, C., Roduit, R., Hardy, S., Joly, E., Dbaibo, G., Rosenberg, L., and Prentki, M. (2003) Saturated fatty acids synergize with elevated glucose to cause pancreatic beta-cell death. *Endocrinology* **144**, 4154-4163
14. Poitout, V., and Robertson, R. P. (2002) Secondary beta-cell failure in type 2 diabetes--a convergence of glucotoxicity and lipotoxicity. *Endocrinology* **143**, 339-342
15. Kameyama, T., Matsushita, F., Kadokawa, Y., and Marunouchi, T. (2011) Myt/NZF family transcription factors regulate neuronal differentiation of P19 cells. *Neurosci Lett* **497**, 74-79
16. Wang, S., Hecksher-Sorensen, J., Xu, Y., Zhao, A., Dor, Y., Rosenberg, L., Serup, P., and Gu, G. (2008) Myt1 and Ngn3 form a feed-forward expression loop to promote endocrine islet cell differentiation. *Dev Biol* **317**, 531-540

Chapter 6: Discussion and conclusions

6.1 Summary of research findings

A decrease of β-cell mass is one of the key contributors to the development and progression of type 2 diabetes [28, 31]. This reduction of β-cell mass could be attributed to an increase of β-cell apoptosis in the islets of type 2 diabetic patients [32-34]. Various factors and processes have been suggested to orchestrate β-cell demise in type 2 diabetes: circulating long chain fatty acids, high blood glucose, Islet Amyloid Polypeptide, pro-inflammatory cytokines, oxidative stress, ER stress as well as mitochondrial dysfunction and fragmentation [31]. Transcription factors play a critical role in mediating the deleterious effects of these diabetogenic conditions.

The Nr4a family of orphan nuclear receptors has been proposed to participate to the pathogenesis of type 2 diabetes. Indeed, *(i)* changes in NR4A expression have been reported in animal models of insulin resistance, diabetes and/or obesity compared to their respective controls; *(ii)* both NR4A expression and activity are under nutritional control; and *(iii)* recent reports demonstrated important roles for NR4A members in the regulation of glucose transport, glucose utilization, insulin sensitivity, gluconeogenesis and insulin secretion [167-169]. Moreover, recent evidence suggests that NR4As might play a role in the regulation of β cell mass as well. Indeed, *(i)* the expression of NR4As is controlled by well established inducers of β-cell death such as palmitate, glucose and pro-inflammatory cytokines; *(ii)* NR4As have been suggested to modulate cell replication and apoptosis in a manner dependent on their subcellular localization; *(iii)* NR4As have been shown to exert an effect on mitochondrial function and dynamics [180, 206, 207, 229].

The neural zinc factor ST18 might exert pro-apoptotic and anti-proliferative actions in breast cancer cells [210]. Moreover, it has been suggested that ST18 is a mediator of TNFα-mediated apoptosis in fibroblasts [211]. These findings support the idea that ST18 might be a mediator of β-cell apoptosis induced by diabetogenic factors such as cytokines.

The overall hypothesis is that Nr4as are key regulators of β-cell mass and more specifically that they mediate the deleterious effects of environmental stresses on β-cells in type 2 diabetes. We also postulate that ST18 is another mediator of stress-induced β-cell apoptosis in type 2 diabetes.

The present thesis therefore aimed to investigate the potential effect of Nr4as on β-cell mass and to perform a first characterization of the expression profiles and roles of Nr4as and ST18 in β-cells. In a second phase, we sought to clarify the mechanisms of action of these 2 families of factors.

The results of our study are listed below. A schematic summary of the pro-apoptotic effects of Nor1 in β-cells is also presented in figure 6.1.

Chapter 3: Evaluation of the effects of Nr4as on β-cell mass and exploration of their role and expression in β-cells.

- $Nor1^{-/-}$ mice but nor $Nur77^{-/-}$ or $Nurr1^{+/-}$ presented larger islets, ~2.5 fold higher β-cell mass, lower non-fasting blood glucose and better glucose tolerance compared to wild type animals. The observations regarding blood glucose and glucose tolerance could not be attributed to changes in weight or food intake.
- β-cell proliferation was increased by 50% in the islets of $Nor1^{-/-}$ mice.
- The expression of Nor1 but not Nur77 or Nurr1 was increased by cytokines (Il1β and IFNγ) in β-cells (INS1 and human islets).
- Nor1 induced a ~3 fold and ~5 fold increase of apoptosis in INS1 cells and human islet's dispersed β-cells respectively. Moreover, Nor1 was shown to be necessary for cytokines-induced apoptosis in β-cells (INS1). More specifically, Nor1 might trigger the intrinsic pathway of apoptosis as it was suggested by the elevated release of cytochrome c in INS1 cells overexpressing Nor1.
- Nor1 expression was increased by a ~ 13 fold in the islets of type 2 diabetic patients (qPCR).

Chapter 4: Mechanism of action of Nor1

- In a diabetogenic environment (10 ng/mL Il1β + IFNγ during 1 hour), Nor1 was translocated to the mitochondria.
- Nor1 reduced glucose oxidation and ATP production in β-cells suggesting that Nor1 hinders mitochondrial function. In addition, we determined that Nor1 increases β-cell lactate production.
- Nor1 increased mitochondrial membrane potential and caused mitochondrial membrane ruptures through which the matrix herniated.
- Nor1 caused mitochondrial fragmentation.
- Nor1 induced mitophagy in β-cells.
- Nor1 did not affect mitochondrial content in β-cells as suggested by the measurement of mitochondrial area by TEM, mtDNA/gDNA content, citrate synthase activity and the expression of the mitochondrial protein VDAC.
- Nor1 did not exert its effects on apoptosis through a colocalization with bcl2 or through modulation of the transcriptional profile of β-cells.

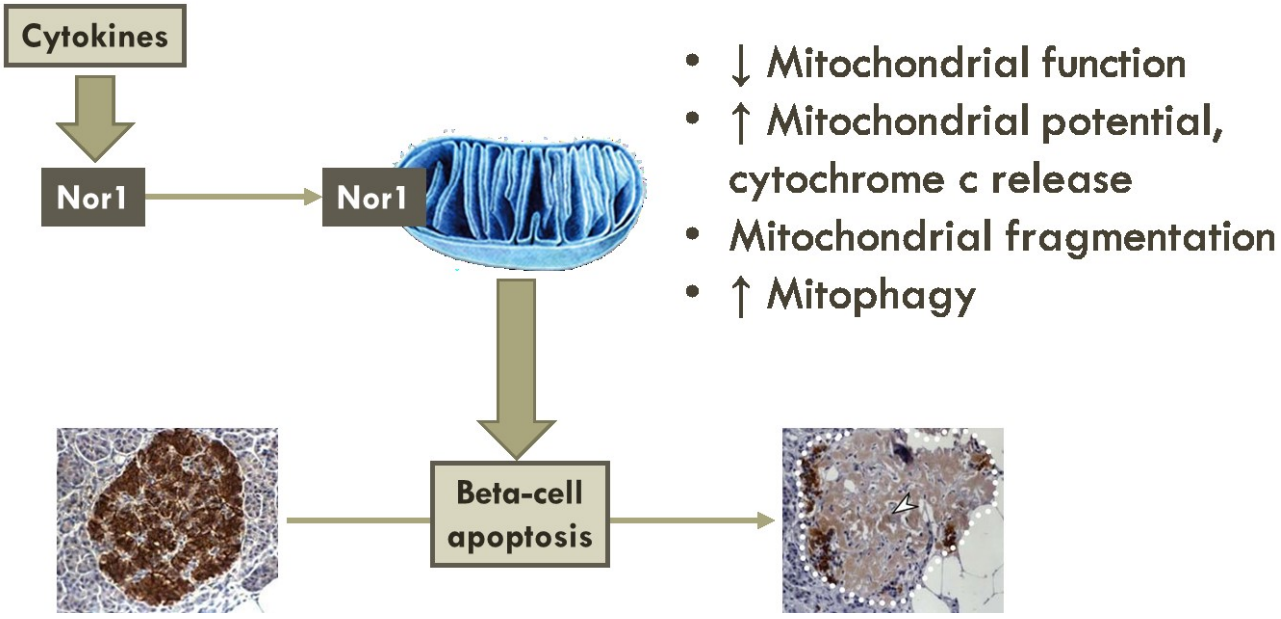


Figure 6.1: Schematic representation of the pro-apoptotic effect of Nor1 in β-cells. Pro-apoptotic cytokines such as Il1β and IFNγ induce the translocation of Nor1 to the mitochondria where it triggers the intrinsic pathway of apoptosis. Nor1 does so by decreasing mitochondrial function,

inducing mitochondrial fragmentation and increasing mitophagy. Nor1 also elevates mitochondrial potential which results in matrix swelling, rupture of the mitochondrial membrane and subsequent release of cytochrome c. The pro-apoptotic action of Nor1 could, along with its negative effect on proliferation, account for its detrimental effect on β-cell mass suggested by the phenotype of $Nor1^{-/-}$ mice.

Chapter 5: Expression profile and role of the transcription factor ST18 in β-cells.

- ST18 expression in the pancreas is restricted to endocrine cells.
- ST18 expression was increased by diabetogenic factors such as palmitate (0.4 mM), oleate (0.4 mM) and cytokines (10 ng/mL Il1β + IFNγ) (INS1 cells). This was confirmed in human islets for palmitate.
- ST18 DNA binding activity was increased by palmitate and cytokines in β-cells (INS1).
- ST18 expression was increased in the pancreas of HFD fed mice.
- ST18 induced a ~ 4-fold increase of β-cell apoptosis (INS1). This was confirmed in human islets. Moreover, ST18 participated to palmitate and cytokines-induced apoptosis in β-cells (INS1).
- ST18 decreased β-cell proliferation by 50% (INS1).
- Palmitate induced the translocation of ST18 to the nucleus (INS1). This translocation coincided with the occurrence of apoptosis in human islet cells.
- ST 18 blunted Glucose and KCl stimulated insulin secretion without altering insulin content.

6.2 Discussion

6.2.1 Nor1 downregulates β-cell mass

We observed that Nor1$^{-/-}$ mice show a ~2.5 - fold increase in β-cell mass compared to control mice, a phenomenon that was not found in mice carrying deletions of the Nur77 or Nurr1 genes. Increased β-cell mass in Nor1$^{-/-}$ animals was accompanied by lower blood glucose levels and improved glucose tolerance (ipGTT). This specific phenotype of Nor1$^{-/-}$ mice resonates with the unique DNA binding properties of Nor1. Indeed, Nur77 and Nurr1 can link the Nur Response Element (NurRE) as homodimers whereas Nor1 displays a much lower affinity for NurRE, resulting in minimal transcriptional activation [163, 164]. In addition, Nur77 and Nurr1, but not Nor1, can form heterodimers with retinoid receptors X (RXR) to target DR5 elements [230, 231]. Thus, Nor1 has DNA binding characteristics and cofactors different from Nur77 and Nurr1. This corresponds to previous reports that demonstrated that Nur77 and Nor1 regulate distinct sets of genes in β-cells [180, 201]. These differences support the idea that Nor1 has unique biological activity among Nr4as.

