The following individuals certify that they have read, and recommend to the Faculty of Graduate and Postdoctoral Studies for acceptance, the dissertation entitled:

The Role of NPAS4 and RGS2 in the Regulation of Pancreatic β-cell Function

submitted by Thilo Speckmann in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Cell and Developmental Biology

Examining Committee:

Dr. Francis C. Lynn
Supervisor

Dr. Dan S. Luciani
Supervisory Committee Member

Supervisory Committee Member

Dr. T. Michael Underhill
University Examiner

Dr. Brian Rodrigues
University Examiner

Additional Supervisory Committee Members:

Dr. Stefan Taubert
Supervisory Committee Member

Dr. C. Bruce Verchere
Supervisory Committee Member
Abstract

Pancreatic β-cells regulate systemic glycemia by releasing the glucose-lowering hormone insulin. Diabetes, a chronic metabolic disease characterized by insulin insufficiency, is linked to β-cell dysfunction with perturbed calcium homeostasis. The activity-induced, calcium-dependent transcription factor, NPAS4, reduced insulin secretion and promoted β-cell health, in part through its target gene, the GTPase-activating protein RGS2. Because our mechanistic understanding of this process remains incomplete, studying the normal physiology of calcium-dependent β-cell function may uncover new avenues for the treatment or prevention of diabetes. The overall goal of my thesis was to establish whether activity-induced NPAS4 and RGS2 expression could optimize β-cell function. Initially, I uncovered a role for CaMKII, calcineurin, and PKB in membrane depolarization-induced Npas4 mRNA and protein expression in MIN6 cells and mouse islets. Calcineurin inhibition and concurrent loss of NPAS4 showed cytotoxic increases in cleaved caspase 3 expression, which was reversed by adenovirally reinstating NPAS4 in MIN6 cells. Co-immunoprecipitation studies in MIN6 cells then uncovered competition between NPAS4 and a related transcription factor, HIF1α, for the shared heterodimerization partner, ARNT. Accordingly, HIF1α target gene expression was lower in human and mouse islets overexpressing Npas4, and higher in β-cell-specific Npas4 knockout mouse islets (N4KO). Because excessive HIF1α signalling compromises β-cell function by switching energy production from oxidative phosphorylation to anaerobic glycolysis, I examined whether N4KO mice developed functional defects. Indeed, N4KO islets showed lower oxygen consumption rate, and HFD-fed N4KO mice developed mild glucose intolerance. To understand how NPAS4 may counteract these defects, I identified shared DNA binding sites of NPAS4 and ARNT in MIN6 cells using ChIP-Seq. Among the shared sites, I observed NPAS4 and ARNT binding near Rgs2, corroborating an earlier study. I then demonstrated that RGS2 is a negative regulator of glucose-stimulated insulin secretion (GSIS), because Rgs2-overexpressing MIN6
cells and mouse islets showed reduced GSIS, due to lower calcium influx and oxygen consumption, whereas *Rgs2* knockout cells exhibited increased GSIS. In sum, I demonstrated that NPAS4 and its target gene, RGS2, are important regulators of β-cell function. This suggests that these two factors could be promising therapeutic targets to promote β-cell health and optimize insulin secretion in diabetes.
Lay Summary

Diabetes is characterized by high blood glucose due to an insufficient effect or amount of insulin, which is released by pancreatic \( \beta \)-cells. The disease may develop following overnutrition and defects in the use of the important cellular signalling molecule, calcium, due to \( \beta \)-cells becoming overworked. Despite available treatments with blood glucose-lowering drugs, blood glucose control may remain inadequate, causing long-term health problems. A potential alternative is to lessen the stresses that nutrient excess places on \( \beta \)-cells, but this requires a better understanding of how \( \beta \)-cells work. Therefore, I studied two proteins which are induced in \( \beta \)-cells following a meal, NPAS4 and RGS2, and their role in insulin secretion. I identified the calcium signals promoting NPAS4 production, and showed that NPAS4 and RGS2 together reduce \( \beta \)-cell stress and optimize insulin release by regulating calcium levels. In summary, they make \( \beta \)-cells work more efficiently, which may be exploited for diabetes treatment.
Preface

Except where indicated otherwise, studies in this thesis were conceived and designed by me and Dr. Francis C. Lynn. I performed experiments, analyzed data, and wrote the manuscripts which form part of this thesis; additionally, Chapters 1, 3, 4, and 6 include work conducted in collaboration with Dr. Paul V. Sabatini (see below). Any element (text, figure, table) originating from previously published manuscripts are re-printed with permission.

All animal work was approved by the University of British Columbia Animal Care Committee under protocols A13-0184, A14-0163 and A17-0158. Human islet work was approved by the BC Children’s Hospital Research Ethics Board under certificate # H09-00676.

Chapter 1:

The parts of Chapter 1 pertaining to calcium signalling (section 1.3) have been published as literature review: Sabatini PV, Speckmann T, Lynn FC. Friend and foe: β-cell Ca\(^{2+}\) signaling and the development of diabetes. Mol Metab. 2019 Mar; 21:1-12. \[1\]

Dr. Paul V. Sabatini, Dr. Francis C. Lynn, and I wrote and edited the manuscript. The manuscript was originally drafted by Dr. Paul V. Sabatini.

Chapter 3:

A version of Chapter 3 has been published as: Speckmann T, Sabatini PV, Nian C, Smith RG, Lynn FC: Npas4 Transcription Factor Expression Is Regulated by Calcium Signaling Pathways and Prevents Tacrolimus-induced Cytotoxicity in Pancreatic β-cells. J Biol Chem. 2016 Feb 5;291(6):2682-95. \[2\]

I designed and performed most of the experiments, analyzed the data, and wrote the manuscript. Dr. Paul V. Sabatini and Dr. Francis C. Lynn contributed to designing, performing
and analyzing experiments. In addition, technical assistance was provided by Cuilan Nian (obtaining islets, cell culture, sample preparation, western blotting) and Riley G. Smith (obtaining islets, sample preparation). All authors subsequently edited the manuscript.

Versions of Figure 2D, 4E&F, 6 and 7, which are used in Chapter 3, represent experiments (gene expression assays, western blot) performed by Dr. Paul V. Sabatini, and published in his doctoral dissertation [3].

**Chapter 4:**


Dr. Paul V. Sabatini and I contributed equally to designing and performing experiments, analyzing the data, and writing the manuscript. Dr. Francis C. Lynn also designed experiments, analyzed the data and wrote the manuscript. The manuscript was originally drafted by Dr. Paul V. Sabatini. In addition, technical assistance was provided by Cuilan Nian (obtaining islets, cell culture, sample preparation, western blotting, genotyping), Dr. Maria M. Glavas (brain dissections), and Chi Kin Wong (metabolic cage studies). All authors subsequently edited the manuscript.

Versions of Figures 1, 5, 6 and S1, which are used in Chapter 4, represent experiments (animal studies, gene expression assays, western blot) performed by, or in collaboration with, Dr. Paul V. Sabatini, and are published in his doctoral dissertation [3].

**Chapter 5:**

I designed and performed most of the experiments and analyzed the data. Figure 5.8 is modified
from experiments and analysis performed by Dr. Paul V. Sabatini. Alexandre Kadhim assisted with ChIP-Seq library construction and analysis of sequencing data.

Chapter 6:

I designed and performed most of the experiments and analyzed the data. Dr. Francis C. Lynn designed and constructed \textit{Rgs2} adenovirus and \textit{Rgs2} CRISPR/Cas9 plasmids. For Figure 6.2, hyperglycemic clamps were performed and pancreatic sections prepared by Dr. Paul V. Sabatini (unpublished); I performed immunostaining and analyzed images. Figure 6.3 is modified from experiments and analysis performed by Dr. Paul V. Sabatini. Further technical assistance was provided by Cuilan Nian (islet isolation, islet picking, cell culture, sample collection, sample processing, western blotting, ELISA, genotyping), Shi Yao Li (islet picking, cell culture, sample collection, sample processing, western blotting, ELISA, genotyping), Riley G. Smith (islet isolation, islet picking, sample collection, sample processing, ELISA), and Lisa Xu (FACS).
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<tbody>
<tr>
<td>aa</td>
<td>amino acid</td>
</tr>
<tr>
<td>AAV</td>
<td>adeno-associated virus</td>
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<tr>
<td>ABCC8</td>
<td>ATP-binding cassette sub-family C member 8</td>
</tr>
<tr>
<td>α,β-MeATP</td>
<td>α,β-methyleneadenosine 5‘-triphosphate</td>
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<tr>
<td>AC</td>
<td>adenylate cyclase</td>
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<td>adenosine diphosphate</td>
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<td>AHR</td>
<td>aryl hydrocarbon receptor</td>
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<td>AHRR</td>
<td>aryl hydrocarbon receptor repressor</td>
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<td>AIP</td>
<td>autocamptide-2 related inhibitor peptide</td>
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<td>Akti-1/2</td>
<td>Akt1/2 kinase inhibitor</td>
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<td>ALDO</td>
<td>fructose-bisphosphate aldolase</td>
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<td>AMP</td>
<td>adenosine monophosphate</td>
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<td>AMPAR</td>
<td>α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor</td>
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<td>AMPK</td>
<td>AMP-activated protein kinase</td>
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<tr>
<td>ANOVA</td>
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<td>AOC</td>
<td>area over the curve</td>
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<td>AP-1</td>
<td>activator protein 1 transcription factor</td>
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<tr>
<td>ARNT</td>
<td>aryl hydrocarbon receptor nuclear translocator</td>
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<td>ARNTL</td>
<td>aryl hydrocarbon receptor nuclear translocator-like protein</td>
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<td>ATF</td>
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<td>CaM</td>
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<td>EGFP</td>
<td>enhanced green fluorescent protein</td>
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<td>EGR</td>
<td>early growth response protein</td>
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<tr>
<td>EGTA</td>
<td>ethylene glycol tetraacetic acid</td>
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<td>ELISA</td>
<td>enzyme-linked immunosorbent assay</td>
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<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>EPAC2</td>
<td>exchange protein directly activated by cAMP 2 (see RAPGEF4)</td>
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</tr>
<tr>
<td>ERK</td>
<td>extracellular signal-regulated kinase (see MAPK)</td>
</tr>
<tr>
<td>et al.</td>
<td>et alii</td>
</tr>
<tr>
<td>EtOH</td>
<td>ethanol</td>
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<td>FBS</td>
<td>fetal bovine serum</td>
</tr>
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<td>free fatty acid</td>
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<td>FFAR1</td>
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<tr>
<td>GLUT2</td>
<td>glucose transporter 2 (see SLC2A2)</td>
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<td>glycerol tolerance test</td>
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<td>G-protein coupled receptor</td>
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<td>GSIS</td>
<td>glucose-stimulated insulin secretion</td>
</tr>
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<td>G alpha subunit</td>
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<td>Gβγ</td>
<td>G beta-gamma complex</td>
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<td>high fat diet</td>
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<td>hypoxia-inducible factor</td>
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<td>human leukocyte antigen</td>
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<tr>
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<td>hepatocyte nuclear factor</td>
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<td>HRE</td>
<td>hypoxia response elements</td>
</tr>
<tr>
<td>i.e.</td>
<td>id est</td>
</tr>
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<td>islet amyloid polypeptide</td>
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<tr>
<td>INS</td>
<td>insulin</td>
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<tr>
<td>IP&lt;sub&gt;3&lt;/sub&gt;</td>
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<tr>
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<td>inositol 1,4,5-trisphosphate receptor</td>
</tr>
<tr>
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<td>intraperitoneal glucose tolerance test</td>
</tr>
<tr>
<td>ITT</td>
<td>insulin tolerance test</td>
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<td>K&lt;sub&gt;ATP&lt;/sub&gt;</td>
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<td>KCNJ11</td>
<td>ATP-sensitive inward rectifier potassium channel 11</td>
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<td>KLF</td>
<td>Krüppel-like factor</td>
</tr>
<tr>
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<tr>
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<td>knockout</td>
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<td>KRBH</td>
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<td>MAF bZIP transcription factor A</td>
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<td>mitochondria-associated membrane</td>
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<tr>
<td>MAP2K</td>
<td>MAP kinase kinase</td>
</tr>
<tr>
<td>MAP3K</td>
<td>MAP kinase kinase kinase</td>
</tr>
<tr>
<td>MAPK</td>
<td>mitogen-activated protein kinase</td>
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<tr>
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<td>monocarboxylate transporter 4 (see SLC16A3)</td>
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<td>mitochondrial calcium uniporter</td>
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<td>myocyte enhancer factor-2</td>
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<td>methanol</td>
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<td>microRNA</td>
</tr>
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<td>maturity onset diabetes of the young</td>
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<td>MOI</td>
<td>multiplicity of infection</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger RNA</td>
</tr>
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<td>Abbreviation</td>
<td>Description</td>
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<td>--------------</td>
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<tr>
<td>N.B.</td>
<td>nota bene</td>
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<tr>
<td>NAD(H)</td>
<td>nicotinamide adenine dinucleotide (reduced)</td>
</tr>
<tr>
<td>NADP(H)</td>
<td>nicotinamide adenine dinucleotide phosphate (reduced)</td>
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<td>mitochondrial Na(^+)/Ca(^2+) exchanger</td>
</tr>
<tr>
<td>NCX</td>
<td>Na(^+)/Ca(^2+) exchanger</td>
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<td>NEUROD1</td>
<td>neurogenic differentiation 1</td>
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<td>nuclear factor of activated T cells</td>
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<td>nerve growth factor</td>
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<td>N-methyl-D-aspartate receptor</td>
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<td>NP-40</td>
<td>nonidet P-40; Octylphenoxy poly(ethyleneoxy)ethanol; IGEPA</td>
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<tr>
<td>ODD</td>
<td>oxygen-dependent degradation</td>
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<td>oral glucose tolerance test</td>
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<td>open reading frame</td>
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<td>OXPHOS</td>
<td>oxidative phosphorylation</td>
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<td>purinergic receptor P2Y1</td>
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<td>Per-Arnt-Sim</td>
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<td>paired box protein</td>
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<td>polymerase chain reaction</td>
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<td>pyruvate dehydrogenase kinase 1</td>
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<td>PDX1</td>
<td>pancreatic and duodenal homeobox 1</td>
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<td>penicillin-streptomycin solution</td>
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<td>paraformaldehyde</td>
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<td>pH</td>
<td>power of hydrogen</td>
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<td>phosphoinositide 3-kinase</td>
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<td>protein kinase B</td>
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<tr>
<td>PKC</td>
<td>protein kinase C</td>
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<td>Full Form</td>
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<td>PLC</td>
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<td>plasma membrane Ca$^{2+}$ ATPase</td>
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<td>quantitative PCR</td>
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<td>RAPGEF4</td>
<td>Rap guanine nucleotide exchange factor 4</td>
</tr>
<tr>
<td>RGS</td>
<td>regulator of G-protein signalling</td>
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<td>RIP</td>
<td>rat insulin promoter</td>
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<td>RNA</td>
<td>ribonucleic acid</td>
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<td>RNase</td>
<td>ribonuclease</td>
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<td>reactive oxygen species</td>
</tr>
<tr>
<td>rpm</td>
<td>revolutions per minute</td>
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<td>RPMI</td>
<td>Roswell Park Memorial Institute 1640 medium with FBS, L-glutamine and pen/strep</td>
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<td>RT</td>
<td>room temperature</td>
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<td>RyR</td>
<td>ryanodine receptor</td>
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<td>scRNA-Seq</td>
<td>single-cell RNA sequencing</td>
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<td>SDS</td>
<td>sodium dodecyl sulfate</td>
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<tr>
<td>SEM</td>
<td>standard error of the mean</td>
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<td>SERCA</td>
<td>sarco/endoplasmic reticulum Ca$^{2+}$ ATPase</td>
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<td>SIM</td>
<td>single-minded</td>
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<tr>
<td>siRNA</td>
<td>silencing</td>
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<td>soluble N-ethylmaleimide-sensitive factor attachment protein receptor</td>
</tr>
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<td>single nucleotide polymorphism</td>
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<td>superoxide dismutase</td>
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<td>SRY-box 9</td>
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<td>somatostatin</td>
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<td>somatostatin receptor 1</td>
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<td>Abbreviation</td>
<td>Description</td>
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<td>-------------</td>
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<tr>
<td>T1D</td>
<td>type 1 diabetes</td>
</tr>
<tr>
<td>T2D</td>
<td>type 2 diabetes</td>
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<td>TAD</td>
<td>transcriptional activation domain</td>
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<td>Tris-acetate-EDTA</td>
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<td>TE</td>
<td>Tris-EDTA</td>
</tr>
<tr>
<td>TF</td>
<td>transcription factors</td>
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<td>tamoxifen</td>
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<tr>
<td>Tris</td>
<td>tris(hydroxymethyl)aminomethane</td>
</tr>
<tr>
<td>TSS</td>
<td>transcriptional start site(s)</td>
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<td>U</td>
<td>enzyme unit</td>
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<td>UDP</td>
<td>uridine diphosphate</td>
</tr>
<tr>
<td>UTP</td>
<td>uridine triphosphate</td>
</tr>
<tr>
<td>UTR</td>
<td>untranslated region</td>
</tr>
<tr>
<td>UV</td>
<td>ultraviolet</td>
</tr>
<tr>
<td>v/v</td>
<td>volume by volume</td>
</tr>
<tr>
<td>VDAC</td>
<td>voltage-dependent anion channel</td>
</tr>
<tr>
<td>VEGFA</td>
<td>vascular endothelial growth factor A</td>
</tr>
<tr>
<td>VHL</td>
<td>von Hippel-Lindau tumor suppressor</td>
</tr>
<tr>
<td>w/v</td>
<td>weight by volume</td>
</tr>
<tr>
<td>WT</td>
<td>wild-type</td>
</tr>
<tr>
<td>x g</td>
<td>x-times gravitational acceleration</td>
</tr>
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</table>
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To my parents and Alexis,

For always encouraging me to strive for my best.
Chapter 1: Introduction

1.1 Regulation of glucose homeostasis

Regulation of whole-body glucose levels is an ancient trait to optimize fuel distribution throughout the body which relies upon the actions of hormones such as insulin. Insulin or insulin-like peptides are found in some invertebrates and virtually all vertebrates [5; 6], whereas its role evolved from a mainly mitogenic growth factor in invertebrates [6] to being a major hormone controlling glucose homeostasis in vertebrates [5].

1.1.1 Islet cytoarchitecture and physiology of glucose homeostasis

In adult vertebrates, one of the main organs regulating systemic glucose homeostasis is the pancreas. The human pancreas can be divided into head, body, and tail, which correspond to the duodenal, gastric, and splenic lobes of the mouse pancreas, respectively [7; 8]. The majority of the pancreas is comprised of acinar and ductal cells, which secrete digestive enzymes and bicarbonate that are drained into the pancreatic ducts [9]. The endocrine compartment only makes up 1-3 % of total pancreatic mass in adult humans [10-13]. It consists of the islets of Langerhans, roughly spherical structures scattered throughout the exocrine tissue [13] that contain specialized endocrine cells secreting blood glucose-regulating hormones into circulation [14]. The five known endocrine cell types are distinguished by their most abundant hormones, namely glucagon-producing α-cells, insulin- and islet amyloid polypeptide (IAPP)-producing β-cells, pancreatic polypeptide-producing PP-cells (γ-cells), somatostatin-producing δ-cells, and ghrelin-producing ε-cells [14-16]. Adult mouse islets are defined by an inner core of β cells (60-80 %) and an outer ring of α- (10-20 %), δ- (5-10 %), and PP-cells (<5 %) [15-20]; ε-cells are rare [21]. In contrast, adult human islets feature a higher number of α-cells (20-40 %) at the expense of β-cells (50-70 %), whereas δ- (~10 %), PP- (<5 %) and ε-cells (<1 %) occur in similar
proportions as in mice [15; 16; 18-20; 22]. The islet has also long been noted for its rich microvasculature of arterioles and venules [23-26], which is relevant for both nutrient and oxygen supply to the islet, as well as hormone transport from the islet into circulation. Lastly, islets are subject to central control, with both sympathetic and parasympathetic innervation modulating hormone secretion [27-30]. Compared to mouse islets, human islet innervation is thought to be more limited [31], although this assertion has been challenged recently [32].

In the postprandial phase after a meal, β-cells sense a rise in blood glucose levels [33] and start secreting insulin [34] to instruct peripheral tissues to take up glucose [35]. Major insulin target tissues include muscle, adipose and liver [36]. Whereas insulin does not increase hepatic glucose uptake [37], it stimulates membrane localisation of the glucose transporter SLC2A4 (GLUT4) in muscle [38; 39] and adipose tissue [40; 41], thus increasing glucose uptake. Insulin also increases glycogenesis in all three tissues, and decreases glycogenolysis and gluconeogenesis in the liver [35; 36]. Through its actions, insulin clears glucose from circulation to avoid hyperglycemia [35; 36]. In contrast, α-cells become active and secrete glucagon when glucose levels fall and β-cells cease insulin secretion [42-44]. Glucagon primarily targets the liver to promote glycogenolysis and gluconeogenesis [45]; countering the effects of insulin on carbohydrate metabolism by increasing hepatic glucose output, and preventing life-threatening hypoglycemia [46-49].

Finally, signals other than glucose regulate glucose homeostasis via fine-tuning of hormonal (insulin, glucagon, somatostatin) secretion. These signals can be paracrine interactions between α-, β-, and δ-cells [50; 51], and para-/sympathetic neurotransmitter release [52; 53], and are often mediated through G-protein coupled receptors [54; 55] (see section 1.5).

1.1.2 Glucose-stimulated insulin secretion

The basic molecular mechanisms controlling glucose-stimulated insulin secretion (GSIS) [56; 57] have been gradually deciphered in the past six decades following the discovery of potassium-
and calcium-dependent electrical activity of β-cells [58, 59]. A postprandial rise in blood glucose triggers glucose uptake into the β-cell through facilitated diffusion by a member of the glucose transporter solute carrier family 2 (SLC2A). In mice, the main glucose transporter is the low-affinity (high Michaelis constant; K_M), high-capacity Slc2a2 (also known as Glut2), which allows efficient uptake proportional to increases in blood glucose concentration [60-63]. Humans express higher levels of the low K_M (high-affinity) transporters SLC2A1 and SLC2A3 (GLUT1/3) [63-66]; although SLC2A2 is present and likely has a functional role [67]. Following uptake into the β-cell, glucose is retained due to phosphorylation by a member of the hexokinase family, glucokinase (GCK). GCK serves as the principal glucose sensor in β-cells, due to its high K_M (low-affinity), preference for glucose as a substrate, and lack of inhibition by its product glucose-6-phosphate [68-72]. Glucose-6-phosphate is then metabolized through glycolysis, and, following shuttling of pyruvate into the mitochondrial matrix, the tricarboxylic acid cycle generates reducing equivalents (NADH+H+). These act on the electron transport chain to establish a proton gradient across the inner mitochondrial membrane. Using this gradient to convert phosphate and adenosine diphosphate (ADP) into adenosine triphosphate ATP [73-77], mitochondrial ATPase raises the ATP:ADP ratio, which leads to the closure of the ATP-sensitive potassium (K_ATP) channel [78-83]. Because the resting β-cell membrane potential is hyperpolarized relative to the extracellular milieu (-80 mV to -60 mV), the intracellular buildup of positively charged potassium ions [84] causes membrane depolarization, and at a membrane potential above -50 mV (-60 mV in human β-cells), action potentials are elicited [77, 79, 85-88]. Membrane depolarization triggers influx of extracellular calcium into the β-cell cytosol, predominantly through L-type voltage-dependent calcium channels (L-VDCCs); although P/Q-type and T-type calcium channels participate in this process [89-92] (Figure 1.1 A-E).
Figure 1.1. Triggering of insulin secretion in pancreatic β-cells.