Our data suggests that the effect of Nor1 on β-cell mass implies an increase in the proliferation of β-cells. Indeed, we detected a 50% increase the staining for the proliferation marker Ki67 in the β-cells of Nor1$^{-/-}$ mice. This finding comes in contrast to those of another study. Indeed, Tessem et al, suggested that Nor1 and Nur77 increase β-cell replication in rat and human islets [201]. The authors used adenoviral encoded genes to study the effect of Nor1 and Nur77 on proliferation. Confirming these results with lentiviral vectors could rule out the possibility that the observed proliferative effects might be due to adenoviral genes. Moreover, the authors did not report the effect that manipulating one Nr4a could have on the expression of the others. It is therefore not certain that their observations are direct consequences of changes of expression of the gene they targeted. The authors confirmed their results with data from Nur77$^{-/-}$ mice but did not analyze Nor1$^{-/-}$ mice. This is of importance because we now know that the phenotypes of these animals can be significantly different.

Our evaluation of the effects of Nor1 by transfecting a Nor1 vector into INS1 cells and human islets revealed that Nor1 is a potent pro-apoptotic agent in β-cells. This is, to our knowledge, the first investigation and first report of an eventual pro-apoptotic effect of Nor1 in β-cells. Indeed,

only Yu et al evaluated the effect of Nur77 overexpression in Min6 cells [192]. They concluded that Nur77 has a protective effect on thapsigargin and palmitate-induced apoptosis in β-cell. If we extrapolate our results and suppose that Nor1 triggers apoptosis *in vivo* as well, we might think that the Nor1$^{-/-}$ animals present reduced apoptosis rates. The contribution of a reduction of apoptosis on the elevation of β-cell mass in these knockouts is questionable. Apoptosis rate has been shown to be relatively low (0.05-0.1%) in human and rodent β-cells, leaving little room for significant reduction [34, 232]. However, evidence suggests that if modifications of β-cell apoptosis rate are sustained over time, it might be sufficient to induce significant variations of β-cell mass. Indeed, Butler et al, showed that type 2 diabetic patients present a lower β-cell mass compared to healthy subjects. This was associated with increased apoptosis but no significant change of β-cell proliferation [34]. Moreover, He et al, reported that in their cyclin D2 mutant transgenic mice, the observed increase of β-cell mass was preceded by a sustained reduction of β-cell apoptosis early in life with no consistent changes in proliferation [232]. The timeline of the evolution of β-cell mass, apoptosis rate and replication in Nor1$^{-/-}$ animals *versus* wild types remains to be established. This would provide valuable clue concerning the potential role of Nor1 in the regulation of β-cell mass.

Our study used Nor1$^{-/-}$ mice. It is therefore unclear whether the effects of Nor1 on pancreatic β-cell mass and blood glucose are direct or secondary to changes in other tissues. To our knowledge, Floxed-Nor1 mice have not yet been generated, which prevents us from studying the effect of specific suppression of Nor1 in β-cells. To address this problem, we investigated the expression and role of Nor1 *in vitro* in INS1 cells and isolated human islets.

6.2.2 Nor1 impairs glucose stimulated insulin secretion

It is tempting to assume that because Nor1$^{-/-}$ animals present elevated β-cell mass compared to controls, their better glucose tolerance can be attributed to higher insulin secretion. Unfortunately, we measured circulating insulin 60 minutes after starting the ipGTT which did not allow us to draw any conclusions regarding insulin secretion in response to glucose in our mice. Indeed, at that point, blood glucose had already been reduced for 45 minutes and did not constitute a strong stimulus for insulin secretion. Plasma insulin was ~ 40% lower in Nor1$^{-/-}$ animals than in wild types. This is presumably a reflect of the significantly lower glycaemia of Nor1$^{-/-}$ animals during the first hour of the ipGTT. However, a study by Weyrich et al previously

helped highlighting a potential role for Nor1 in the regulation of insulin secretion in humans [182]. They showed in 2 independent cohorts comprising 1495 and 5265 patients, that the minor allele of Single Nucleotide Polymorphism (SNP) rs12686676 located in the Nor1 locus was associated with significantly higher insulin secretion. Interestingly, no other member of the Nr4a family had an association with insulin secretion in humans [178]. Despite the association of rs12686676 with insulin secretion, no correlation could be made with the prevalence of either diabetes, impaired glucose tolerance or impaired fasting glucose in the study group.

In our study, we have resorted to evaluate the effect of Nor1 on glucose stimulated insulin secretion (GSIS) in INS1 cells. Our data suggests that Nor1 reduces GSIS by ~50%. This is in accordance with work from Gao et al that reported a significant decrease of GSIS in Min6 cells transfected with Nor1 [233]. Conversely, other evidence suggests that Nor1 might upregulate GSIS [178, 179]. These teams however used Nor1 silencing in β-cells, which induced a reduction of GSIS. Unfortunately, they did not evaluate the effect of modulating Nor1 on the expression of Nur77 and Nurr1. We can therefore not exclude that downregulating Nor1 induces an upregulation of Nur77 which has been shown to impair GSIS [180].

6.2.3 Nor1 as a mediator a diabetogenic stresses

We demonstrated that pro-apoptotic cytokines rapidly increased Nor1 expression by a ~ 13-fold and ~ 3-fold in INS1 cells and human islets respectively. The expression of *Nor1* was also increased by high glucose (25 mM) in our study although the effect of glucose was lesser and later than that of cytokines. Interestingly, it has been suggested that Il1β expression was increased in β-cells after incubation in the presence of high glucose. This induction of Il1β has been shown to be necessary for the pro-apoptotic effect of long-term exposure to high glucose [81]. Il1β is the major contributor to the increase of *Nor1* expression by cytokines. We therefore propose that high glucose increases *Nor1* expression via its effect on Il1β. This would explain why the peak of *Nor1* expression was shifted to 8h instead of 4h in β-cells treated with 25 mM glucose *versus* direct exposure to cytokines.

Palmitate induces early beta-cell apoptosis but did not significantly increase *Nor1* expression in our study. These findings come in contradiction with earlier results from Briand et al [180]. Indeed, their data shows that the expression of all 3 Nr4as is increased by palmitate and

cytokines in Min6 cells as well as in human and mouse islets. However, the effect of palmitate on Nor1 expression observed by Briand et al appears later (48h) and is lesser than the effect on Nur77 and Nurr1. Furthermore, the subcellular localisation of Nor1 as well as its potential contribution to the pro-apoptotic effects of environmental stress in β-cells has to our knowledge never been investigated.

The expression of *Nor1* has also been suggested to be increased by the ER stress inducer and pro-apoptotic agent thapsigargin in β-cells [233].

Evidence that Nor1 is upregulated by diabetogenic factors is building up. It supports our own data showing that Nor1 exerts pro-apoptotic actions and more specifically is a key mediator of cytokines-induced apoptosis in β-cells.

6.2.4 Regulation of Nr4as

Evidence concerning the actions of Nor1 in β-cells remain inconclusive. Indeed, there is a discordance in the literature and compared to our data regarding the effects of Nor1 on β-cell replication and death as well as on insulin secretion. It might thus be judicious here to discuss the regulation of Nr4as' activity and report what we know on the impact of post-translational modifications and subcellular localization on the effects of Nr4as.

The natural ligands for the NR4A nuclear receptors remain to be identified and the NR4As are therefore designated as "orphan receptors". Moreover, Nr4as present two distinct structural characteristics that distinguish them from the rest of the nuclear receptors superfamily: *(i)* the absence of a ligand binding cavity and *(ii)* the absence of a classical binding site for coactivators [16]. These observations suggest a ligand-independent function for NR4A nuclear receptors. Their activity could be instead regulated by expression, posttranslational modifications and subcellular modifications. Consistently, Nurr1 has been shown to be constitutively active [154, 155] and its transcriptional activity agrees with its proteasome-dependent turnover [156]. Additionally, all three NR4As can be phosphorylated by kinases such as JNK, RSK and MAPK [157-159]. However, the consequences of these posttranslational modifications on Nr4as activity and effects remain little investigated. Finally, NR4As can be sumoylated, which has been proposed to hinder their transcriptional activity [160, 161].

Subtle differences in the environment of cells could have a big impact on the post-translational modifications that regulate Nr4as' actions and lead to substantial differences in terms of effects. For example, PKCδ-induced phosphorylation of Nur77 and Nor1 that occurs in thymocytes in response to IL1β is the signal that drives the translocation of these Nr4as to the mitochondria where they interact with bcl2 to trigger intrinsic apoptosis [204, 206]. The induction of apoptosis following Nr4as' translocation has been well established in various immune and cancer cells [202, 203, 205, 234]. In cancer cells, subcellular localization of Nur77 has been shown to be a key regulator of Nur77's actions. Indeed, Zhang et al described that in a wide range of cancer cells, nuclear localization of Nur77 promotes cell growth but that in response to pro-apoptotic stimuli, Nur77 is translocated to the mitochondria where it triggers apoptosis [207].

Because Nr4as are nuclear receptors and thereby expected to act as transcription factors, the study of their mechanism of action in β-cells as been primarily limited to the evaluation of their transcriptional effects. Briand et al report that palmitate induces the nuclear translocation of Nur77 and that overexpressing Nur77 reduces the expression of *MafA*, *PDX1*, *NeuroD1*, *Ins1* and *Ins2* in Min6 cells [180]. This could explain the downregulatory effect Nur77 has on GSIS in their study. Nor1 has also been suggested to affect GSIS through modulation of genes involved in insulin biosynthesis and exocytosis [178, 233]. Moreover, Nor1 has been shown to affect β-cell replication by regulating the expression of genes that control cell cycle (*Ube2c*, *p21*) [201]. However, evidence showing the presence of Nor1 in the nucleus and in some instances direct binding of Nor1 to the promoters of the genes it is supposed to be regulating is lacking. Finally, Nur77 has been shown to translocate to the nucleus in β-cells exposed to thapsigargin and palmitate and regulate the expression of ER stress and pro-apoptotic genes [192].

These studies helped characterizing the mechanisms of action of Nr4as in β-cells. However, given the importance of subcellular localization in the regulation of the effects of Nr4as, it is unfortunate that it has not been more extensively explored. In particular, before our work, there was no evaluation of the localization of Nor1 in response to pro-apoptotic signalling in β-cells. Our results demonstrate that cytokines induce the translocation of Nor1 to the mitochondria where it can induce cytochrome c release. This is especially relevant to our understanding of the role and mechanisms of actions of Nor1 in β-cells because one of the characteristics of Nor1 is that it has very limited effect on the transcriptome compared to Nur77 and Nurr1. Our own RNA

sequencing experiment did not allow us to identify any transcriptional targets of Nor1. This echoes the work of others, who studied the transcriptional effects of Nur77, Nurr1 and Nor1 in β-cells and systematically found that the effect of Nor1 was significantly lesser than that of Nur77 or Nurr1 [178, 180, 201]. Briand et al conducted a microarray on RNA from Min6 cells transfected with Nur77, Nurr1 or Nor1 *versus* control. They found 1277 genes regulated by Nur77 versus 313 for Nurr1 and 90 for Nor1 [180].

6.2.5 Mitochondrial effects of Nor1

The canonical pathway of intrinsic apoptosis involves the dissipation of the mitochondrial membrane potential [235]. However, our data suggests that Nor1 increases mitochondrial transmembrane potential. This constitutes a hint that Nor1 induces non-canonical pathways in β-cells. Several groups suggested that pro-apoptotic stimuli could induce mitochondrial hyperpolarization followed by an osmotic imbalance leading to outer mitochondrial membrane rupture, release of intermembrane space proteins and cell death [68, 236, 237]. Our results suggest that such a cascade could be induced by Nor1 in β-cells.

We also determined that Nor1 impairs mitochondrial function and energy production in β-cells. Glucose oxidation was reduced by ~ 50% and ATP production by ~ 30% in our INS1 cells transfected with Nor1 compared to controls. Appropriate ATP production by the mitochondria following glucose uptake and glycolysis is essential for proper coupling of glucose sensing and insulin secretion in β-cells [14]. It is therefore not surprising that Nor1 has a negative effect on glucose stimulated insulin secretion in our model. Reynolds et al recently published results that were opposite to ours. Indeed, they show that silencing Nor1 reduces oxygen consumption and ATP production in INS1 cells [179]. Logically, glucose stimulated insulin secretion is concomitantly reduced in their study. The main reason for this discrepancy could be the transfection method. Indeed, Reynolds et al used adenoviral transfection of their ShNor1 while I used nucleofection to transfect Nor1flag. The nucleofection damages membranes substantially more than adenoviral transfection. We indeed used 0.6 µM of digitonin to permeabilize the cells *versus* 8 µM for the Reynolds team. These differences in membrane integrity could explain the differences between the respirometry results obtained by Reynolds et al and ours. Moreover, they report that their cells stayed transfected during 3 weeks before their first experiments. In these conditions, one can hardly rule out indirect effects of silencing Nor1.

The question of the specific point where Nor1 exerts its effects on the glucose metabolism and ATP production pathway remains unanswered. Our metabolomics analysis shows that Nor1 induces an increase of the intermediates of the Krebs cycle and glycolysis as well as of lactate and the metabolites of the pentose pathway. Moreover, our results reveal an increase of NADH following Nor1 overexpression in INS1 cells. This suggests an impairment of the electron transfer chain causing accumulation of the electron donor NADH as well as the upstream metabolites. Interestingly, our NADH results resonate with those of Reynolds et al who found an increase of the NAD^+/NADH ratio when Nor1 is silenced in INS1 cells [179]. They attribute these results to a downregulation of isocitrate dehydrogenase.

Mitochondrial dynamics govern the maintenance of optimal mitochondrial function [238]. It has indeed been established that disrupting the mitochondrial network leads to reduced glucose oxidation and ATP production [111]. It is therefore not surprising, given the negative effect that Nor1 exerts on mitochondrial function that we also observed a predominance of small, fragmented mitochondria in β-cells transfected with Nor1. Interestingly, Nor1 has been suggested to modulate mitochondrial fusion and function in skeletal muscle as well [173]. Mitochondrial division is a prerequisite to a normal rate of cytochrome c release. Moreover, apoptosis always features fragmented mitochondria [113]. In addition, it has been suggested that Drp1, a key factor in the mitochondrial fission machinery, promotes cytochrome c release [105, 115, 239]. The mitochondrial fragmentation we show in β-cells overexpressing Nor1 therefore fits with a potential induction of apoptosis by Nor1. Whether the fragmentation participates to the induction of apoptosis or accompanies it remains to be determined.

Mitochondrial morphology also dictates the removal of these organelles by mitophagy [110]. Small, rounded, fragmented mitochondria are indeed targeted by autophagosomes. We thus quantified mitophagy and concluded that Nor1 induces an accumulation of double-membraned vacuoles targeting the mitochondria. Interestingly, the number of autophagosomes is increased in the β-cells of diabetic patients compared to non-diabetic subjects [130]. Furthermore, autophagy has been associated to cell death but whether it constitutes an independent pathway or accompanies apoptosis remains unknown [128]. Nur77 has been suggested to induce autophagic cell death following mitochondrial translocation [204, 240]. However, in β-cells transfected with Nor1, the nuclei showed signs of chromatin condensation which is a hallmark of apoptotic cell

death (data not shown). This could indicate that Nor1 triggers apoptosis in β-cells along and not through an induction of mitochondrial autophagy.

6.2.6 The pro-apoptotic effects of ST18

Very little is known about the biological functions of the transcription factor ST18. Our study is the first to evaluate the expression of ST18 in the pancreas and to characterize the role of ST18 in β-cells.

We have demonstrated here that the expression and activity of ST18 are enhanced by prolonged exposure to free fatty acids and cytokines. In addition, using ST18-specific siRNAs, we suggest that ST18 is a mediator of the pro-apoptotic action of free fatty acids and cytokines in β-cells.

The pro-apoptotic action of ST18 is compatible with its role as a tumor suppressor as well as a mediator of TNFα-induced apoptosis. Put together, these data support that ST18 plays a primordial role in the regulation of cell death. However, the biological role of ST18 is likely to be more complex. A recent publication reported that the expression of ST18 was increased during neuronal differentiation *in vitro* and *in vivo* [241]. The authors showed that overexpression of ST18 in P19 cells (a model of neuronal progenitor cells) caused differentiation. Thus, the biological role of ST18 may depend on the context.

The role of ST18 in neuronal differentiation recalls the role of MyT1 in the differentiation of endocrine cells. Wang et al showed that MyT1 is necessary for endocrine cell differentiation [212]. This raises questions about possible redundancy between the three NZF factors. However, substantial evidence suggests that NZF members may have different actions: (i) NZF members display different DNA binding affinities [209]; (ii) ST18 acts as a transcription repressor whereas MyT1 and NZF2 act as transcription activators [209]; (iii) ST18 lacks the acidic and serine/threonine-rich regulatory domains present in the 2 other NZFs [209]; (iv) and finally, the three NZF members are not equipotent to induce neuronal differentiation [241].

6.3 Future directions

This first exploration of the expression and roles of Nor1 and ST18 in β-cells allowed us to gather valuable information and reveal their potential importance in the molecular machinery that regulates β-cell life and death. Questions remain to be answered however in order to more finely characterize the effects of Nor1 and ST18 in vivo as well as their mechanism of action. A thorough understanding of these factor's regulation, effects and mechanisms of action is necessary to determine if they would constitute realistic targets for the treatment of diabetes.

Here are a few directions that could help us further our understanding of the role of Nor1 and ST18 in β-cells:

Nor1

Generating β-cell specific Nor1$^{-/-}$ mice by crossing MIP-CRE mice with floxed Nor1 mice would allow us to determine the contribution of the effect of Nor1 on β-cells to the phenotype of the whole body knockout animals. It would also tell us if the upregulation of β-cell mass we observe is solely due to an effect of Nor1 on the β-cells or if regulatory factors from other tissues were involved. Furthermore, studying β-cell mass, apoptosis, proliferation and glucose tolerance in β-cell specific Nor1$^{-/-}$ embryos as well as at birth and different stages of their development could help drawing a timeline of the effects of Nor1 on β-cells. It would be useful to determine when β-cell mass starts to be significantly different and what are the relative contributions of proliferation and apoptosis.

Another future direction could be evaluating the impact of high fat diet on the glucose tolerance of Nor1$^{-/-}$ animals *versus* wild types. This would help determining if impairing Nor1 could be an interesting protective strategy against high fat diet-induced glucose intolerance and diabetes.

In addition, identifying the signalling pathways involved in the increase of Nor1 expression by cytokines would lead to the identification of interesting therapeutic targets to modulate Nor1 expression and potentially affect β-cell life and death.

Also, determining the mechanisms that lead to the translocation of Nor1 to the mitochondria in response to cytokines would be an interesting project. Indeed, my data suggests that the translocation of Nor1 to the mitochondria is a key event in the induction of apoptosis and the

downregulation of GSIS by Nor1. Therefore, impairing that translocation would be an interesting pharmacological approach. I would start by studying the phosphorylation of Nor1 in β-cells exposed to cytokines and interfering with it with specific kinase inhibitors to see if it is necessary for translocation and apoptosis induction. Sumoylation has been shown to influence Nr4as sub-cellular localisation. It also has a protective effect against cytokines-induced β-cell apoptosis [242]. I would thus study Nor1 sumoylation in response to cytokines as well. Then, I would modulate it using transfection with the SUMO inhibitor SENP1 and study the effect on β-cell apoptosis.

Finally, further investigation of the effect of Nor1 SNP rs12686676 on β-cell function, proliferation and apoptosis could lead to interesting therapeutic approaches as well. This is especially true if we think of the possibility of transfecting β-cells with a mutated version of Nor1 that boosts GSIS prior to transplant. I would generate plasmids bearing Nor1 with the SNP and transfect them in INS1 cells to study GSIS, apoptosis and proliferation *versus* control. If the results are conclusive, mice bearing the polymorphism could be generated.

ST18

Generating β-cell specific $ST18^{-/-}$ animals to characterize their β-cell mass, proliferation and apoptosis as well as their metabolic profile would be the next step. In a second stage, their response to high fat diet could be studied.

Another future direction could be the identification of the transcriptional targets of ST18 in β-cells. In order to do so, I would combine two genome wide approaches such as RNA-sequencing and chromatin immunoprecipitation-on-chip. This would identify the genes that are regulated by ST18 and its DNA binding sites across the whole genome. This could be a first step towards a better understanding of its mechanisms of action.

6.4 Relevance of the work and conclusions

Evidence presented in the current thesis identifies the orphan nuclear receptor Nor1 and the NZF transcription factor ST18 as potent regulators of β-cell life and death as well as β-cell secretory function. *In vivo*, genetic deletion of Nor1 results in increased β-cell mass, elevated β-cell proliferation and better glycaemic control. *In vitro*, cytokines upregulate Nor1 expression in a dose-dependent manner and induce its translocation to the mitochondria. Therefore, not surprisingly, evidence described here suggests that Nor1 induces apoptosis and is a necessary mediator of cytokines-induced apoptosis in β-cells. Importantly, *Nor1* expression is increased in the islets of type 2 diabetic patients thus unveiling a possible implication of Nor1 in the development of the disease. Furthermore, possible mechanisms of action of Nor1 echoing its cytokine-induced translocation to the mitochondria are revealed here. Indeed, impaired mitochondrial function, increased lactate production, mitochondrial fragmentation and finally an increase of mitophagy are reported in β-cells overexpressing Nor1. In addition to these results concerning Nor1, other work presented here established a first characterization of the expression of ST18 in β-cells. The diabetogenic factors palmitate and cytokines increase ST18 expression and activity. Similarly to what was showed for Nor1, a pro-apoptotic and anti-proliferative role was uncovered for ST18. Finally, the data collected here demonstrates that both factors also hinder glucose stimulated insulin secretion which in the case of Nor1 is consistent with its negative effect on ATP production.