(A) Glucose is transported into β-cells through a member of the solute carrier family 2 (SLC2A1 in humans, SLC2A2 in mice), and retained due to phosphorylation by glucokinase (GCK). (B) Glucose-6-phosphate is then metabolized through glycolysis, and resulting pyruvate shuttled into mitochondria. (C) Oxidative phosphorylation (OXPHOS) generates ATP from ADP. (D) The increase in the ATP:ADP ratio causes the closure of the K<sub>ATP</sub> channel, and subsequent buildup of potassium ions (K<sup>+</sup>) leads to membrane depolarization (Ψ<sub>m</sub> = membrane potential). (E) This triggers influx of extracellular calcium (Ca<sup>2+</sup>) through L-type voltage-dependent calcium channels (L-VDCCs). (F) The rise in cytosolic calcium (Ca<sup>2+</sup><sub>cyt</sub>) then triggers insulin release via effects on the exocytotic machinery. (G) Glucose, fatty acid, and amino acid metabolism generate additional metabolic coupling factors (MCF) enhancing calcium influx and exocytosis. FFA = free fatty acid; AA = amino acid; NADPH = nicotinamide adenine dinucleotide phosphate (reduced); ROS = reactive oxygen species.

The rise in cytosolic calcium (Ca<sup>2+</sup><sub>cyt</sub>) levels triggers the release of insulin from pre-docked granules into circulation<sup>[93-97]</sup> (Figure 1.1 F). Briefly, this involves the stages of insulin granule docking, priming, and fusion, mediated by the actions of a quaternary sec1/Munc18-like (SM) / soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) protein
complex [77; 98; 99] (consisting of STXBP1 [100], STX1A [101], SNAP25 [102], VAMP2 [103; 104]). An increase in Ca\textsuperscript{2+}\textsubscript{cyt} is detected by calcium-binding synaptotagmins (e.g. SYT7), which likely induce conformational changes in the SM/SNARE complex, thus triggering insulin granule fusion with the plasma membrane, i.e. insulin secretion [99; 105; 106].

The kinetics of insulin secretion can be divided into two phases [107]. Following a stimulus such as elevated blood glucose, first-phase insulin secretion is characterized by a steep rise and fall in circulating insulin levels, with a peak at 1-5 min and duration of about 5-15 min [108-110]. It depends on the release of pre-docked vesicles of the readily releasable pool [97; 101; 111-113]; although newcomer vesicles from a reserve pool also make up a significant amount of first-phase insulin secretion [114-116]. First-phase insulin release is followed by sustained second-phase insulin secretion, which mainly relies on the release of newcomer insulin granules [112-114; 116]. Circulating insulin levels during second-phase secretion tend to be lower than during first-phase [107; 108], but can be sustained for a few hours if blood glucose levels remain elevated [77; 99].

In summary, GSIS involves triggering β-cell depolarization, calcium influx, and insulin secretion downstream of glucose metabolism. A wide array of other stimuli (section 1.3 and 1.5) sustain or amplify insulin secretion directly or indirectly via enhancing energy metabolism, Ca\textsuperscript{2+}\textsubscript{cyt} influx, and/or insulin granule exocytosis [76; 110]; for example, lipids, amino acids, or their derivatives provide metabolic coupling factors (Figure 1.1 G). Perturbations in these essential β-cell processes underlie the pathophysiology of impaired glucose homeostasis and diabetes.

### 1.2 Diabetes mellitus

*Diabetes mellitus* is a chronic metabolic disease characterized by insufficient blood glucose homeostasis as the result of an absolute lack of insulin or relative insufficiency of insulin action [117]. As a consequence, target tissues throughout the body exhibit reduced glucose uptake, leading to hyperglycemia [118]. To understand how a disease of excess blood glucose came to be
the story of β-cells and insulin secretion, one may take a look at the history of diabetes over the past millenia.

### 1.2.1 History of diabetes – from sweet urine to insulin

In untreated cases of diabetes, excess blood glucose is excreted in urine, resulting in frequent urination (polyuria). The first record of a disease of polyuria comes from an ancient Egyptian papyrus dated to 1552 B.C. [119; 120], whereas a first reference to sweet urine (glycosuria) is provided in an Indian text from the 5th century B.C. [121]. Yet it took two millennia before researchers started to gain insight into the physiology of the endocrine pancreas and its dysfunction in diabetes. A first comprehensive description of diabetes mellitus comes from Aretaeus of Cappadocia around the 2nd century AD [122]; alluding to the symptom of polyuria, he contributed to the term diabetes, from Greek “to pass through” [123]; whereas English physician Thomas Willis later added Latin *mellitus*, for “honey-sweet”, in reference to glycosuria [124; 125]. Treatment options for this terminal disease (in the case of absolute lack of insulin) had essentially been limited to protein-rich and carbohydrate-reduced diet [126], due to a lack of knowledge of the underlying defects.

Clues for associating diabetes with a defect in the pancreas came from Cawley in 1788, in which he identified pancreatic calculi, obstructions of the pancreatic duct, in a post-mortem autopsy of a diabetic man [127]. Pancreatectomy experiments performed by von Mering and Minkowski in 1889 showed that dogs became diabetic following the procedure [128], while pancreas replacement rescued the diabetic phenotype [129]. While this suggested an important role for the pancreas in glucose homeostasis, it remained unknown which cells were ultimately responsible for the effect.

Histological evidence for the existence of islets was presented by Langerhans in his dissertation [130]. Unable to attribute them a function, Langerhans falsely suggested the islets
could be nerve fibres, and for some time, they were disregarded as lymphatic follicles [23; 24] or exhausted acini [131]. The preservation of islets was noted following duct ligation, which failed to cause diabetes despite the destruction of the exocrine pancreas [132]. Yet, the implication of islets was not realized until Schulze [133] and Ssobolew [134] performed their own duct ligation experiments, and proposed that absence of glycosuria was a result of islet survival. In support of this, Dieckhoff [135] and Ssobolew [134] noted an unusually low number of islets in the pancreas of diabetic patients. In the interval, Laguesse had initiated a series of studies on “les ilôts de Langerhans” [136-140], and correctly deduced their endocrine nature [139; 140], supported in this view by Diamare [141]. Opie then suggested it was the islets which were directly responsible for the regulation of glucose metabolism, as he observed lesions restricted to islets in diabetic patients [142]. In conclusion, these studies were suggestive that the pancreatic islet is an endocrine tissue exerting glucose-lowering action, which is defective in diabetes.

Over the next two decades, many attempts were made to extract the glucose-lowering secreted substance for the treatment of diabetes, starting with Zülzer, who achieved partial success with his pancreatic extract “Acomatrol” in humans; yet, side effects were intolerable [143-145]. It was not until 1921 that an islet extract of adequate purity for the successful use in human patients was developed by Banting, Best, Collip and MacLeod [146]. Banting and MacLeod were awarded the Nobel Prize for Physiology or Medicine in 1923 “for the discovery of insulin”, named so after its origin from the islet (from Latin “insula”) [145]. While insulin therapy transformed diabetes from a fatal to a survivable disease, patients still suffered from reduced quality of life due to side-effects and complications. To quote Banting from his Nobel lecture: “Insulin is not a cure for diabetes; it is a treatment.” [147].
1.2.2 Diagnosis and symptoms

The 2018 clinical practice guidelines of Diabetes Canada recommend a diagnosis of diabetes if any of four criteria exceeds a threshold, which is ≥11.1 mM for random glycemia, ≥7.0 mM for fasting glycemia, ≥11.1 mM for two-hour glycemia during 75 g OGTT, or ≥6.5 % for HbA1c[148]. As diabetes is a disease of impaired glucose uptake, indicative symptoms include increased thirst (polydipsia), polyuria with glycosuria, increased hunger (polyphagia) and weight loss, which may be accompanied by fatigue, blurred vision, recurring infections, and decreased wound healing [117; 118; 149]. Poorly controlled chronic hyperglycemia results in an overall reduced life-span, due to cardiovascular complications, which accounts for the main cause of death, as well as damage to the kidneys (nephropathy), eyes (retinopathy), nerves (neuropathy), and limbs (amputations) [117; 118; 149].

1.2.3 Etiology and pathophysiology

Historically, two main types of diabetes are distinguished [150], defined on the basis of symptoms and the underlying genetic and environmental cause. Type 1 diabetes is a predominantly autoimmune disorder in which T cells attack β-cells, resulting in absolute insulin deficiency due to β-cell dysfunction and destruction [151-153]. It is estimated that T1D makes up 7-12 % of all cases of diabetes in high income countries [149]. A variety of genetic risk factors and environmental triggers have been identified, including HLA polymorphisms, viral infection, and diet [154]; however, the exact mechanism precipitating T1D in each case remains a matter of debate, making disease prediction challenging.

The vast majority of all diabetes cases (estimated at 87-91 %) are classified as type 2 diabetes (T2D), which is characterized by relative insulin deficiency [149]. T2D pathophysiology is multifaceted; there is no singular cause of T2D, but studies suggest a considerable genetic component in combination with environmental stresses [155-157]. Although not all of those at risk
or with T2D are obese or insulin-resistant [158; 159], the overall model is that a metabolically challenging environment (excess nutrients; sedentary lifestyle; age) causes a combination of β-cell dysfunction and insulin resistance in genetically predisposed individuals [118; 155; 160; 161]; a process involving all glucoregulatory tissues [162]. Overnutrition and obesity increase insulin demand and secretion, precipitating or hastening β-cell dysfunction [163-167]. At the same time, obesity is a major determinant for insulin resistance [168], due to defects in liver (increased hepatic glucose production [169]), muscle (decreased muscle glucose uptake [170; 171]), adipose tissue (increased free fatty acid release [172; 173], perturbed adipokine secretion [174-177], inflammation [178-180]), α-cells (increased glucagon secretion), kidney (increased glucose re-uptake), intestines (perturbed incretin secretion), and the brain (increased appetite) [162].

Initially, glucose homeostasis is maintained and increased insulin demand met by an expansion of β-cell mass [181-185] and augmented insulin output [186-190]. If metabolic challenges persist, insulin resistance and β-cell dysfunction worsen, eventually progressing to β-cell failure and T2D with overt hyperglycemia (impaired fasting glycemia and glucose intolerance) [153; 155]. β-cell failure is characterized by a 50-97 % decrease in insulin secretion [191-194] (diminished first- and second-phase insulin secretion [108; 195]) and a 24-65 % reduction in β-cell mass, likely due to apoptosis [181; 196-199]. It is caused by a combination of endoplasmatic reticulum (ER) stress, glucomitotoxicity, oxidative stress, excitotoxicity (excessive calcium influx), amyloid deposits, O-glycosylation, inflammation, disruption of β-cell connectivity, and dedifferentiation [167; 200-202]. Altogether, T2D presents as a heterogenous disease [162; 168], warranting further research into the molecular origins of β-cell failure (such as calcium-dependent factors, see section 1.3).

Gestational diabetes is a special form of diabetes resembling T2D wherein β-cells are unable to compensate for increased insulin demand during pregnancy [203]. The disease is transitional and recedes in most cases, but 40 % of affected women are at risk of developing T2D later on [204].
Lastly, some rare forms of diabetes are caused by monogenic mutations, such as maturity-onset diabetes of the young or neonatal diabetes [205-207]. Notably, a majority of mutations causing monogenic diabetes affects transcription factors (HNF1α, HNF1β, HNF4α, KLF11, NEUROD1, PAX4, PDX1) or factors involved in insulin exocytosis (GCK, ABCC8, KCNJ11, INS) [205-207], highlighting the importance of transcriptional regulation and stimulus-secretion coupling in maintaining glucose homeostasis.

1.2.4 Treatments

The principal treatment option for T1D is the use of exogenous insulin [146; 208] and, in severe cases, islet transplantation [209; 210]; whereas additional and combinatorial approaches are increasingly explored [208; 211]. Treatment options for T2D are as varied as disease pathophysiology, including weight loss and exercise [212], bariatric surgery [213; 214], insulin secretagogues (sulfonylureas [215], incretin analogues and dipeptidyl peptidase IV inhibitors [216-218]), insulin sensitizers (biguanides [219; 220], thiazolidinediones [221; 222]), glycosurics (SGLT2 inhibitors [223]), insulin [224], or a combination of the aforementioned [118; 225]. Each of these carries advantages and disadvantages which need to be weighed carefully. For example, exogenous insulin or oral glucose-lowering agents can cause hypoglycemia, which may manifest in the form of increased perspiration, paleness (pallor), rapid breathing (tachypnea), behavioural changes, and, if not treated promptly, hypoglycemic coma and death [118]. Use of insulin secretagogues is also associated with hastening β-cell exhaustion [226; 227].

As of 2017, the prevalence of diabetes kept on rising - there were an estimated 424.9 million people worldwide affected by a form of diabetes, with numbers projected to increase to 628.6 million by 2045 [149]. Therefore, continued research into the underlying biology of pancreatic β-cell function is needed to find alternative treatment options. Depending on the type of diabetes, these may be administered in combination with, or in place of, insulin, with the
ultimate goal of preserving or restoring β-cell function, β-cell mass, or insulin sensitivity in peripheral tissues.

1.3 Calcium-dependent signalling in β-cells

The divalent cation calcium acts as a potent signalling molecule in most cell types, including the insulin-producing pancreatic β-cell [1]. Extracellular calcium influx and other signals activate a host of signalling pathways, mobilize additional calcium from intracellular stores, and induce gene expression changes [228]. Calcium and its downstream effectors, mainly kinases and transcription factors, therefore play a pivotal role in β-cells, with considerable leverage over β-cell function, survival, proliferation, and fate [1]. This means that calcium levels must be tightly regulated to diminish potentially damaging effects, such as inadequately high insulin release. In this context, a growing body of literature demonstrates that perturbances underlie many of the defects seen in diabetes [1].

1.3.1 Intracellular calcium handling

Ca\(^{2+}\)\(_{\text{cyt}}\) levels in unstimulated β-cells are around 100 nM [229], as opposed to the millimolar range in the extracellular fluid [230]. Glucose-stimulated membrane depolarization triggers the opening of L-VDCCs, and extracellular calcium influx raises Ca\(^{2+}\)\(_{\text{cyt}}\) to 300-1000 nM [229-231]; although there are concentration gradients and differences between organelles [230; 232-234]. In addition to this extracellular source, β-cells contain a range of membrane-bound intracellular calcium stores, namely the endoplasmatic reticulum, Golgi, vesicles, mitochondria, and the nucleus [235; 236], which can be mobilized upon stimulation and contribute to insulin exocytosis and other signalling events. Calcium release from these stores can be triggered by external signals, such as hormones (insulin [237; 238]; incretins [239-243]) or nutrients (FFAs [244]), and their downstream second messengers and metabolites, including inositol 1,4,5-trisphosphate (IP\(_3\)) [245], long chain acyl CoA [246], various adenine nucleotides [243; 247; 248], and calcium [249; 250] itself. Based on their
sensitivity to these downstream effectors, several intracellular calcium stores can be distinguished (Figure 1.2).

Figure 1.2. Integrated view of insulin secretion and calcium handling in β-cells.

(A-F) Glucose is taken up by β-cells through SLC2A1/2 (A), and complete oxidation through glycolysis (B) and OXPHOS (C) generates ATP; other substrates for ATP generation include free fatty acids (FFA) or amino acids (AA). The increase in the ATP:ADP ratio leads to K\textsubscript{ATP} channel closure and plasma membrane depolarization (D), triggering calcium influx through L-VDCCs (E) and insulin exocytosis (F). (G) Insulin secretion is modulated via G-protein coupled receptors (GPCRs). When ligand binds its cognate GPCR,
Ga exchanges GDP for GTP, leading to the dissociation of Ga and Gβγ subunits and downstream signalling. (H) Regulator of G-protein signalling (RGS) proteins inactivate GPCR signalling by promoting Ga-intrinsic GTP hydrolysis. (I) Gaα-coupled receptors (e.g. glucagon-like peptide 1 receptor, GLP1R) activate adenylyl cyclases (ACs), which convert ATP into cAMP and thus activate protein kinase A (PKA). PKA promotes insulin release through phosphorylation of members of the exocytotic machinery and L-VDCC. (J) Gaα-coupled receptors (e.g. somatostatin receptor 1, SSTR1) reduce insulin release by inhibiting ACs. (K) Gaαq-coupled receptors (e.g. free fatty acid receptor 1, FFAR1; muscarinic acetylcholine receptor M3, M3R; purinergic receptor P2Y1, P2Y1R) activate phospholipase C (PLC), which converts phosphatidylinositol 4,5-bisphosphate (PIP2) into inositol 1,4,5-trisphosphate (IP3) and diacylglycerol (DAG). Alternatively, PLC is activated by Ca2+cyt. IP3 then binds the inositol 1,4,5-trisphosphate receptor (IP3R) to induce calcium release from intracellular stores, whereas DAG-dependent PKC activation directly promotes exocytosis. (L&M) In addition, Ca2+cyt is sensed by calmodulin (CaM) (L). CaM and its targets, such as CaMKII, enhance insulin secretion through direct effects on the exocytotic machinery (F), or induce calcium signalling pathways (e.g. CaMK, CaN) and gene expression changes through downstream transcription factors (TFs) (M). (N) Ca2+cyt- and CaM-dependent processes trigger calcium-induced calcium release (CICR) from intracellular stores through ryanodine receptors (RyRs) and IP3R. (O) Following ER calcium release, for example through CICR or metabolic coupling factors such as ROS, microdomains between the ER and mitochondria (mitochondria-associated membranes, MAMs) facilitate ER-mitochondrial calcium transfer. Mitochondrial calcium uptake through voltage-dependent anion channels (VDACs) and the mitochondrial calcium uniporter (MCU) promotes ATP generation. (P) Ca2+cyt is cleared from the cytosol through re-uptake into intracellular stores via sarco/endoplasmic reticulum Ca2+ ATPase (SERCA). (Q&R) Calcium is exported from the cell via plasma membrane Ca2+ ATPase (PMCA) (Q) and Na+/Ca2+ exchanger (NCX) (R). Mitochondrial calcium export is mediated by the mitochondrial Na+/Ca2+ exchanger (NCLX) (R). Modified from Sabatini et al. (2018) [1].

The IP3-sensitive pool is accessed downstream of phospholipase C (PLC). Gaαq-coupled GPCRs, such as muscarinic, purinergic, or FFA receptors, activate PLC [251]. PLC can also be activated GPCR-independently by high intracellular calcium levels [252; 253]. PLC then breaks down the phospholipid phosphatidylinositol 4,5-bisphosphate (PIP2) into DAG and IP3, both of which have signalling functions. The IP3 receptor (IP3R) is a calcium channel expressed on the ER [254], insulin granules, and Golgi [255; 256], which upon IP3 binding releases calcium into the cytosol (Figure 1.2).

The ryanodine-sensitive pool [257] refers to the high affinity of ryanodine receptors (RyRs) to the toxic diterpenoid ryanodine. Physiological RyR ligands include long chain acyl CoA, ATP, cAMP and cyclic ADP ribose (cADPR) [247; 258]. RyRs are expressed at low levels in human islets [259], and proposed to localize to the ER [260], insulin granules [261], endosomes [257] and the plasma membrane [262]. RyR channels mediate calcium-induced calcium release (CICR) [261], a mechanism by which calcium release from intracellular stores, such as the ER, occurs
downstream of calcium influx through L-VDCCs and glucagon-like peptide 1 (GLP1) -PKA signalling [240; 249; 263]. IP3Rs contribute to this process [250]. In β-cells, CICR can be triggered by glucose-dependent activation of CaMKII, which phosphorylates and thus activates RyR2 [264] (Figure 1.2).

Mitochondria are a notable site for calcium handling [265]. The principal roles of mitochondrial calcium are the regulation of cellular energy production, buffering Ca\textsuperscript{2+}\textsubscript{cyt}, and regulation of apoptosis [266]. Higher matrix calcium levels enhance the activity of some mitochondrial enzymes, such as α-ketoglutarate dehydrogenase and isocitrate dehydrogenase, thus increasing the availability of metabolic substrates and optimizing ATP production [265; 267-269]. Transport of calcium across the double-membrane into the mitochondrial matrix is achieved through voltage-dependent anion channels (VDACs) [270] in the outer mitochondrial membrane and the mitochondrial calcium uniporter (MCU) complex [271; 272] in the inner mitochondrial membrane [266; 273; 274]. Notably, mitochondrial calcium entry from the cytosol is limited by the low affinity of the MCU, but following activity-induced IP3R- [276] or RyR-mediated [247; 277] ER calcium release, ER-mitochondrial microdomains (mitochondria-associated membranes; MAMs) facilitate the transport of large quantities of calcium into mitochondria [266; 267; 273; 274]. Thus, the MCU is not very calcium-conducive under basal conditions, but facilitates mitochondrial calcium influx and ATP production during metabolic demand, when cytosolic or MAM calcium levels are high [266]. Toxic effects of calcium overload, such as excessive reactive oxygen species (ROS) production, can be prevented by calcium extrusion from the mitochondria via the mitochondrial Na\textsuperscript{+}/Ca\textsuperscript{2+} exchanger (NCLX) [278]. Together, these studies illustrate the importance of MAMs and mitochondrial calcium homeostasis for β-cell stimulus-secretion coupling [234; 274; 279; 280] (Figure 1.2).
Calcium is cleared from the cytosol either through re-uptake into intracellular stores, such as into the ER via sarco/endoplasmic reticulum Ca\textsuperscript{2+} ATPase (SERCA), or exported from the cell via the actions of plasma membrane Ca\textsuperscript{2+} ATPase (PMCA) and Na\textsuperscript{+}/Ca\textsuperscript{2+} exchanger (NCX) \[281\] (Figure 1.2). Notably, insulin receptor signalling in β-cells from isolated islets increases their Ca\textsuperscript{2+}_{cyt} levels in an autocrine-paracrine positive feedback loop via inhibition of SERCA \[282\], representing another layer of control over calcium signalling.

### 1.3.2 Calcium-dependent signalling and transcription factors

Aside from directly triggering exocytosis and enhancing mitochondrial metabolism, calcium influx induces a variety of signalling pathways. For some calcium-binding proteins, it is a direct effector molecule. This applies to some protein kinase C (PKC) isoforms, which are Ser/Thr kinases activated upon binding of calcium and DAG \[283; 284\]. Among other signalling functions, PKC activity promotes insulin secretion \[285-287\] through phosphorylation of SM/SNARE proteins \[288\], transcriptional changes \[289\], or insulin granule recruitment \[290\] (Figure 1.2).

Other calcium binding proteins essentially act as intracellular calcium receptors, translating intracellular calcium levels into biological activity via their downstream targets. The EF-hand family protein calmodulin (CaM) falls into this category, and is a prominent calcium sensor in β-cells \[291\]. Calcium-bound CaM undergoes conformational changes, enabling it to bind and modulate target activity \[292; 293\]. Currently, over 120 CaM-interacting proteins are known \[294; 295\], and in β-cells these include the Ca\textsuperscript{2+}/CaM-dependent kinases (CaMKs), the phosphatase calcineurin (CaN), the calcium channels L-VDCC, IP3R and RyR, and the PMCA \[1; 295; 296\]. Since CaM is such a widely used calcium sensor, a large range of cellular outcomes can be encoded through binding and modulation of diverse targets, affecting nearly every aspect of β-cell biology either directly or indirectly \[1\] (Figure 1.3).
Figure 1.3. Calcium-induced signalling pathways and transcription factors in β-cells.