In the context of overweight and obesity, increased circulating fatty acids such as palmitate and plasma cytokines are some of the factors incriminated in the elevation of β-cell apoptosis that leads to progressive decrease of β-cell mass and the eventual development of type 2 diabetes [28, 31]. This is particularly concerning given the dramatic rise of overweight and obesity worldwide [243]. The obvious solution to defeat obesity and its complications such as diabetes would be a joint effort at the individual and institutional level to reduce palmitate-rich food consumption and portion sizes and promote physical activity. However, it is a long-term endeavor bound to encounter personal, cultural and economical barriers. Finding mediators of diabetogenic factors-induced β-cell apoptosis and understanding their effects and mechanisms of action in the ultimate goal of discovering if they could constitute good pharmaceutical targets would provide a solution against obesity-associated diabetes that leaves room for human error. Our work identified Nor1 as a new diabetogenic gene that is increased in the islets of type 2 diabetic

patients. Moreover, we were the first to show that Nor1 induces β-cell apoptosis and downregulates β-cell mass. In addition, we show that Nor1 impairs β-cell function. A reduction of β-cell mass and impaired β-cell function are the 2 elements that constitute β-cell failure, the turning point event in the pathophysiology of type 2 diabetes. We therefore revealed a new promising therapeutic target for the prevention of the development of type 2 diabetes. Our study of the mechanism of action of Nor1 provides useful hints that interfering with Nor1's subcellular localization might be an interesting pharmacological approach to prevent cytokines-induced β-cell apoptosis.

References

1. Patutina, O., et al., *Inhibition of metastasis development by daily administration of ultralow doses of RNase A and DNase I.* Biochimie, 2011. **93**(4): p. 689-96.
2. Bourne, R.R., et al., *Causes of vision loss worldwide, 1990-2010: a systematic analysis.* Lancet Glob Health, 2013. **1**(6): p. e339-49.
3. Saran, R., et al., *US Renal Data System 2015 Annual Data Report: Epidemiology of Kidney Disease in the United States.* Am J Kidney Dis, 2016. **67**(3 Suppl 1): p. Svii, S1-305.
4. Sarwar, N., et al., *Diabetes mellitus, fasting blood glucose concentration, and risk of vascular disease: a collaborative meta-analysis of 102 prospective studies.* Lancet, 2010. **375**(9733): p. 2215-22.
5. Moxey, P.W., et al., *Lower extremity amputations--a review of global variability in incidence.* Diabet Med, 2011. **28**(10): p. 1144-53.
6. ADA, *2. Classification and Diagnosis of Diabetes.* Diabetes Care, 2016. **39**(Supplement 1): p. S13-S22.
7. Cheng, A.Y., *Canadian Diabetes Association 2013 clinical practice guidelines for the prevention and management of diabetes in Canada. Introduction.* Can J Diabetes, 2013. **37 Suppl 1**: p. S1-3.
8. Kim, A., et al., *Islet architecture: A comparative study.* Islets, 2009. **1**(2): p. 129-36.
9. Seino, *pancreatic beta cell in health and disease*2008: Bell.
10. Scharfmann, R., et al., *Beta cells within single human islets originate from multiple progenitors.* PLoS One, 2008. **3**(10): p. e3559.
11. Pocock, G., *Human physiology: the basis of medicine*. 3rd ed, ed. O.U. press2006, Oxford.
12. Wu, X. and W.T. Garvey, *Insulin Action*, in *Textbook of Diabetes*2010, Wiley-Blackwell. p. 104-125.
13. Costanzo, L., *Endocrine Physiology*, in *Physiology*, Saunders, Editor 2014. p. 383-446.
14. Henquin, J.C., *Triggering and amplifying pathways of regulation of insulin secretion by glucose.* Diabetes, 2000. **49**(11): p. 1751-60.
15. Henquin, J.C., D. Dufrane, and M. Nenquin, *Nutrient control of insulin secretion in isolated normal human islets.* Diabetes, 2006. **55**(12): p. 3470-7.
16. Song, S.H., et al., *Direct measurement of pulsatile insulin secretion from the portal vein in human subjects.* J Clin Endocrinol Metab, 2000. **85**(12): p. 4491-9.
17. Goodner, C.J., et al., *Insulin, glucagon, and glucose exhibit synchronous, sustained oscillations in fasting monkeys.* Science, 1977. **195**(4274): p. 177-9.
18. Stagner, J.I., E. Samols, and G.C. Weir, *Sustained oscillations of insulin, glucagon, and somatostatin from the isolated canine pancreas during exposure to a constant glucose concentration.* J Clin Invest, 1980. **65**(4): p. 939-42.
19. Alberti, K.G.M.M., *The Classification and Diagnosis of Diabetes Mellitus*, in *Textbook of Diabetes*2010, Wiley-Blackwell. p. 24-30.
20. Gough, S. and P. Narendran, *Insulin and Insulin Treatment*, in *Textbook of Diabetes*2010, Wiley-Blackwell. p. 425-439.
21. Farney, A.C., D.E. Sutherland, and E.C. Opara, *Evolution of Islet Transplantation for the Last 30 Years.* Pancreas, 2016. **45**(1): p. 8-20.
22. Ma, R.C.W. and P.C.Y. Tong, *Epidemiology of Type 2 Diabetes*, in *Textbook of Diabetes*2010, Wiley-Blackwell. p. 45-68.
23. Stene, L.C., et al., *Epidemiology of Type 1 Diabetes*, in *Textbook of Diabetes*2010, Wiley-Blackwell. p. 31-44.

24. Alsahli, M. and J.E. Gerich, *Abnormalities of Insulin Secretion and β-Cell Defects in Type 2 Diabetes*, in *Textbook of Diabetes*2010, Wiley-Blackwell. p. 160-173.
25. Yki-Järvinen, H., *Insulin Resistance in Type 2 Diabetes*, in *Textbook of Diabetes*2010, Wiley-Blackwell. p. 174-190.
26. Kasuga, M., *Insulin resistance and pancreatic beta cell failure.* J Clin Invest, 2006. **116**(7): p. 1756-60.
27. Prentki, M. and C.J. Nolan, *Islet beta cell failure in type 2 diabetes.* J Clin Invest, 2006. **116**(7): p. 1802-12.
28. Rhodes, C.J., *Type 2 diabetes-a matter of beta-cell life and death?* Science, 2005. **307**(5708): p. 380-4.
29. Lang, D.A., et al., *Brief, irregular oscillations of basal plasma insulin and glucose concentrations in diabetic man.* Diabetes, 1981. **30**(5): p. 435-9.
30. Brunzell, J.D., et al., *Relationships between fasting plasma glucose levels and insulin secretion during intravenous glucose tolerance tests.* J Clin Endocrinol Metab, 1976. **42**(2): p. 222-9.
31. Weir, G.C. and S. Bonner-Weir, *Islet beta cell mass in diabetes and how it relates to function, birth, and death.* Ann N Y Acad Sci, 2013. **1281**: p. 92-105.
32. Rahier, J., et al., *Pancreatic beta-cell mass in European subjects with type 2 diabetes.* Diabetes Obes Metab, 2008. **10 Suppl 4**: p. 32-42.
33. Yoon, K.H., et al., *Selective beta-cell loss and alpha-cell expansion in patients with type 2 diabetes mellitus in Korea.* J Clin Endocrinol Metab, 2003. **88**(5): p. 2300-8.
34. Butler, A.E., et al., *Beta-cell deficit and increased beta-cell apoptosis in humans with type 2 diabetes.* Diabetes, 2003. **52**(1): p. 102-10.
35. Costes, S., et al., *beta-Cell failure in type 2 diabetes: a case of asking too much of too few?* Diabetes, 2013. **62**(2): p. 327-35.
36. Porte, D., Jr., *Banting lecture 1990. Beta-cells in type II diabetes mellitus.* Diabetes, 1991. **40**(2): p. 166-80.
37. Porte, D., Jr. and S.E. Kahn, *beta-cell dysfunction and failure in type 2 diabetes: potential mechanisms.* Diabetes, 2001. **50 Suppl 1**: p. S160-3.
38. Ferrannini, E. and G. Mingrone, *Impact of different bariatric surgical procedures on insulin action and beta-cell function in type 2 diabetes.* Diabetes Care, 2009. **32**(3): p. 514-20.
39. Guidone, C., et al., *Mechanisms of recovery from type 2 diabetes after malabsorptive bariatric surgery.* Diabetes, 2006. **55**(7): p. 2025-31.
40. Malandrucco, I., et al., *Very-low-calorie diet: a quick therapeutic tool to improve beta cell function in morbidly obese patients with type 2 diabetes.* Am J Clin Nutr, 2012. **95**(3): p. 609-13.
41. Lim, E.L., et al., *Reversal of type 2 diabetes: normalisation of beta cell function in association with decreased pancreas and liver triacylglycerol.* Diabetologia, 2011. **54**(10): p. 2506-14.
42. Cnop, M., et al., *The long lifespan and low turnover of human islet beta cells estimated by mathematical modelling of lipofuscin accumulation.* Diabetologia, 2010. **53**(2): p. 321-30.
43. Hanley, S.C., et al., *{beta}-Cell mass dynamics and islet cell plasticity in human type 2 diabetes.* Endocrinology, 2010. **151**(4): p. 1462-72.
44. Ritzel, R.A., et al., *Relationship between beta-cell mass and fasting blood glucose concentration in humans.* Diabetes Care, 2006. **29**(3): p. 717-8.
45. Meier, J.J., et al., *Functional assessment of pancreatic beta-cell area in humans.* Diabetes, 2009. **58**(7): p. 1595-603.
46. Matveyenko, A.V. and P.C. Butler, *Beta-cell deficit due to increased apoptosis in the human islet amyloid polypeptide transgenic (HIP) rat recapitulates the metabolic defects present in type 2 diabetes.* Diabetes, 2006. **55**(7): p. 2106-14.

47. Meier, J.J., et al., *Pancreatic diabetes manifests when beta cell area declines by approximately 65% in humans.* Diabetologia, 2012. **55**(5): p. 1346-54.
48. Meier, J.J. and R.C. Bonadonna, *Role of reduced beta-cell mass versus impaired beta-cell function in the pathogenesis of type 2 diabetes.* Diabetes Care, 2013. **36 Suppl 2**: p. S113-9.
49. Menge, B.A., et al., *Partial pancreatectomy in adult humans does not provoke beta-cell regeneration.* Diabetes, 2008. **57**(1): p. 142-9.
50. Tarabra, E., S. Pelengaris, and M. Khan, *A simple matter of life and death-the trials of postnatal Beta-cell mass regulation.* Int J Endocrinol, 2012. **2012**: p. 516718.
51. Bonner-Weir, S., et al., *Beta-cell growth and regeneration: replication is only part of the story.* Diabetes, 2010. **59**(10): p. 2340-8.
52. Jonas, J.C., et al., *Chronic hyperglycemia triggers loss of pancreatic beta cell differentiation in an animal model of diabetes.* J Biol Chem, 1999. **274**(20): p. 14112-21.
53. Talchai, C., et al., *Pancreatic beta cell dedifferentiation as a mechanism of diabetic beta cell failure.* Cell, 2012. **150**(6): p. 1223-34.
54. Bonner-Weir, S., et al., *Compensatory growth of pancreatic beta-cells in adult rats after short-term glucose infusion.* Diabetes, 1989. **38**(1): p. 49-53.
55. Swenne, I., *The role of glucose in the in vitro regulation of cell cycle kinetics and proliferation of fetal pancreatic B-cells.* Diabetes, 1982. **31**(9): p. 754-60.
56. Yi, P., J.S. Park, and D.A. Melton, *Betatrophin: a hormone that controls pancreatic beta cell proliferation.* Cell, 2013. **153**(4): p. 747-58.
57. Buteau, J., *GLP-1 receptor signaling: effects on pancreatic beta-cell proliferation and survival.* Diabetes Metab, 2008. **34 Suppl 2**: p. S73-7.
58. Matveyenko, A.V. and P.C. Butler, *Relationship between beta-cell mass and diabetes onset.* Diabetes Obes Metab, 2008. **10 Suppl 4**: p. 23-31.
59. Pollard, T., *Programmed Cell Death*, in *Cell Biology*, Elsevier, Editor 2008. p. 833-850.
60. Hotchkiss, R.S., et al., *Cell death.* N Engl J Med, 2009. **361**(16): p. 1570-83.
61. Abnova. *Apoptosis*. 2017 January 16 2017]; Available from: http://www.abnova.com/support/resources.asp?switchfunctionid=%7BCBB86AB6-2EA6-422F-BBBD-1CB8B9DCE6FA%7D.
62. Elmore, S., *Apoptosis: a review of programmed cell death.* Toxicol Pathol, 2007. **35**(4): p. 495-516.
63. Locksley, R.M., N. Killeen, and M.J. Lenardo, *The TNF and TNF receptor superfamilies: integrating mammalian biology.* Cell, 2001. **104**(4): p. 487-501.
64. Kischkel, F.C., et al., *Cytotoxicity-dependent APO-1 (Fas/CD95)-associated proteins form a death-inducing signaling complex (DISC) with the receptor.* EMBO J, 1995. **14**(22): p. 5579-88.
65. Kroemer, G., B. Dallaporta, and M. Resche-Rigon, *The mitochondrial death/life regulator in apoptosis and necrosis.* Annu Rev Physiol, 1998. **60**: p. 619-42.
66. Bonora, M., et al., *Molecular mechanisms of cell death: central implication of ATP synthase in mitochondrial permeability transition.* Oncogene, 2015. **34**(12): p. 1608.
67. Kroemer, G., L. Galluzzi, and C. Brenner, *Mitochondrial membrane permeabilization in cell death.* Physiol Rev, 2007. **87**(1): p. 99-163.
68. Vander Heiden, M.G., et al., *Outer mitochondrial membrane permeability can regulate coupled respiration and cell survival.* Proc Natl Acad Sci U S A, 2000. **97**(9): p. 4666-71.
69. Eguchi, K. and I. Manabe, *Macrophages and islet inflammation in type 2 diabetes.* Diabetes Obes Metab, 2013. **15 Suppl 3**: p. 152-8.
70. Richardson, S.J., et al., *Islet-associated macrophages in type 2 diabetes.* Diabetologia, 2009. **52**(8): p. 1686-8.

71. Marselli, L., et al., *beta-Cell inflammation in human type 2 diabetes and the role of autophagy.* Diabetes Obes Metab, 2013. **15 Suppl 3**: p. 130-6.
72. Pickup, J.C., et al., *NIDDM as a disease of the innate immune system: association of acute-phase reactants and interleukin-6 with metabolic syndrome X.* Diabetologia, 1997. **40**(11): p. 1286-92.
73. Pickup, J.C. and M.A. Crook, *Is type II diabetes mellitus a disease of the innate immune system?* Diabetologia, 1998. **41**(10): p. 1241-8.
74. Spranger, J., et al., *Inflammatory cytokines and the risk to develop type 2 diabetes: results of the prospective population-based European Prospective Investigation into Cancer and Nutrition (EPIC)-Potsdam Study.* Diabetes, 2003. **52**(3): p. 812-7.
75. Pradhan, A.D., et al., *C-reactive protein, interleukin 6, and risk of developing type 2 diabetes mellitus.* JAMA, 2001. **286**(3): p. 327-34.
76. Donath, M.Y. and S.E. Shoelson, *Type 2 diabetes as an inflammatory disease.* Nat Rev Immunol, 2011. **11**(2): p. 98-107.
77. Marchetti, P., *Islet inflammation in type 2 diabetes.* Diabetologia, 2016. **59**(4): p. 668-72.
78. Eizirik, D.L. and T. Mandrup-Poulsen, *A choice of death--the signal-transduction of immune-mediated beta-cell apoptosis.* Diabetologia, 2001. **44**(12): p. 2115-33.
79. Donath, M.Y., et al., *Cytokines and beta-cell biology: from concept to clinical translation.* Endocr Rev, 2008. **29**(3): p. 334-50.
80. Berchtold, L.A., et al., *Cytokines and Pancreatic beta-Cell Apoptosis.* Adv Clin Chem, 2016. **75**: p. 99-158.
81. Maedler, K., et al., *Glucose-induced beta cell production of IL-1beta contributes to glucotoxicity in human pancreatic islets.* J Clin Invest, 2002. **110**(6): p. 851-60.
82. Mikkelsen, H.B. and L. Thuneberg, *Op/op mice defective in production of functional colony-stimulating factor-1 lack macrophages in muscularis externa of the small intestine.* Cell Tissue Res, 1999. **295**(3): p. 485-93.
83. Ehses, J.A., et al., *Increased number of islet-associated macrophages in type 2 diabetes.* Diabetes, 2007. **56**(9): p. 2356-70.
84. Eguchi, K. and R. Nagai, *Islet inflammation in type 2 diabetes and physiology.* J Clin Invest, 2017. **127**(1): p. 14-23.
85. Donath, M.Y., *Targeting inflammation in the treatment of type 2 diabetes: time to start.* Nat Rev Drug Discov, 2014. **13**(6): p. 465-76.
86. El Muayed, M., et al., *Acute cytokine-mediated downregulation of the zinc transporter ZnT8 alters pancreatic beta-cell function.* J Endocrinol, 2010. **206**(2): p. 159-69.
87. Taylor-Fishwick, D.A., et al., *Production and function of IL-12 in islets and beta cells.* Diabetologia, 2013. **56**(1): p. 126-35.
88. Weaver, J.R., et al., *Integration of pro-inflammatory cytokines, 12-lipoxygenase and NOX-1 in pancreatic islet beta cell dysfunction.* Mol Cell Endocrinol, 2012. **358**(1): p. 88-95.
89. Donath, M.Y., et al., *Inflammatory mediators and islet beta-cell failure: a link between type 1 and type 2 diabetes.* J Mol Med (Berl), 2003. **81**(8): p. 455-70.
90. Lundh, M., et al., *Small-molecule inhibition of inflammatory beta-cell death.* Diabetes Obes Metab, 2013. **15 Suppl 3**: p. 176-84.
91. Cetkovic-Cvrlje, M. and D.L. Eizirik, *TNF-alpha and IFN-gamma potentiate the deleterious effects of IL-1 beta on mouse pancreatic islets mainly via generation of nitric oxide.* Cytokine, 1994. **6**(4): p. 399-406.
92. Imai, Y., et al., *Interaction between cytokines and inflammatory cells in islet dysfunction, insulin resistance and vascular disease.* Diabetes Obes Metab, 2013. **15 Suppl 3**: p. 117-29.
93. Chang, Y.C. and L.M. Chuang, *The role of oxidative stress in the pathogenesis of type 2 diabetes: from molecular mechanism to clinical implication.* Am J Transl Res, 2010. **2**(3): p. 316-31.

94. Parish, R. and K.F. Petersen, *Mitochondrial dysfunction and type 2 diabetes.* Curr Diab Rep, 2005. **5**(3): p. 177-83.
95. Brownlee, M., *A radical explanation for glucose-induced beta cell dysfunction.* J Clin Invest, 2003. **112**(12): p. 1788-90.
96. Tiedge, M., et al., *Relation between antioxidant enzyme gene expression and antioxidative defense status of insulin-producing cells.* Diabetes, 1997. **46**(11): p. 1733-42.
97. Tanaka, Y., et al., *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, 2002. **99**(19): p. 12363-8.
98. Chandra, J., A. Samali, and S. Orrenius, *Triggering and modulation of apoptosis by oxidative stress.* Free Radic Biol Med, 2000. **29**(3-4): p. 323-33.
99. Hou, N., et al., *Reactive oxygen species-mediated pancreatic beta-cell death is regulated by interactions between stress-activated protein kinases, p38 and c-Jun N-terminal kinase, and mitogen-activated protein kinase phosphatases.* Endocrinology, 2008. **149**(4): p. 1654-65.
100. Sakuraba, H., et al., *Reduced beta-cell mass and expression of oxidative stress-related DNA damage in the islet of Japanese Type II diabetic patients.* Diabetologia, 2002. **45**(1): p. 85-96.
101. Pi, J., et al., *Reactive oxygen species as a signal in glucose-stimulated insulin secretion.* Diabetes, 2007. **56**(7): p. 1783-91.
102. Valko, M., et al., *Free radicals and antioxidants in normal physiological functions and human disease.* Int J Biochem Cell Biol, 2007. **39**(1): p. 44-84.
103. Leibowitz, G., et al., *Glucose regulation of beta-cell stress in type 2 diabetes.* Diabetes Obes Metab, 2010. **12 Suppl 2**: p. 66-75.
104. Orlova, D.D., et al., *[The role of mitochondrial dynamics in cell death].* Tsitologiia, 2015. **57**(3): p. 184-91.
105. Detmer, S.A. and D.C. Chan, *Functions and dysfunctions of mitochondrial dynamics.* Nat Rev Mol Cell Biol, 2007. **8**(11): p. 870-9.
106. Cipolat, S., et al., *OPA1 requires mitofusin 1 to promote mitochondrial fusion.* Proc Natl Acad Sci U S A, 2004. **101**(45): p. 15927-32.
107. Chen, H., A. Chomyn, and D.C. Chan, *Disruption of fusion results in mitochondrial heterogeneity and dysfunction.* J Biol Chem, 2005. **280**(28): p. 26185-92.
108. Smirnova, E., et al., *Dynamin-related protein Drp1 is required for mitochondrial division in mammalian cells.* Mol Biol Cell, 2001. **12**(8): p. 2245-56.
109. Rovira-Llopis, S., et al., *Mitochondrial dynamics in type 2 diabetes: Pathophysiological implications.* Redox Biol, 2017. **11**: p. 637-645.
110. Twig, G., et al., *Fission and selective fusion govern mitochondrial segregation and elimination by autophagy.* EMBO J, 2008. **27**(2): p. 433-46.
111. Bach, D., et al., *Mitofusin-2 determines mitochondrial network architecture and mitochondrial metabolism. A novel regulatory mechanism altered in obesity.* J Biol Chem, 2003. **278**(19): p. 17190-7.
112. van der Bliek, A.M., Q. Shen, and S. Kawajiri, *Mechanisms of mitochondrial fission and fusion.* Cold Spring Harb Perspect Biol, 2013. **5**(6).
113. Suen, D.F., K.L. Norris, and R.J. Youle, *Mitochondrial dynamics and apoptosis.* Genes Dev, 2008. **22**(12): p. 1577-90.
114. Karbowski, M., et al., *Spatial and temporal association of Bax with mitochondrial fission sites, Drp1, and Mfn2 during apoptosis.* J Cell Biol, 2002. **159**(6): p. 931-8.
115. Frank, S., et al., *The role of dynamin-related protein 1, a mediator of mitochondrial fission, in apoptosis.* Dev Cell, 2001. **1**(4): p. 515-25.
116. Molina, A.J.A., et al., *Mitochondrial Networking Protects β-Cells From Nutrient-Induced Apoptosis.* Diabetes, 2009. **58**(10): p. 2303-2315.