(A) Low energy status, such as during fasting, raises the ratio of adenosine monophosphate (AMP) and ADP over ATP, which activates serine/threonine kinase 11 (STK11) and AMP-activated protein kinase (AMPK). AMPK reduces forkhead box protein O1 (FOXO1) expression, and modulates translation via
inhibition of the mammalian target of rapamycin (MTOR) pathway. (B) β-cell activity induces calcium influx through L-VDCCs. Rising Ca\(^{2+}\) levels are sensed by CaM. (C) CaM activates the phosphatase CaN, which dephosphorylates nuclear factor of activated T cells (NFAT) or myocyte enhancer factor-2 (MEF2). NFAT subsequently translocates to the nucleus, and MEF2 transcriptional activity is de-repressed due to displacement of histone deacetylases (HDAC). Re-phosphorylation by glycogen synthase kinase 3 beta (GSK3β) and dual specificity tyrosine-phosphorylation-regulated kinase 1A (DYRKIA) excludes NFAT from the nucleus. (D) CaM-dependent kinase kinase (CaMKK) phosphorylates downstream CaMKIV (or CaMKI), although CaM also directly activates CaMKIV. CaMKIV promotes transcription through inhibitory phosphorylation of HDAC, or phosphorylation of cAMP response element binding protein (CREB). CaMKK also activates AMPK. (E) CaM-stimulated CaMKII perpetuates its own activity via autophosphorylation, promotes insulin secretion via L-VDCC activation and insulin granule recruitment, or dampens CREB activity. (F) Activation of growth factor receptors (GFR) and CaM stimulates Ras protein-specific guanine nucleotide-releasing factors (RASGRF). RASGRF stimulates GDP to GTP exchange at Rat sarcoma (RAS) GTPases; signals from the insulin receptor (InsR) co-activate RAS. RAS then activates rapidly accelerated fibrosarcoma (RAF) proteins, aided by CaN relieving inhibitory feedback phosphorylation from MAPK3/1. RAF initiates the MAPK cascade through MAP2K1/2, MAPK3/1, and ribosomal protein S6 kinase A1/3 (RPS6KA1/3), culminating in the phosphorylation of CREB. (G) Activation of the InsR stimulates phosphoinositide 3-kinase (PI3K), which in turn triggers a downstream kinase cascade through 3-phosphoinositide-dependent protein kinase-1 (PDPK1), protein kinase B (PKB), MTOR, and ribosomal protein S6 kinase beta-1 (P70S6K), regulating translation and nuclear exclusion of FOXO1. (H) Ga\(_{i}^{-}\)- or Ga\(_{q}^{-}\)-stimulated PKA or PKC phosphorylate CREB, respectively. (I) Dephosphorylation of CREB regulated transcription coactivator 2 (CRTC2) by CaN induces nuclear translocation, promoting CREB-mediated transcription. Inhibitory phosphorylation by microtubule affinity regulating kinase 2 (MARK2) and salt inducible kinase 2 (SIK2) causes nuclear export of CRTC2. Modified from Sabatini et al. (2019) [1]. Dashed lines indicate multiple step events.

The CaMK family of Ser/Thr protein kinases is activated upon Ca\(^{2+}\)/CaM binding and subsequently phosphorylates numerous substrates (Figure 1.3). Pancreatic β-cells express most CaMK [297] family members, including CaMKK1 [298], CaMKK2 [299], traces of CaMKI isoforms (α, γ, δ) [296; 300; 301], all CaMKII isoforms (α, β, γ, δ) [302; 303] and CaMKIV [298]. CaMKKs phosphorylate and enhance the activities of CaMKI, CaMKIV [298; 304; 305], AMP-activated protein kinase (AMPK) [305-307], protein kinase B (PKB; also known as AKT) [308] and, likely indirectly through CaMKIV, mitogen-activated protein kinases (MAPks) [309] (Figure 1.3). Thus, CaMKKs are intersecting with kinase pathways not directly or primarily regulated by calcium. CaMKII isoforms are not activated by CaMKK, but can autophosphorylate themselves [310], which maintains them in an activated state even after calcium levels fall [303]. This effect was suggested to be part of forming a “metabolic memory” in β-cells [311].

Nuclear calcium spikes [233; 312; 313] and CaM-CaMK signalling cascades often converge on the activation of transcription factors, thereby transmitting β-cell activity-induced changes in
calcium levels into a transcriptional response (Figure 1.3). One such transcription factor family are the cAMP response element-binding proteins (CREB), which bind DNA as homodimers, recognizing the cAMP response element (CRE; 5'-'TGACGTCA-3') \(^{[314]}\). Under non-stimulatory conditions, when Ca\(^{2+}\)\(_{cyt}\) is low, unphosphorylated CREB is nuclear and bound to CREs \(^{[315]}\), but not transcriptionally active. Glucose- and growth factor-stimulated calcium signalling events activate CaMKIV, PKA, PKB, PKC, and MAPKs, all of which phosphorylate CREB at Ser133, activating it \(^{[314; 316-323]}\). CaMKII, despite phosphorylating CREB at Ser133, also phosphorylates an inhibitory residue at Ser142, which dampens CREB activity \(^{[324]}\). Ser133 phosphorylation attracts interaction of CREB with its co-factors CREB regulated transcription coactivator 2 (CRTC2) and CREB binding protein (CBP), which enhance CREB transcriptional activity \(^{[318]}\).

The Ca\(^{2+}/CaM\)-activated phosphatase CaN positively regulates CRTC2 nuclear translocation \(^{[325]}\), whereas microtubule affinity regulating kinase 2 (MARK2) \(^{[326]}\) and salt inducible kinase 2 (SIK2) \(^{[325]}\) cause nuclear export, further fine-tuning CREB transcriptional activity (Figure 1.3).

Aside from kinases, CaM binding activates the phosphatase CaN (Figure 1.3) \(^{[327]}\). Among the CaN substrates, nuclear factor of activated T cells (NFAT) family members reside in the cytoplasm in their phosphorylated state \(^{[328-330]}\). Upon dephosphorylation by CaN, NFAT translocates into the nucleus and binds DNA as a monomer at its consensus motif (5'-WGGAAA-3') \(^{[329-332]}\), although cooperative DNA binding of NFAT with other transcription factors (AP-1 \(^{[333; 334]}\), MEF2 \(^{[335]}\), MAF \(^{[336; 337]}\)) is also well established \(^{[338; 339]}\). Re-phosphorylation of NFAT by kinases DYRK1A and GSK3β causes nuclear export and terminates transcription \(^{[340]}\).

β-cells express most MAPKs \(^{[300; 341]}\), some of which are in part modulated by a rise in intracellular calcium levels (Figure 1.3). Following calcium influx, Ca\(^{2+}/CaM\) contributes to the activation of guanine nucleotide exchange factor (GEF) RASGRF1 \(^{[342]}\), which in turn activates
RAS family GTPases[^343;344]. These RAS proteins then activate RAF kinases, such as the MAP3K BRAF, aided by CaN relieving inhibitory feedback phosphorylation of BRAF by MAPK3/1 (also known as ERK1/2) [337; 341; 345-347]. The subsequent MAPK cascade sees successive phosphorylation of MAP2K1/2 (also known as MEK1/2), MAPK3/1, and ribosomal protein S6 kinase A1/3 (RPS6KA1/3; also known as RSK1/2) [341; 346; 348]. RPS6KA1/3 translocate to the nucleus where they promote transcription by phosphorylating and activating CREB [321; 349-351]. MAPK3/1 activity is further enhanced by calcium-dependent phosphorylation, which relies on L-VDCCs, CaN, and Gs-stimulated PKA activity downstream of glucose and GLP1/gastric inhibitory polypeptide (GIP) [341; 352].

Several other kinases and transcription factors are affected by aforementioned signalling events. CaMK and CaN signalling converge on the activation of myocyte enhancer factor-2 (MEF2) family members (Figure 1.3). MEF2 has been implicated in neuronal synapse development, potentially via upregulation of Npas4 expression [353; 354]. Under basal conditions, MEF2 resides in the nucleus and is bound to DNA (consensus motif: 5'-TGTTACTWWAAATAGAW-3' [355]), acting as a transcriptional repressor due to association with histone deacetylases (HDACs) [356; 357]. Activity-induced calcium influx de-represses MEF2 transcriptional activity via 1) CaM binding disrupting MEF2:HDAC complexes [358] or inhibitory phosphorylation of HDACs by CaMKIV [359; 360], 2) phosphorylation of MEF2 by MAPKs [361-363], and 3) dephosphorylation of MEF2 by CaN [357; 364; 365].

Lastly, activation of the insulin receptor tyrosine kinase stimulates phosphoinositide 3-kinase (PI3K), leading to downstream activation of protein kinase B (PKB) and mammalian target of rapamycin (MTOR) [366]. Activation of this pathway causes nuclear exclusion of forkhead box protein O1 (FOXO1), and promotes translation, insulin secretion and β-cell survival [367; 368]. The
role of AMPK in β-cells is less well established \[369\], but it may intersect with these pathways by inhibiting MTOR activation \[370\], and repressing FOXO1 expression or activity \[371\] (Figure 1.3).

As evident by this extensive list of calcium-dependent signalling pathways (Figure 1.3), calcium acts as a universal second messenger to induce transcriptional changes and modulate myriad cellular processes. The importance of many of these signalling pathways for β-cell function and survival has been established, and will be reviewed in the following sections.

1.3.3 Calcium signalling stimulates insulin production

There are different strategies for β-cells to cope with metabolic challenges. Apart from β-cell mass expansion, increased insulin content and β-cell function are observed in non-diabetic obesity and the early, compensatory stage of T2D \[153; 372; 373\]. Furthermore, elevated blood glucose levels are a major stimulus for insulin secretion; and while β-cells release only 2-20 granules per minute during peak first-phase insulin secretion \[77; 97; 374\], corresponding to 0.0002-0.002% of their roughly 10,000 granules \[97; 375; 376\], insulin content eventually needs to be replenished. As such, adaptations have evolved to link elevated glucose levels to enhanced insulin transcription and translation \[377-380\]. Because higher glucose levels and insulin demand coincide with increased intracellular calcium levels, calcium is an ideal second messenger for this task. Indeed, when calcium influx from the extracellular milieu through L-VDCC is blocked, glucose-stimulated insulin production is diminished \[381\].

An important role in the adaptation of insulin expression to metabolic stimuli befits the CaMK and CaN pathways. In mouse islets, NFAT activity downstream of CaN is sufficient to increase expression of the \textit{Ins1} and \textit{Ins2} genes, as was shown in an overexpression model of constitutively active NFATC2 \[382\]. Moreover, glucose stimulation increases NFATC2 binding to the insulin promoter \[383\], while CaN inhibition diminishes promoter activity \[384\], supporting a role for the CaN-NFAT axis in insulin transcription. The involvement of CaMK signalling in
insulin transcription has been demonstrated in INS-1 cells overexpressing either CaMKIV with defective kinase function, which reduced the amount of insulin message, or constitutively active CaMKIV, which enhanced insulin transcription \[298\]. Notably, glucose exposure is also associated with increased insulin mRNA half-life \[385\] and translation \[386\], although the significance of calcium signalling in both processes needs to be established.

### 1.3.4 Calcium signalling stimulates insulin secretion

While there is some evidence to the contrary \[387; 388\], enhanced β-cell function, evident as increased intracellular calcium levels and improved insulin secretion, is an important β-cell adaptation to short-term high glucose exposure \[389\]. In addition to direct calcium sensing by the exocytic machinery, calcium signalling events take on a vital role during insulin secretion. This is best illustrated in models of altered calcium signalling \[390-393\], which impair insulin secretion and systemic glucose homeostasis.

Transgenic mice expressing inactive CaM become hyperglycemic due to low serum insulin, as glucose- and KCl-stimulated insulin release from their islets is reduced \[390\]. Although insulin content is also diminished, the muscarinic receptor agonist carbachol, which mobilizes intracellular calcium stores, could restore insulin secretion to normal \[390\]; indicating that the defect in insulin secretion may originate from the L-VDCC. Interestingly, transgenic mice with overexpression of CaM also became hyperglycemic and abolished GSIS, due to a defect in glucose metabolism \[394; 395\]. These seemingly disparate results, while implicating disturbances in intracellular calcium signalling in β-cell dysfunction, specify the need to differentiate between the individual contributions of CaM targets.

The CaM-dependent CaN phosphatase pathway is of clinical relevance, as immunosuppressive drugs tacrolimus (FK-506) and cyclosporin A, CaN inhibitors, acutely reduce glucose- and KCl-stimulated insulin exocytosis in human islets \[396; 397\]. In keeping with these observations,
overexpression of constitutively active CaN substrates, NFATC1 and NFATC2, enhanced glucose- and KCl-stimulated insulin exocytosis in mouse islets without any changes to insulin content [398]. These studies implicate CaN-NFAT signalling in promoting insulin release. However, future studies need to address whether these are direct effects on exocytosis, or related to independent roles of CaN-NFAT in β-cell replication [382; 398] and survival [397; 399].