117. Yu, T., J.L. Robotham, and Y. Yoon, *Increased production of reactive oxygen species in hyperglycemic conditions requires dynamic change of mitochondrial morphology.* Proceedings of the National Academy of Sciences of the United States of America, 2006. **103**(8): p. 2653-2658.
118. Lee, Y.J., et al., *Roles of the mammalian mitochondrial fission and fusion mediators Fis1, Drp1, and Opa1 in apoptosis.* Mol Biol Cell, 2004. **15**(11): p. 5001-11.
119. Brooks, C., et al., *Bak regulates mitochondrial morphology and pathology during apoptosis by interacting with mitofusins.* Proc Natl Acad Sci U S A, 2007. **104**(28): p. 11649-54.
120. Parone, P.A., et al., *Inhibiting the mitochondrial fission machinery does not prevent Bax/Bak-dependent apoptosis.* Mol Cell Biol, 2006. **26**(20): p. 7397-408.
121. Estaquier, J. and D. Arnoult, *Inhibiting Drp1-mediated mitochondrial fission selectively prevents the release of cytochrome c during apoptosis.* Cell Death Differ, 2007. **14**(6): p. 1086-94.
122. Liesa, M. and Orian S. Shirihai, *Mitochondrial Dynamics in the Regulation of Nutrient Utilization and Energy Expenditure.* Cell Metabolism. **17**(4): p. 491-506.
123. Kelley, D.E., et al., *Dysfunction of Mitochondria in Human Skeletal Muscle in Type 2 Diabetes.* Diabetes, 2002. **51**(10): p. 2944-2950.
124. Shirihai, O.S., M. Song, and G.W. Dorn, *How Mitochondrial Dynamism Orchestrates Mitophagy.* Circulation Research, 2015. **116**(11): p. 1835-1849.
125. Ashrafi, G. and T.L. Schwarz, *The pathways of mitophagy for quality control and clearance of mitochondria.* Cell Death Differ, 2013. **20**(1): p. 31-42.
126. Mizushima, N. and M. Komatsu, *Autophagy: Renovation of Cells and Tissues.* Cell. **147**(4): p. 728-741.
127. Choe, S.C., A. Hamacher-Brady, and N.R. Brady, *Autophagy capacity and sub-mitochondrial heterogeneity shape Bnip3-induced mitophagy regulation of apoptosis.* Cell Commun Signal, 2015. **13**: p. 37.
128. Galluzzi, L., et al., *Cell death modalities: classification and pathophysiological implications.* Cell Death Differ, 2007. **14**(7): p. 1237-43.
129. Shimizu, S., et al., *Role of Bcl-2 family proteins in a non-apoptotic programmed cell death dependent on autophagy genes.* Nat Cell Biol, 2004. **6**(12): p. 1221-8.
130. Masini, M., et al., *Autophagy in human type 2 diabetes pancreatic beta cells.* Diabetologia, 2009. **52**(6): p. 1083-6.
131. Lee, Y., et al., *Increased lipogenic capacity of the islets of obese rats: a role in the pathogenesis of NIDDM.* Diabetes, 1997. **46**(3): p. 408-13.
132. Jacqueminet, S., et al., *Inhibition of insulin gene expression by long-term exposure of pancreatic beta cells to palmitate is dependent on the presence of a stimulatory glucose concentration.* Metabolism, 2000. **49**(4): p. 532-6.
133. Maedler, K., et al., *Distinct effects of saturated and monounsaturated fatty acids on beta-cell turnover and function.* Diabetes, 2001. **50**(1): p. 69-76.
134. Cnop, M., et al., *Inverse relationship between cytotoxicity of free fatty acids in pancreatic islet cells and cellular triglyceride accumulation.* Diabetes, 2001. **50**(8): p. 1771-7.
135. Shimabukuro, M., et al., *Lipoapoptosis in beta-cells of obese prediabetic fa/fa rats. Role of serine palmitoyltransferase overexpression.* J Biol Chem, 1998. **273**(49): p. 32487-90.
136. Poitout, V. and R.P. Robertson, *Minireview: Secondary beta-cell failure in type 2 diabetes--a convergence of glucotoxicity and lipotoxicity.* Endocrinology, 2002. **143**(2): p. 339-42.
137. Prentki, M., et al., *Malonyl-CoA signaling, lipid partitioning, and glucolipotoxicity: role in beta-cell adaptation and failure in the etiology of diabetes.* Diabetes, 2002. **51 Suppl 3**: p. S405-13.
138. El-Assaad, W., et al., *Saturated fatty acids synergize with elevated glucose to cause pancreatic beta-cell death.* Endocrinology, 2003. **144**(9): p. 4154-63.

139. Ruderman, N. and M. Prentki, *AMP kinase and malonyl-CoA: targets for therapy of the metabolic syndrome.* Nat Rev Drug Discov, 2004. **3**(4): p. 340-51.
140. Lupi, R., et al., *Prolonged exposure to free fatty acids has cytostatic and pro-apoptotic effects on human pancreatic islets: evidence that beta-cell death is caspase mediated, partially dependent on ceramide pathway, and Bcl-2 regulated.* Diabetes, 2002. **51**(5): p. 1437-42.
141. Shao, S., et al., *Transcription factors involved in glucose-stimulated insulin secretion of pancreatic beta cells.* Biochem Biophys Res Commun, 2009. **384**(4): p. 401-4.
142. Fujimoto, K. and K.S. Polonsky, *Pdx1 and other factors that regulate pancreatic beta-cell survival.* Diabetes Obes Metab, 2009. **11 Suppl 4**: p. 30-7.
143. Jonsson, J., et al., *Insulin-promoter-factor 1 is required for pancreas development in mice.* Nature, 1994. **371**(6498): p. 606-9.
144. Stoffers, D.A., et al., *Pancreatic agenesis attributable to a single nucleotide deletion in the human IPF1 gene coding sequence.* Nat Genet, 1997. **15**(1): p. 106-10.
145. Bernardo, A.S., C.W. Hay, and K. Docherty, *Pancreatic transcription factors and their role in the birth, life and survival of the pancreatic beta cell.* Mol Cell Endocrinol, 2008. **294**(1-2): p. 1-9.
146. Kitamura, T., et al., *The forkhead transcription factor Foxo1 links insulin signaling to Pdx1 regulation of pancreatic beta cell growth.* J Clin Invest, 2002. **110**(12): p. 1839-47.
147. Kulkarni, R.N., et al., *PDX-1 haploinsufficiency limits the compensatory islet hyperplasia that occurs in response to insulin resistance.* J Clin Invest, 2004. **114**(6): p. 828-36.
148. Okamoto, H., et al., *Role of the forkhead protein FoxO1 in beta cell compensation to insulin resistance.* J Clin Invest, 2006. **116**(3): p. 775-82.
149. Robertson, R.P., *Chronic oxidative stress as a central mechanism for glucose toxicity in pancreatic islet beta cells in diabetes.* J Biol Chem, 2004. **279**(41): p. 42351-4.
150. Close, A.F., C. Rouillard, and J. Buteau, *NR4A orphan nuclear receptors in glucose homeostasis: a minireview.* Diabetes Metab, 2013. **39**(6): p. 478-84.
151. Committee, N.R.N., *A unified nomenclature system for the nuclear receptor superfamily.* Cell, 1999. **97**(2): p. 161-3.
152. Saucedo-Cardenas, O., et al., *Cloning and structural organization of the gene encoding the murine nuclear receptor transcription factor, NURR1.* Gene, 1997. **187**(1): p. 135-9.
153. Zhao, Y. and D. Bruemmer, *NR4A orphan nuclear receptors: transcriptional regulators of gene expression in metabolism and vascular biology.* Arterioscler Thromb Vasc Biol, 2010. **30**(8): p. 1535-41.
154. Castro, D.S., et al., *Activity of the Nurr1 carboxyl-terminal domain depends on cell type and integrity of the activation function 2.* J Biol Chem, 1999. **274**(52): p. 37483-90.
155. Wang, Z., et al., *Structure and function of Nurr1 identifies a class of ligand-independent nuclear receptors.* Nature, 2003. **423**(6939): p. 555-60.
156. Volakakis, N., et al., *Characterization of the Nurr1 ligand-binding domain co-activator interaction surface.* J Mol Endocrinol, 2006. **37**(2): p. 317-26.
157. Han, Y.H., et al., *Regulation of Nur77 nuclear export by c-Jun N-terminal kinase and Akt.* Oncogene, 2006. **25**(21): p. 2974-86.
158. Wingate, A.D., et al., *Nur77 is phosphorylated in cells by RSK in response to mitogenic stimulation.* Biochem J, 2006. **393**(Pt 3): p. 715-24.
159. Kovalovsky, D., et al., *Activation and induction of NUR77/NURR1 in corticotrophs by CRH/cAMP: involvement of calcium, protein kinase A, and MAPK pathways.* Mol Endocrinol, 2002. **16**(7): p. 1638-51.
160. Galleguillos, D., et al., *PIASgamma represses the transcriptional activation induced by the nuclear receptor Nurr1.* J Biol Chem, 2004. **279**(3): p. 2005-11.

161. Arredondo, C., et al., *PIASgamma enhanced SUMO-2 modification of Nurr1 activation-function-1 domain limits Nurr1 transcriptional synergy.* PLoS One, 2013. **8**(1): p. e55035.
162. Wilson, T.E., et al., *Identification of the DNA binding site for NGFI-B by genetic selection in yeast.* Science, 1991. **252**(5010): p. 1296-300.
163. Wilson, T.E., T.J. Fahrner, and J. Milbrandt, *The orphan receptors NGFI-B and steroidogenic factor 1 establish monomer binding as a third paradigm of nuclear receptor-DNA interaction.* Mol Cell Biol, 1993. **13**(9): p. 5794-804.
164. Maira, M., et al., *Heterodimerization between members of the Nur subfamily of orphan nuclear receptors as a novel mechanism for gene activation.* Mol Cell Biol, 1999. **19**(11): p. 7549-57.
165. Forman, B.M., et al., *Unique response pathways are established by allosteric interactions among nuclear hormone receptors.* Cell, 1995. **81**(4): p. 541-50.
166. Perlmann, T. and L. Jansson, *A novel pathway for vitamin A signaling mediated by RXR heterodimerization with NGFI-B and NURR1.* Genes Dev, 1995. **9**(7): p. 769-82.
167. Pei, L., et al., *NR4A orphan nuclear receptors are transcriptional regulators of hepatic glucose metabolism.* Nat Med, 2006. **12**(9): p. 1048-55.
168. Fu, Y., et al., *NR4A orphan nuclear receptors modulate insulin action and the glucose transport system: potential role in insulin resistance.* J Biol Chem, 2007. **282**(43): p. 31525-33.
169. Kanzleiter, T., et al., *Regulation of the nuclear hormone receptor nur77 in muscle: influence of exercise-activated pathways in vitro and obesity in vivo.* Biochim Biophys Acta, 2009. **1792**(8): p. 777-82.
170. Chao, L.C., et al., *Skeletal muscle Nur77 expression enhances oxidative metabolism and substrate utilization.* J Lipid Res, 2012.
171. Chao, L.C., et al., *Nur77 coordinately regulates expression of genes linked to glucose metabolism in skeletal muscle.* Mol Endocrinol, 2007. **21**(9): p. 2152-63.
172. Chao, L.C., et al., *Insulin resistance and altered systemic glucose metabolism in mice lacking Nur77.* Diabetes, 2009. **58**(12): p. 2788-96.
173. Pearen, M.A., et al., *The nuclear receptor, Nor-1, markedly increases type II oxidative muscle fibers and resistance to fatigue.* Mol Endocrinol, 2012. **26**(3): p. 372-84.
174. Zhu, X., et al., *Prostaglandin A2 Enhances Cellular Insulin Sensitivity via a Mechanism that Involves the Orphan Nuclear Receptor NR4A3.* Horm Metab Res, 2012. **45**(3): p. 213-20.
175. Wang, S.C., et al., *Nr4a1 siRNA expression attenuates alpha-MSH regulated gene expression in 3T3-L1 adipocytes.* Mol Endocrinol, 2011. **25**(2): p. 291-306.
176. Perez-Sieira, S., et al., *Female nur77-deficient mice show increased susceptibility to diet-induced obesity.* PLoS One, 2013. **8**(1): p. e53836.
177. Pols, T.W., et al., *Nur77 modulates hepatic lipid metabolism through suppression of SREBP1c activity.* Biochem Biophys Res Commun, 2008. **366**(4): p. 910-6.
178. Ordelheide, A.M., et al., *Nor-1, a novel incretin-responsive regulator of insulin genes and insulin secretion.* Mol Metab, 2013. **2**(3): p. 243-55.
179. Reynolds, M.S., et al., *beta-Cell deletion of Nr4a1 and Nr4a3 nuclear receptors impedes mitochondrial respiration and insulin secretion.* Am J Physiol Endocrinol Metab, 2016. **311**(1): p. E186-201.
180. Briand, O., et al., *The nuclear orphan receptor Nur77 is a lipotoxicity sensor regulating glucose-induced insulin secretion in pancreatic beta-cells.* Mol Endocrinol, 2012. **26**(3): p. 399-413.
181. Gao, W., et al., *Elevation of NR4A3 expression and its possible role in modulating insulin expression in the pancreatic beta cell.* PLoS One, 2014. **9**(3): p. e91462.
182. Weyrich, P., et al., *Common polymorphisms within the NR4A3 locus, encoding the orphan nuclear receptor Nor-1, are associated with enhanced beta-cell function in non-diabetic subjects.* BMC Med Genet, 2009. **10**: p. 77.

183. Nonogaki, K., et al., *Serotonin 5-HT2C receptor-independent expression of hypothalamic NOR1, a novel modulator of food intake and energy balance, in mice.* Biochem Biophys Res Commun, 2009. **386**(2): p. 311-5.
184. Veum, V.L., et al., *The nuclear receptors NUR77, NURR1 and NOR1 in obesity and during fat loss.* Int J Obes (Lond), 2012. **36**(9): p. 1195-202.
185. Chimienti, F., et al., *In vivo expression and functional characterization of the zinc transporter ZnT8 in glucose-induced insulin secretion.* J Cell Sci, 2006. **119**(Pt 20): p. 4199-206.
186. Fu, Y., et al., *Down-regulation of ZnT8 expression in INS-1 rat pancreatic beta cells reduces insulin content and glucose-inducible insulin secretion.* PLoS One, 2009. **4**(5): p. e5679.
187. Nicolson, T.J., et al., *Insulin storage and glucose homeostasis in mice null for the granule zinc transporter ZnT8 and studies of the type 2 diabetes-associated variants.* Diabetes, 2009. **58**(9): p. 2070-83.
188. Wijesekara, N., et al., *Beta cell-specific Znt8 deletion in mice causes marked defects in insulin processing, crystallisation and secretion.* Diabetologia, 2010. **53**(8): p. 1656-68.
189. Petersen, A.B., et al., *siRNA-mediated knock-down of ZnT3 and ZnT8 affects production and secretion of insulin and apoptosis in INS-1E cells.* APMIS, 2011. **119**(2): p. 93-102.
190. Navarro, M.A., et al., *Trans-10,cis-12-CLA dysregulate lipid and glucose metabolism and induce hepatic NR4A receptors.* Front Biosci (Elite Ed), 2010. **2**: p. 87-97.
191. Roche, E., et al., *Palmitate and oleate induce the immediate-early response genes c-fos and nur-77 in the pancreatic beta-cell line INS-1.* Diabetes, 1999. **48**(10): p. 2007-14.
192. Yu, C., et al., *The Orphan Nuclear Receptor NR4A1 Protects Pancreatic beta-Cells from Endoplasmic Reticulum (ER) Stress-mediated Apoptosis.* J Biol Chem, 2015. **290**(34): p. 20687-99.
193. Maxwell, M.A., et al., *Nur77 regulates lipolysis in skeletal muscle cells. Evidence for cross-talk between the beta-adrenergic and an orphan nuclear hormone receptor pathway.* J Biol Chem, 2005. **280**(13): p. 12573-84.
194. Eaton, S., K. Bartlett, and P.A. Quant, *Carnitine palmitoyl transferase I and the control of beta-oxidation in heart mitochondria.* Biochem Biophys Res Commun, 2001. **285**(2): p. 537-9.
195. Fritz, I.B., S.K. Schultz, and P.A. Srere, *Properties of partially purified carnitine acetyltransferase.* J Biol Chem, 1963. **238**: p. 2509-17.
196. Bruce, C.R., et al., *Overexpression of carnitine palmitoyltransferase-1 in skeletal muscle is sufficient to enhance fatty acid oxidation and improve high-fat diet-induced insulin resistance.* Diabetes, 2009. **58**(3): p. 550-8.
197. Gao, X.F., et al., *Enhanced susceptibility of Cpt1c knockout mice to glucose intolerance induced by a high-fat diet involves elevated hepatic gluconeogenesis and decreased skeletal muscle glucose uptake.* Diabetologia, 2009. **52**(5): p. 912-20.
198. Ishizawa, M., H. Kagechika, and M. Makishima, *NR4A nuclear receptors mediate carnitine palmitoyltransferase 1A gene expression by the rexinoid HX600.* Biochem Biophys Res Commun, 2012. **418**(4): p. 780-5.
199. Susini, S., et al., *Glucose and glucoincretin peptides synergize to induce c-fos, c-jun, junB, zif-268, and nur-77 gene expression in pancreatic beta(INS-1) cells.* FASEB J, 1998. **12**(12): p. 1173-82.
200. Zhang, L., C. Paine, and R. Dip, *Selective regulation of nuclear orphan receptors 4A by adenosine receptor subtypes in human mast cells.* J Cell Commun Signal, 2010. **4**(4): p. 173-83.
201. Tessem, J.S., et al., *Nkx6.1 regulates islet beta-cell proliferation via Nr4a1 and Nr4a3 nuclear receptors.* Proc Natl Acad Sci U S A, 2014. **111**(14): p. 5242-7.
202. Kiss, B., et al., *Retinoids induce Nur77-dependent apoptosis in mouse thymocytes.* Biochim Biophys Acta, 2015. **1853**(3): p. 660-70.
203. Chen, H.Z., et al., *Akt phosphorylates the TR3 orphan receptor and blocks its targeting to the mitochondria.* Carcinogenesis, 2008. **29**(11): p. 2078-88.