Another signalling cascade important for promoting insulin secretion is the CaMK pathway, and CaMKII in particular [303]. Overexpression of a CaMKII inhibitory peptide impairs L-VDCC calcium conductance, and thus reduces both glucose- and KCl-stimulated calcium influx [391]. In consequence, mice are more susceptible to high fat diet (HFD) -induced glucose intolerance [391]. The proposed mechanism was enhanced L-VDCC calcium conductance following phosphorylation by CaMKII. CaMKII also stimulates insulin secretion through insulin granule recruitment and promoting exocytosis [400]. Another mechanism by which CaMKII promotes insulin secretion may be through the formation of what is called “metabolic memory” [311]. Upon CaM binding and CaMKII autophosphorylation, activated CaMKII can retain CaM binding and kinase activity even after the initial stimulus is removed. This formed the basis for experiments of exposing human islets to repeated high glucose stimuli, interspersed by low glucose recovery periods [311]. Glucose pulse-treated islets showed increased insulin secretion following a subsequent glucose stimulus when compared to islets cultured in low glucose [311]. As predicted, pulse-treatment correlates with higher levels of phosphorylated CaMKII in conjunction with increases in GCK, L-VDCC subunit α1C (CACNA1C), synaptosomal-associated protein 25 (SNAP25), and MAFA expression, which are major proteins involved in stimulus-secretion coupling and exocytosis [311]. Co-culture with KN-93, a CaMK inhibitor, abolished expression changes and the increased insulin secretion induced by glucose pulse treatment, suggesting the effects were due to CaMKII [311]. Moreover, CaMKII phosphorylates SYN1 [401; 402] and RyR2 [264] in response to glucose stimulation. Since SYN1 is involved in insulin granule docking, and RyR
in intracellular calcium mobilization, this is another potential mechanism by which CaMKII enhances insulin exocytosis. Studying the transcription factors downstream of CaMKII, female Creb1 knockout (KO) mice became mildly glucose intolerant on HFD due to reduced GLP1-stimulated insulin secretion. Knockout of its co-activator Crtc2 reduced cAMP-dependent insulin secretion, due to a loss of MafA expression. Together, these studies provide further support for a role of CaMKII in insulin secretion.

Finally, calcium signalling also affects β-cell mitochondrial activity through “calcium-metabolic coupling”. Since insulin production, secretion and pumping of ions are energetically demanding processes, mitochondrial activity needs to be optimized to generate adequate levels of ATP. This is achieved via a process involving L-VDCC-dependent calcium influx and CaMKII activity, and directly couples oxygen consumption to insulin exocytosis.

1.3.5 The role of calcium in β-cell survival and T2D

Not only does calcium promote β-cell function, but also β-cell survival. Defects in calcium handling occur when levels of free fatty acids (FFAs) within β-cells increase, which gradually depletes ER calcium, cause ER stress, apoptosis, and diabetes. In contrast, lower apoptosis during short-term glucose culture depends on depolarization-induced calcium influx through L-VDCCs, since both the KATP channel opener diazoxide as well as the L-VDCC blocker nifedipine prevented the cytoprotective effect of high glucose culture. Both CaN and CaMKs mediate the effects on β-cell survival, as outlined below.

CaN inhibition with FK-506 induces β-cell apoptosis, measured as cleaved caspase 3 expression, and leads to hyperglycemia due to graft failure of human islets transplanted into diabetic mice. Increased apoptosis is reflected in human biopsies from transplant recipients who underwent CaN inhibitor treatment, and the same immunosuppressive regimen is associated with a risk of developing impaired glucose homeostasis and diabetes.
Moreover, decreased β-cell survival following FK-506 treatment was likely due to decreased NFAT binding at the Irs2 promoter, as reduced Irs2 expression decreases pro-survival insulin signalling through PI3K/PKB \(^{422}\).

Reduced apoptosis in MIN6 cells cultured in high glucose depends on CaMKIV-mediated activation of CREB, because constitutively active CaMKIV mimicked the effects of high glucose under low glucose conditions, whereas dominant-negative CREB blocked such pro-survival effects \(^{423}\). In agreement, loss of CREB transcriptional activity impaired β-cell survival and resulted in diabetes due to β-cell apoptosis \(^{316; 424}\). This loss correlated with stark reductions in IRS2 and phospho-PKB, important pro-survival proteins in the insulin signalling pathway \(^{316}\).

Together, these studies demonstrate the direct role of calcium, CaN-NFAT and CaMKIV-CREB in β-cell survival. As discussed earlier, calcium signalling is also beneficial to human and mouse β-cell function (section 1.3) \(^{389}\). Perturbed β-cell function or viability are part of the pleiotropic defects seen in T2D; yet, evidence of potentially pathological T2D risk variants involved in calcium signalling is only just emerging in recent genome-wide association studies (GWAS) \(^{156; 425}\). Several genes encoding kinases involved in calcium signalling, including CDC123/CAMK1D \(^{426}\), CAMKK2 \(^{427}\), and genes associated with MAPK signalling (RASGRP1 \(^{428}\), MAP3K11 \(^{429}\), MAP3KI \(^{430}\)), carry variants increasing the risk of developing T2D. Notably, variant rs11257655 at the CDC123/CAMK1D locus is a likely causal risk variant \(^{425}\) and is associated with lower GSIS in humans \(^{431; 432}\), but the exact mechanism of this defect is not known. However, regulatory regions in 40 % of all T2D susceptibility loci show linkage to the CaN substrate NFATC2 \(^{398}\). Expression of these risk genes is changed when NFATC2 or NFATC1 are overexpressed in mouse or human islets \(^{398}\). Lastly, β-cell MAMs are reduced in patients with T2D \(^{433}\), which likely impairs mitochondrial calcium homeostasis and
β-cell metabolism. Together, these studies support the argument that altered calcium influx and signalling are risk factors for T2D development.

The associations made in many of the above studies are correlative, and as such need experimental validation, for example in mouse models. To closely mirror T2D pathophysiology, these models have to replicate the harmful effects of chronic hyperglycemia, such as reduced insulin expression, secretion and apoptosis. Indeed, transgenic mice with β-cell specific overexpression of the chicken Calm1 gene, Camk2a or constitutively active CaN (Ppp3ca subunit), all became hyperglycemic due to progressive β-cell apoptosis. In human T2D patients, it was shown that these negative effects could be rescued by promoting a reprieve from chronically elevated calcium. Promoting “β-cell rest” in the diabetic milieu is beneficial in the short term, as several days of treatment with the K<sub>ATP</sub> channel opener diazoxide, to prevent β-cell depolarization and calcium influx, improved insulin secretion in patients.

Conversely, while treatment of T2D patients with K<sub>ATP</sub> channel-blocking sulfonylureas as insulin secretagogues leads to initial reductions in HbA<sub>1c</sub> level, glycemic control eventually worsens and β-cell function declines during long-term use; supporting a beneficial role for reducing β-cell workload.

In conclusion, calcium influx and signalling pathways are important for β-cell maintenance, survival, and function. In the early stages of hyperglycemic challenge, elevated calcium provides a beneficial adaptation supporting the aforementioned processes. During this period, loss of calcium signalling elements such as CaN, CamKII or CREB has deleterious effects. However, prolonged hyperglycemia and ensuing chronic elevation in intracellular calcium during T2D promote β-cell stress, apoptosis and loss of function and maturity. This suggests that a balance needs to be struck between stimulating and limiting β-cell activity and calcium signalling during normal and pathological conditions to maintain β-cell viability and
function \[^{227}\]. A recurring theme in the preceding sections was the induction of transcriptional changes through calcium-dependent signalling cascades via activation of transcription factors. This process allows β-cell adaptation in a changing environment and provides feedback to a wide variety of cellular events. To better understand pathological perturbances of calcium signalling during T2D, it will be informative to study transcription factors which show calcium-dependent activity.

1.4 bHLH-PAS domain transcription factors

Transcription factors are proteins that bind to a specific DNA sequence, thus regulating gene expression \[^{443}\]. All non-ribosomal, protein-coding genes are transcribed by RNA polymerase II, which, together with at least six general transcription factors and the Mediator complex, assembles as preinitiation complex (PIC) at the transcriptional start site (TSS) of a gene’s core promoter \[^{444; 445}\]. Specific transcription factors recognize and bind defined DNA sequences in enhancer or promoter regions, and regulate transcription of a gene through direct interactions with the PIC, or via recruitment of transcriptional co-regulators \[^{443; 444}\]. While some transcription factors are constitutively expressed and active, for example to maintain housekeeping gene expression, others are regulated more dynamically \[^{446; 447}\], and are therefore particularly suited for adaption to changes in cellular environment.

The bHLH (basic helix-loop-helix) superfamily of transcription factors have important functions in both organismal development and cellular function \[^{448}\], with a subclass of bHLH transcription factors containing a PAS (PER, ARNT, and SIM) domain \[^{449}\]. The role of these bHLH-PAS proteins is broadly defined as environmental sensors \[^{449}\] and, based on phylogenetic conservation of their PAS domains, several subgroups of bHLH-PAS transcription factors can be identified and biological function predicted \[^{449}\]. These include 1) the xenobiotic compound response factor Aryl hydrocarbon receptor (AHR) and its antagonist AHR repressor (AHRR); 2)
circadian rhythm proteins Circadian locomoter output cycles kaput (CLOCK) and Neuronal PAS domain protein 2 (NPAS2); 3) regulators of neuronal growth and function, NPAS1, 3 and 4, and Single-minded 1 and 2 (SIM1/2); 4) hypoxia response proteins Hypoxia inducible factor 1, 2 and 3 alpha (HIF1α, 2α, 3α); and 5) dimerization partners AHR nuclear translocator (ARNT), ARNT2, ARNT-like (ARNTL), and ARNTL2 [449].

These groups highlight the various aspects of cellular biology and response to environmental cues mediated by bHLH-PAS transcription factors. Given that the fundamental purpose of pancreatic β-cells is to properly regulate insulin secretion, perception and response to intra- and extracellular signals is of prime importance. This places bHLH-PAS domain proteins at the forefront of adaption to environmental stimuli in the β-cell.

1.4.1 Domain structure and classification

Generally, bHLH-PAS proteins contain three major domains which are essential to their function (Figure 1.4). At the N-terminal end, the bHLH domain contains a stretch of DNA-binding basic amino acids adjacent to a dimerization domain of two α-helices bridged by a loop [448]. Transcription factors with this domain recognize E-box-like motifs (5'-CANNTG-3') [450–454]. Adjacent to the bHLH, the eponymous PAS domain features two repeats of around 70 amino acids each, called PAS A and PAS B, which participate in homo- and heterotypic [450; 455; 456] protein-protein interactions (PAS A, PAS B) and binding of small molecules [457–459] (mainly PAS B) [449; 454].
Figure 1.4. Domain structure of bHLH-PAS transcription factors.

(A) The general structure of bHLH-PAS transcription factors consists of three main elements. The DNA-binding basic helix-loop-helix (bHLH; purple) domain, the Per-Arnt-Sim (PAS; blue) domain for transcription factor heterodimerization (PAS A, PAS B and PAC motif) or ligand binding (PAS B), and the transcriptional activation domain (TAD; green) for interaction with the transcription preinitiation complex (either directly or via co-regulators). NH$_2$ = amino-terminus, COOH = carboxy-terminus. (B-D) Examples of bHLH-PAS transcription factors; numbers indicate amino acid positions of the human protein. ARNT (B) is a Class II general partner factor with the potential to heterodimerize with Class I factors, such as HIF1α (C) or NPAS4 (D). HIF1α contains an N-terminal and a C-terminal TAD. Its oxygen-dependent degradation (ODD) domain is involved in protein stability. The exact amino acid positions of the NPAS4 TAD are unknown, but are located in the C-terminal region.

Although primary amino acid sequence conservation between PAS domains of different bHLH-PAS members are relatively low, the quaternary structure of bHLH-PAS heterodimers is mostly conserved. Two distinct architectural groups emerge based on whether complexes contain ARNT or ARNTL as a dimerization partner. This indicates that only a few key amino acids are needed to determine conserved folding and dimerization ability. The conserved heterodimer structure is probably functionally important, because bHLH-PAS transcription factors bind DNA as obligate heterodimers; without PAS-dependent interactions, their ability to regulate transcription is impaired. Furthermore, a C-terminal transcriptional activation domain (TAD) promotes transcriptional activation (Figure 1.4). This occurs directly through
binding of the PIC, or indirectly through recruitment of co-activators or co-repressors to act as a bridge between DNA-binding bHLH-PAS transcription factors and the PIC \cite{444,445}.

Based on their dimerization potential, mammalian bHLH-PAS transcription factors are classified as either belonging to Class I or Class II. Because bHLH-PAS proteins are obligate heterodimers, specific Class I factors must bind a Class II general partner factor \cite{449}. Of the Class II proteins, ARNTL and ARNTL2 dimerize with the circadian regulator Class I proteins, whereas ARNT (Figure 1.4) or ARNT2 act as general partner factors for all other Class I bHLH-PAS proteins \cite{449,462,463}. Although ARNT homodimers have been observed, their functional significance is unclear \cite{455,456}.

### 1.4.2 Expression and function of bHLH-PAS domain transcription factors in pancreatic islets and β-cells

Several bHLH-PAS proteins are expressed in pancreatic islets or insulinoma cell lines, including AHR, ARNT, ARNT2, ARNTL, CLOCK, HIF1α, HIF2α, and NPAS4 \cite{464}. Almost all of these proteins have a role in glucose homeostasis in animals in vivo, such as CLOCK and ARNTL \cite{465}. These two bHLH-PAS proteins are core components of the circadian clock \cite{466-468}, and their heterodimerization and transcriptional activity \cite{469} forms the basis for diurnal patterns in energy metabolism \cite{470}, glycemia \cite{471}, glucose tolerance \cite{472}, and insulin secretion \cite{473} among others \cite{474}. When Arntl is genetically ablated from β-cells using Pdx1-Cre \cite{475} or RIP-Cre \cite{476} drivers, impaired glucose tolerance ensues due to decreased insulin secretion \cite{475,476}. The metabolic phenotype of mice with a mutation in the Clock gene mirrors that of Arntl KO mice in terms of impaired glucose tolerance and reduced insulin secretion \cite{475,477}.

Ahr mRNA is detectable in MIN6 cells, mouse, and human islets, and siRNA-mediated knockdown in MIN6 cells results in decreased basal and stimulated insulin release \cite{478}. In addition, AHR is reduced in islets from T2D donors \cite{478}. Female germline Ahr KO mice
developed fasting hypoinsulinemia and insulin resistance dependent on age and pregnancy status [479]. However, pregnant animals did not exhibit hyperglycemia or glucose intolerance despite changes in insulin secretion and sensitivity. In the aged cohort, a small group (about 23%) of females developed severe glucose intolerance without signs of hyperglycemia or elevated levels of HbA1c [479]. Of note, immunoprecipitation (IP) of AHR in MIN6 precipitated ARNT from whole cell extracts, but not nuclear extracts, raising the question whether AHR:ARNT complexes are functionally active in β-cells [480].

HIF2α [296; 478] and ARNT2 [296; 481] are present in human β-cells, whereas HIF3α is not [296]. Their roles in β-cells remain unclear. HIF2α heterodimerizes with ARNT when its expression is stabilized by deferoxamine treatment [480], enhances hepatic insulin sensitivity [482], and is involved in embryonic pancreas development [483]. However, it may not be expressed in mature murine islets [483; 484], and Pdx1-Cre-driven overexpression in mouse β-cells failed to uncover any role for HIF2α in glucose homeostasis, islet mass, or insulin sensitivity regardless of diet, suggesting it is dispensable [485]. Although ARNT2 germline KO led to lethal defects in the formation of secretory neurons [486], a role in β-cells was not reported.

Of particular importance for this thesis are the general partner factor, ARNT; the master regulator of the hypoxic response, HIF1α; and NPAS4 (Figure 1.4). Hence, these will be examined in more detail in the following paragraphs.

1.4.3 Role of ARNT in the islet

ARNT is expressed constitutively in many tissues including the pancreas and β-cells [4], though ARNT message and protein expression increase in response to hypoxia in certain cell lines [487; 488] and human islets [480]. Because it acts as the main Class II partner factor for bHLH-PAS heterodimerization, animal studies have tried to address what role ARNT plays in β-cells. However, conflicting reports have highlighted some problems using KO models of Arnt.
Initially, studies using the Cre-lox system in mice uncovered a role for ARNT in β-cell insulin secretion [478]. Using the RIP-Cre model, β-Arnt KO mice developed impaired glucose tolerance due to defects in GSIS within the first 2-3 months of life. This correlated with the loss of mature β-cell markers, although insulin content was unchanged. Mechanistically, it is difficult to ascertain what drives this phenotype. Since ARNT dimerizes with most bHLH-PAS domain factors, any of them could be responsible for the effects. However, individual knockdown of either Hif1α, Hif2α, or Ahr in MIN6 cells did not fully recapitulate the effects [478], indicating that the effect in β-Arnt KO mice may be a synergistic effect of the loss of all factors. A drawback of this study is the use of the Magnusson lab RIP-Cre driver [489], as mice with this transgene develop glucose intolerance and impaired insulin secretion [490]. This was due to artificial expression of active human growth hormone, which negatively impacts β-cell function in transgenic mouse lines [491; 492]. Nonetheless, further evidence for a role of ARNT in β-cell function was uncovered in a transplant model. Arnt KO islets transplanted into diabetic recipient mice failed to return blood glucose control to normal levels, which was not due to reduced β-cell survival; suggesting a functional defect [493].

Although these findings highlight that ARNT does play a role in β-cells, they leave open the question whether it is correlates with diabetes development in humans. Although ARNT is found (1q21.3) [494] in the T2D susceptibility locus 1q21-q24 [495; 496], single nucleotide polymorphisms (SNPs) in ARNT did not appear to significantly affect its expression or confer a significant risk of developing T2D; despite association with slightly altered insulin secretion in non-diabetic individuals [496]. When testing whether ARNT expression is associated with diabetes disease onset or development, a 2005 study [478] noticed decreased levels of ARNT in T2D donor islets, along with a decrease in other bHLH-PAS factors AHR, ARNTL, and HIF1α. The decrease in ARNT was not reproduced in mouse models of obesity and diabetes, which could be a limitation of the animal model. However, later studies on human T2D islet donor
tissue did not find significantly lower ARNT levels \cite{296; 481}. Other differences between these studies are that Gunton noticed a decrease in many glycolytic genes including aldolase, while the latter found no differences except for an increase in ALDOB and LDHA in T2D islets \cite{481}.

In summary, ARNT has a role in β-cell function, but the contributions of its individual partner factors remain to be defined. It is possible that there are perturbances in ARNT expression during diabetes, but whether they are driving disease progression or are secondary to disease progression is unclear.

### 1.4.4 Role of oxygen and HIF1α in the islet

Of the hypoxia-inducible factors, HIF1α is expressed in both murine and human β-cells and likely the main regulator of the hypoxic response \cite{464; 480; 481; 497}. While HIF1α mRNA, like ARNT \cite{453; 498; 499}, is relatively stable even during hypoxia \cite{500; 501}, HIF1α protein is tightly regulated at the post-translational level \cite{487; 502; 503} (Figure 1.5). In the presence of molecular oxygen (O₂), α-ketoglutarate, and Fe²⁺, HIF1α is hydroxylated by oxygen-dependent prolyl hydroxylase domain (PHD) enzymes at prolines 402 and 564 of its oxygen-dependent degradation (ODD) domain \cite{454; 504-508}. VHL, which is part of an E3 ubiquitin ligase complex \cite{509; 510}, recognizes and binds to these hydroxylated prolines and induces polyubiquitination, leading to subsequent proteasomal degradation of HIF1α \cite{503; 505; 506; 511}. When O₂ levels are low, PHD enzyme activity is inhibited, which stabilizes HIF1α by preventing hydroxylation and ubiquitination \cite{503; 505; 511}. The production of ROS, for example from active glucose metabolism, also contributes to the stabilization of HIF1α by inhibiting PHD enzymes \cite{500; 503; 512-515}. Following its stabilization, HIF1α translocates to the nucleus and heterodimerizes with ARNT \cite{454}. There, the HIF1α:ARNT heterodimer regulates gene expression by binding to hypoxia response elements (HRE; 5’-TACGTG-3’) at target gene promoters \cite{464; 516} (Figure 1.5).
Figure 1.5. Regulation of HIF1α protein stability.

Under normoxic conditions, prolyl hydroxylase domain (PHD) enzymes use O₂, α-ketoglutarate (α-KG), and Fe²⁺ as substrate for HIF1α hydroxylation at prolines 402 and 564 of its oxygen-dependent degradation (ODD) domain (1). The hydroxylated prolines are recognized by the E3 ubiquitin ligase VHL Complex, which in turn ubiquitinates HIF1α (2) to target it for proteasomal degradation (3). Low levels of the PHD enzyme substrate O₂, or inhibition of PHD enzyme activity by high levels of reactive oxygen species (ROS), leads to reduced HIF1α hydroxylation (4). In consequence, HIF1α is stabilized, allowing it to heterodimerize with its partner factor ARNT in the nucleus (5). HIF1α:ARNT binds hypoxia response element (HRE) -containing DNA sequences, leading to up- or downregulation of HIF1α target genes (7). Modified from Sabatini and Lynn (2015) [464] and Sabatini et al. (2018) [4].

In general, validated HIF1α target genes are involved in promoting anaerobic glycolysis and vascularization [464; 480; 517-521]. Thus, HIF1α limits energy expenditure by causing a switch from aerobic oxidative phosphorylation (OXPHOS) to anaerobic glycolytic glucose utilization, and restores or optimizes oxygen supply by promoting blood vessel growth. HIF1α target genes promoting these changes are Ldha, Pdk1, Aldoa, Slc16a3 (Mct4), Gapdh, Pfk, and Vegfa [464; 518]. Under normal conditions, these factors are expressed at low levels, as β-cells are optimized for heavy glucose utilization through OXPHOS for maximal ATP generation and insulin
secretion [404; 484; 522]. When HIF1α is stabilized due to oxidative stress or hypoxia, 1) PDK1 reduces the amount of pyruvate entering mitochondria by suppressing pyruvate dehydrogenase; 2) LDHA catalyzes the conversion of pyruvate into lactate, and 3) SLC16A3 facilitates lactate removal from the cell [484; 522-524]. In this manner, immediate survival under stress is ensured at the expense of optimal β-cell function [514].

Because it is difficult to measure actual oxygen levels in vivo, what is physiologically relevant hypoxia is a field of open debate. Generally, it is accepted that due to the need of being metabolically active to fulfill their function, β-cells have a high oxygen demand [404], which is reflected in the rich vascularization of the islet. It is estimated that islets receive 10 % of total pancreatic blood flow while only constituting 1-2 % of mass in the adult animal [525]. Yet, the normal islet microenvironment is moderately hypoxic in vivo (pO₂ 31-37 mm Hg) [526]. This has led to the hypothesis that during a metabolic challenge, such as a high glucose bolus, oxygen depletion through aerobic OXPHOS may lead to local hypoxia in the islet, which stabilizes HIF1α and limits β-cell function due to limitations in ATP production [527; 528]. This interpretation contrasts with another hypothesis that it is increased mitochondrial ROS production which stabilizes HIF1α during relatively hypoxic periods, but not when O₂ is complete absent [529; 530]. Although these studies were not conducted in islet cells, they provide evidence that redox products such as ROS (superoxide and peroxide), which are generated as by-products of mitochondrial metabolism, inhibit PHD enzymes and thus stabilize HIF1α [500; 503; 512-515; 529; 531]. Despite being metabolically active, β-cells express relatively low levels of antioxidant defense enzymes [532-534], rendering them susceptible to ROS buildup [535]. This suggests that aside from harnessing the benefits of ROS for insulin secretion [536], β-cells must be equipped with mechanisms to limit aberrant ROS-induced HIF1α signalling.
Physiologically, β-cell-specific loss of HIF1α in the RIP-Cre mouse causes glucose intolerance, reduced insulin secretion, and impaired ATP production, which was associated with reduced expression of *Glut2* and glycolytic genes \(^{[480]}\). In agreement, HIF1α expression was reduced in islets from T2D donors, and mild stabilization of HIF1α with an iron chelator improved glucose tolerance in control but not *Hif1α* KO mice \(^{[480]}\). However, several studies using *Vhl* KO mice to stabilize HIF1α likewise found glucose intolerance and decreased insulin secretion \(^{[497; 520; 537]}\), which could be prevented by dual loss of *Vhl* and *Hif1α* \(^{[497]}\). Intriguingly, both HIF1α stabilization as well as β-cell specific knockout reduced *Gck* and *Glut2* expression \(^{[480; 519]}\), leaving the question of HIF1α’s role in regulating β-cell glucose supply unresolved. Together, these studies suggest that a balance in HIF1α signalling is needed, since both aberrant activation as well as loss of *Hif1α* impaired β-cell function \(^{[480]}\).