204. Wang, A., et al., *Phosphorylation of Nur77 by the MEK-ERK-RSK cascade induces mitochondrial translocation and apoptosis in T cells.* J Immunol, 2009. **183**(5): p. 3268-77.
205. Yang, H., et al., *Induction and intracellular localization of Nur77 dictate fenretinide-induced apoptosis of human liver cancer cells.* Biochem Pharmacol, 2010. **79**(7): p. 948-54.
206. Thompson, J., et al., *Protein kinase C regulates mitochondrial targeting of Nur77 and its family member Nor-1 in thymocytes undergoing apoptosis.* Eur J Immunol, 2010. **40**(7): p. 2041-9.
207. Zhang, X.K., *Targeting Nur77 translocation.* Expert Opin Ther Targets, 2007. **11**(1): p. 69-79.
208. Wang, W.J., et al., *Orphan nuclear receptor TR3 acts in autophagic cell death via mitochondrial signaling pathway.* Nat Chem Biol, 2014. **10**(2): p. 133-40.
209. Yee, K.S. and V.C. Yu, *Isolation and characterization of a novel member of the neural zinc finger factor/myelin transcription factor family with transcriptional repression activity.* J Biol Chem, 1998. **273**(9): p. 5366-74.
210. Jandrig, B., et al., *ST18 is a breast cancer tumor suppressor gene at human chromosome 8q11.2.* Oncogene, 2004. **23**(57): p. 9295-302.
211. Yang, J., et al., *The transcription factor ST18 regulates proapoptotic and proinflammatory gene expression in fibroblasts.* FASEB J, 2008. **22**(11): p. 3956-67.
212. Wang, S., et al., *Loss of Myt1 function partially compromises endocrine islet cell differentiation and pancreatic physiological function in the mouse.* Mech Dev, 2007. **124**(11-12): p. 898-910.
213. Kim, J.G., et al., *Myelin transcription factor 1 (Myt1) of the oligodendrocyte lineage, along with a closely related CCHC zinc finger, is expressed in developing neurons in the mammalian central nervous system.* J Neurosci Res, 1997. **50**(2): p. 272-90.
214. Gu, G., et al., *Global expression analysis of gene regulatory pathways during endocrine pancreatic development.* Development, 2004. **131**(1): p. 165-79.
215. Shoelson, S.E., L. Herrero, and A. Naaz, *Obesity, inflammation, and insulin resistance.* Gastroenterology, 2007. **132**(6): p. 2169-80.
216. Jayasimhan, A., K.P. Mansour, and R.M. Slattery, *Advances in our understanding of the pathophysiology of Type 1 diabetes: lessons from the NOD mouse.* Clin Sci (Lond), 2014. **126**(1): p. 1-18.
217. Anderson, M.S. and J.A. Bluestone, *The NOD mouse: a model of immune dysregulation.* Annu Rev Immunol, 2005. **23**: p. 447-85.
218. Ikegami, H., S. Makino, and H. Taniguchi, *The NOD Mouse and Its Related Strains*, in *Animal Models of Diabetes, Second Edition*2007, CRC Press. p. 41-60.
219. Sindelar, D., et al., *Animal Models to Study Obesity and Type 2 Diabetes Induced by Diet*, in *Animal Models of Diabetes, Second Edition*2007, CRC Press. p. 349-357.
220. Tschop, M. and M.L. Heiman, *Rodent obesity models: an overview.* Exp Clin Endocrinol Diabetes, 2001. **109**(6): p. 307-19.
221. Pejnovic, N.N., et al., *Galectin-3 deficiency accelerates high-fat diet-induced obesity and amplifies inflammation in adipose tissue and pancreatic islets.* Diabetes, 2013. **62**(6): p. 1932-44.
222. Hohmeier, H.E., et al., *Isolation of INS-1-derived cell lines with robust ATP-sensitive K+ channel-dependent and -independent glucose-stimulated insulin secretion.* Diabetes, 2000. **49**(3): p. 424-30.
223. Buteau, J., et al., *Metabolic diapause in pancreatic beta-cells expressing a gain-of-function mutant of the forkhead protein Foxo1.* J Biol Chem, 2007. **282**(1): p. 287-93.
224. Cho, W.J., et al., *Matrix metalloproteinase-2, caveolins, focal adhesion kinase and c-Kit in cells of the mouse myocardium.* J Cell Mol Med, 2007. **11**(5): p. 1069-86.
225. Katragadda, D., et al., *Epoxyeicosatrienoic acids limit damage to mitochondrial function following stress in cardiac cells.* J Mol Cell Cardiol, 2009. **46**(6): p. 867-75.
226. Eskelinen, E.L., *Fine structure of the autophagosome.* Methods Mol Biol, 2008. **445**: p. 11-28.

227. Hassinen, I., *Mitochondrial metabolism*, in *Mitochondria*, M.-S.S. Stephen Schaffer, Editor 2007, Springer: New York.
228. Gnaiger, E. *Mitochondrial Pathways and Respiratory Control: An Introduction to OXPHOS Analysis*. 2012 March 23 2017]; Available from: http://www.bioblast.at/index.php/Gnaiger_2012_MitoPathways.
229. Pearen, M.A., et al., *Transgenic muscle-specific Nor-1 expression regulates multiple pathways that effect adiposity, metabolism, and endurance.* Mol Endocrinol, 2013. **27**(11): p. 1897-917.
230. Kurakula, K., et al., *NR4A nuclear receptors are orphans but not lonesome.* Biochim Biophys Acta, 2014. **1843**(11): p. 2543-2555.
231. Gissendanner, C.R., et al., *The Caenorhabditis elegans NR4A nuclear receptor is required for spermatheca morphogenesis.* Dev Biol, 2008. **313**(2): p. 767-86.
232. He, L.M., et al., *Cyclin D2 protein stability is regulated in pancreatic beta-cells.* Mol Endocrinol, 2009. **23**(11): p. 1865-75.
233. Gao, W., et al., *Elevation of NR4A3 Expression and Its Possible Role in Modulating Insulin Expression in the Pancreatic Beta Cell.* PLoS One, 2014. **9**(3).
234. Rapak, A., et al., *Apoptosis of lymphoma cells is abolished due to blockade of cytochrome c release despite Nur77 mitochondrial targeting.* Apoptosis, 2007. **12**(10): p. 1873-8.
235. Lemasters, J.J., et al., *The mitochondrial permeability transition in cell death: a common mechanism in necrosis, apoptosis and autophagy.* Biochim Biophys Acta, 1998. **1366**(1-2): p. 177-96.
236. Giovannini, C., et al., *Mitochondria hyperpolarization is an early event in oxidized low-density lipoprotein-induced apoptosis in Caco-2 intestinal cells.* FEBS Lett, 2002. **523**(1-3): p. 200-6.
237. Leal, A.M., et al., *Violacein induces cell death by triggering mitochondrial membrane hyperpolarization in vitro.* BMC Microbiol, 2015. **15**: p. 115.
238. Morciano, G., et al., *Intersection of mitochondrial fission and fusion machinery with apoptotic pathways: Role of Mcl-1.* Biol Cell, 2016. **108**(10): p. 279-293.
239. Oettinghaus, B., et al., *DRP1-dependent apoptotic mitochondrial fission occurs independently of BAX, BAK and APAF1 to amplify cell death by BID and oxidative stress.* Biochim Biophys Acta, 2016. **1857**(8): p. 1267-76.
240. Bouzas-Rodriguez, J., et al., *The nuclear receptor NR4A1 induces a form of cell death dependent on autophagy in mammalian cells.* PLoS One, 2012. **7**(10): p. e46422.
241. Kameyama, T., et al., *Myt/NZF family transcription factors regulate neuronal differentiation of P19 cells.* Neurosci Lett, 2011. **497**(2): p. 74-9.
242. Hajmrle, C., et al., *SUMOylation protects against IL-1beta-induced apoptosis in INS-1 832/13 cells and human islets.* Am J Physiol Endocrinol Metab, 2014. **307**(8): p. E664-73.
243. Malik, V.S., W.C. Willett, and F.B. Hu, *Global obesity: trends, risk factors and policy implications.* Nat Rev Endocrinol, 2013. **9**(1): p. 13-27.

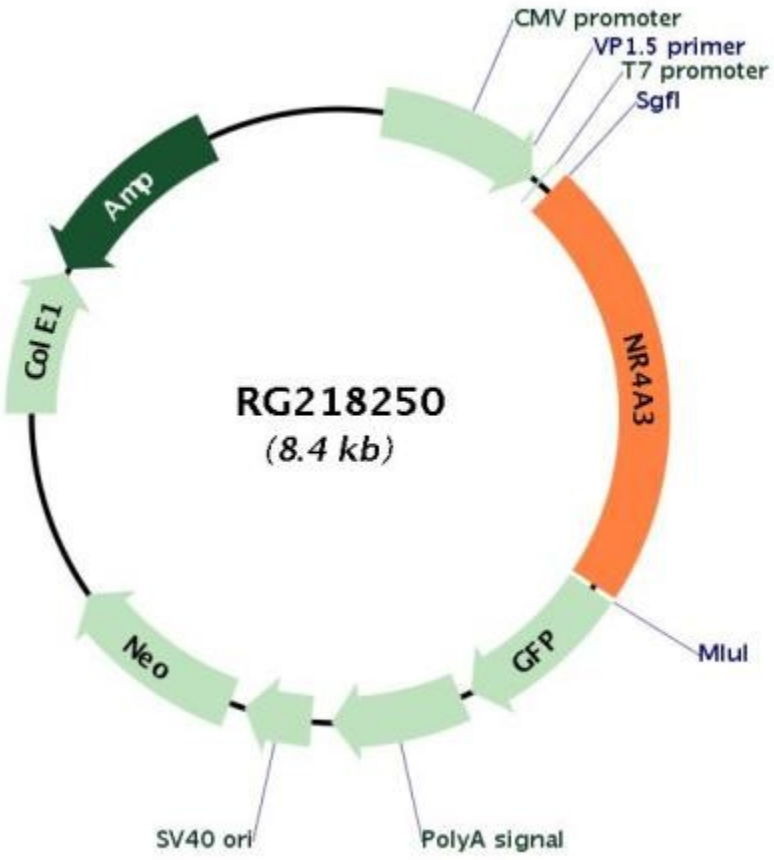
Annexe 1: T2D islets

	Donor number	Cts Nor1	average Cts Nor1	Cts Actin	average Cts actin	ΔCt	ΔΔCt (compared to average ND)	fold change
ND	H1759	32.868946	31.377059	26.520636	24.9505265	6.42653	0.0805835	0.94567509
		29.885172		23.380417				
	R073	31.493755	31.478235	24.335732	24.598387	6.87985	0.533899	0.69068558
		31.462715		24.861042				
	R075	30.842196	30.2543545	24.380222	24.127512	6.12684	-0.2191065	1.16401246
		29.666513		23.874802				
	R081	31.19104	31.3730575	25.598686	25.4224845	5.95057	-0.395376	1.31528551
		31.555075		25.246283				
					average	6.34595		
T2D	R080	27.646408	26.6524965	24.887129	24.653346	1.99915	-4.3467985	20.347766
		25.658585		24.419563				
	R131	28.704355	28.9682245	25.895414	26.258009	2.71022	-3.6357335	12.42982
		29.232094		26.620604				
	R137	28.80285	28.82336	26.125446	25.889077	2.93428	-3.411666	10.6417684
		28.84387		25.652708				
	R154	30.359505	30.4272785	26.624393	26.7533635	3.67392	-2.672034	6.37327097
		30.495052		26.882334				
	HP15258-01T2D	26.130596	26.115898	24.996332	24.885968	1.22993	-5.116019	34.6796877
		26.1012		24.775604				
	R155	31.542042	31.309711	27.877708	27.824987	3.48472	-2.861225	7.2663205
		31.07738		27.772266				
	HP16023-01T2D	29.272482	29.436246	27.199091	27.038864	2.39738	-3.948567	15.4396378
		29.60001		26.878637				

	BMI	Age (years)	HbA1C (%)	Medication
R080	28.0	56	5.8	Metformin
R131	26.0	52	6.8	Metformin
R137	36.7	57	5.5	Metformin
R154	40.8	57	7.4	
HP15258-01T2D	43.0		6.5	
R155	34.0	56	6.3	
HP16023-01T2D	24.4	51	6.9	

Annexe 2: Plasmids

1. pCMV-Nor1-GFP



2. pCMV-Nor1-Flag