### 1.4.5 The neuronal PAS domain protein 4 (NPAS4)

In 2004, two groups independently cloned *Npas4* after discovering it in neuronal tissues, and hence originally named it LE-PAS (limbic enriched PAS) \(^{[538]}\) and NXF (neuronal expressed/transcription factor) \(^{[539]}\). When initially characterized, it was noted for its low sequence identity with other vertebrate bHLH-PAS family members \(^{[449; 538; 539]}\). In mice and humans, the gene consists of 8 coding exons interspersed by 7 introns, resulting in a transcript of 3,277 bp and 3,303 bp, respectively \(^{[540; 541]}\). This includes a 5’-UTR (mice: 155 bp; humans: 176 bp) and a 3’-UTR (humans: 718 bp; mice: 713 bp) \(^{[540; 541]}\). Its genomic location in humans is on the forward strand of chromosome 11 (band 11q13.2), while in mice it is found on the reverse strand of chromosome 19 (band 19; 19A) \(^{[542; 543]}\). Humans have several splice variants whose significance has not been characterized \(^{[540; 541]}\).

The main transcript is translated into an 802 amino acid (aa) protein with a predicted molecular weight of 87 kDa, but it is detected at around 110 kDa in immunoblots \(^{[544]}\). A larger 200 kDa
band is also detected, but decreases with the induction of the 110 kDa band \[^{544}\]. The NPAS4 protein features the characteristic bHLH (aa 1-53), PAS A (aa 70-144), and PAS B domain (human aa 203-273; mouse aa 203-275) \[^{464; 545}\] (Figure 1.4). A reporter assay indicates the presence of a C-terminal transcriptional activation domain (TAD) in the region of aa 597-802, although its exact sequence has not been determined \[^{539}\] (Figure 1.4).

**1.4.5.1 NPAS4 expression pattern**

Npas4 is primarily expressed in neuronal tissues, particularly in the limbic system (such as cortex, hippocampus, and olfactory bulb) \[^{538}\], but traces of mRNA were also found in skeletal muscle and kidney \[^{539}\], and a higher molecular weight band in murine testis \[^{538; 544; 546}\]. Furthermore, NPAS4 mRNA and protein is found in human endothelial cells \[^{547}\], as well as murine and human islets \[^{446}\]. One of the difficulties with the detection of NPAS4 expression is that its basal expression is usually very low or absent. It was noted soon after its discovery that it is induced by certain stimuli, such as seizure \[^{548}\], cerebral ischemia \[^{544}\], or membrane depolarization \[^{354}\]. Because gene expression in tissues is often assayed under basal, unstimulated conditions, NPAS4 expression is likely underappreciated, especially in tissues responsive to NPAS4-inducing stimuli such as depolarization \[^{446}\]. Npas4 mRNA stability is regulated by microRNAs (miRs) and a factor suppressing neural genes in non-neuronal tissues \[^{549}\], which contributes to its more restricted expression.

**1.4.5.2 NPAS4 expression is activity- and calcium-dependent**

Npas4 expression is induced in an activity-dependent manner, specifically downstream of membrane depolarization and calcium influx \[^{354; 446}\]. Because Npas4 mRNA expression could be induced without the need for de novo protein synthesis, it was established as an immediate early gene \[^{354; 446}\]. Npas4 mRNA expression can be induced by many stimuli in murine neurons and the rat neuroblastoma cell line PC12, including KCl, bicuculline, Metrazol, glutamate, NGF, and
BDNF [354; 550]. Npas4 expression in the pars tuberalis is regulated in a circadian fashion through the melatonin GPCR [551; 552], and visual stimuli following a period of dark rearing in mice [354]. In the hippocampus, Npas4 can be suppressed by stress due to social isolation or restraining [553; 554]. Induction by bicuculline is partly NMDAR- & AMPAR-dependent [354], whereas induction by NGF depends on PI3K and PKB signalling, but not PKC [550]. Notably, Lin et al. (2008) [354] did not see NPAS4 protein induction using forskolin, or a lower concentration of NGF and BDNF, suggesting either a dose-dependence or different mechanisms of Npas4 mRNA and protein expression. Furthermore, Npas4 is induced by a variety of cellular stresses in PC12 cells, including ER stress with the SERCA inhibitor thapsigargin, oxidative stress with the ROS generator SIN-1, and osmotic stress with sorbitol [555]. The common denominator in these processes is a rise in intracellular calcium levels, as thapsigargin and ROS elevate $\text{Ca}^{2+}_{\text{cyt}}$ by blocking ER calcium re-uptake or trigger ER calcium release [556; 557], and neuronal activity and osmotic stress induces membrane depolarization and extracellular calcium influx [354; 558; 559].

Findings about Npas4 expression in neurons were corroborated in pancreatic islets, where it is expressed as an immediate early gene in a calcium-dependent manner following high glucose exposure, membrane depolarization by KCl, or depletion of ER calcium by thapsigargin [446]. The exact calcium-dependent signalling pathways controlling Npas4 induction were not uncovered in this work in $\beta$-cells; their identification might contribute to a better understanding of the regulation of Npas4 expression in pancreatic $\beta$-cells.

1.4.5.3 Npas4 expression dynamics

NPAS4 expression is regulated predominantly at the transcriptional level. Basal mRNA expression in most tissues is either low or undetectable [560; 561]; hence, the same applies to protein. However, upon receiving an adequate stimulus, cells rapidly and substantially increase their NPAS4 expression [560]. Depending on cell type and stimulus, both mRNA and protein are
detected within 5-45 min, which, after reaching a peak between 0.5-2 h, decline almost as quickly as they were induced [354; 446; 544; 562; 563]. Even among immediate early genes, this makes NPAS4 one of the most rapidly induced and dynamically regulated genes in excitable cells [560]. Furthermore, NPAS4 protein does not appear to require prior activation or stabilization, as is for example the case for HIF1α [503; 505; 506; 511]; despite certain post-translational modifications, such as phosphorylation [550], further enhancing its transcriptional activity. Altogether, its attributes make NPAS4 an optimal transcription factor for rapidly translating a stimulus into transcriptional changes.

1.4.5.4 NPAS4 function and disease association in neuronal cells

The function of Npas4 has been well characterized in murine neurons [560]. Npas4 promotes somatic, but not dendritic, formation and maintenance of inhibitory synapses on excitatory neurons, and excitatory synapse formation on inhibitory neurons [354; 564; 565]; providing overall negative neuronal feedback in the form of inhibitory postsynaptic potentials. This effect was achieved via cell-type specific transcriptional regulation of activity-dependent genes [565]. In contrast, transcriptional suppression of Npas4 due to social isolation or chronic restraint stress impairs spatial memory, neurogenesis, and increases male aggression [533; 554; 566]. This was also associated with a loss of NPAS4 transcriptionally upregulating immediate early genes Fos, Arc, and Egr1, which are involved in contextual fear memory formation [562]. Furthermore, Npas4 is induced in response to cerebral ischemia [544] and protects neuronal cells against various excitotoxic cellular stresses [555; 567]. Consequently, germline KO mice show significantly increased mortality due to apoptosis-dependent neurodegeneration from 3 months of age [555]. Treatment with an excitotoxic drug, kainate, accelerated the neurodegenerative process in Npas4 KO compared to control mice [555]. Another phenotype of germline Npas4 null mice is reduced angiogenesis, which was attributed to loss of transcriptional regulation of the Cdh5
gene by Npas4 in endothelial cells [547]. Altogether, these studies established NPAS4 as an activity-induced regulator of neuronal and vascular growth and function.

While animal models helped define the physiological and molecular functions performed by NPAS4, few studies associate single nucleotide polymorphisms (SNPs) or other mutations in NPAS4 with a phenotype in humans. Among other genes, NPAS4 localizes to the bipolar disorder susceptibility locus ODZ4 [568]. Another study found deletion of an area of chromosome 11 (11q13.2), encompassing NPAS4, in a case with mild intellectual disability [569]. In addition, a human variant of NPAS4, F417S, leads to a complete loss of transcriptional activity; it thus seems possible that loss of NPAS4 activity is a risk factor for developing neuropsychiatric or neurodegenerative disorders [570]. These associations need to be tested in further studies.

1.4.5.5 DNA binding of NPAS4 in neuronal cells

Mechanistically, NPAS4 forms a heterodimeric transcription factor with Class II bHLH-PAS proteins ARNT, ARNT2, or ARNTL to bind DNA [539; 571]. In neuronal cells in vitro, the interaction of NPAS4 with ARNT2 was the strongest, followed by ARNT, although transcriptional activity between the two was similar [539; 571]. A weak interaction was also seen with ARNTL, but NPAS4 does not heterodimerize with ARNTL2, SIM2, or CLOCK, nor does it form homodimers [539]. When the transcriptional co-activator EP300 is overexpressed, the activity of the NPAS4 C-terminal TAD is enhanced [539]. EP300 interacts with many other transcription factors including ARNT [572], suggesting that EP300 could bridge NPAS4:ARNT complexes with other important transcription factors to promote co-operative regulation of transcription. Post-translational modifications of the NPAS4 protein influence its transcriptional activity, as NGF treatment leads to MAPK-dependent NPAS4 phosphorylation, enhancing its transcriptional activity [550].
NPAS4 preferentially binds to enhancer regions in neurons \[^{315}\]. Regarding its consensus binding motif, NPAS4 is unusually promiscuous among the bHLH-PAS proteins. While it recognizes the core bHLH-PAS motif CGTG, the rest of its DNA binding sequence is less defined, as the NPAS4:ARNT2 heterodimer is able to drive reporter gene expression in decreasing order of preference from TCGTG, GCGTG, and CCGTG motifs \[^{539}\]. NPAS4:ARNT expresses reporter constructs with the TCGTG element with equal efficiency as NPAS4:ARNT2 \[^{571}\]. Furthermore, NPAS4:ARNT recognizes the central midline element ACGTG \[^{538}\], which forms part of the hypoxia response element (HRE) TACGTG \[^{502}\]. The Drosophila homolog of NPAS4:ARNT, dysfusion:tango, also shows variable sequence preferences, in decreasing order of TCGTG, GCGTG, ACGTG, and CCGTG \[^{573}\]. Consequently, NPAS4 may be able to compete with other bHLH-PAS domain factors for target gene binding, as has been shown between NPAS4 and SIM2 at the Dbn1 gene \[^{539}\]. The preferred DNA binding motif and complete list of target genes of NPAS4 in β-cells have not been revealed.

### 1.4.5.6 Role of NPAS4 in pancreatic β-cells

Previous research demonstrates a role for Npas4 in murine pancreatic β-cells \[^{446}\]. As mentioned earlier, it is highly expressed in glucose-stimulated mouse and human islets, as well as by membrane depolarization or palmitate in MIN6 cells \[^{446}\]. Functionally, Npas4 has at least three different effects in β-cells. First, Npas4 overexpression diminishes Ins1 and Ins2 expression and insulin content in mouse islets, whereas knockdown with siRNA increases insulin gene expression \[^{446}\]. This was associated with an enrichment at both insulin promoters following membrane depolarization in MIN6 cells, suggesting NPAS4 controls insulin transcription \[^{446}\]. Furthermore, protein levels of important insulin-regulating transcription factors PDX1 and NEUROD1 were decreased in Npas4-overexpressing MIN6 cells, whereas Mafa mRNA as well as protein were increased \[^{446}\]. Second, amplification of GSIS by the GLP1R agonist exendin-4
was significantly reduced when Npas4 was overexpressed in MIN6 cells and islets, suggesting NPAS4 controls β-cell function [446]. This is possibly due to transcriptional upregulation of Rgs2, a regulator of G-protein signalling, since NPAS4 was enriched at a Rgs2 enhancer region and Npas4 overexpression caused an increase in Rgs2 message and protein [446]. Third, Npas4 expression is upregulated by ER stress inducers thapsigargin and palmitate in MIN6 cells and islets [446]. This indicated that NPAS4 may be involved in the cellular stress response. Indeed, when Npas4 was overexpressed, both thapsigargin- and palmitate-induced levels of the ER stress marker Ddit3 were markedly reduced in MIN6 cells and islets [446]. This correlated with an increase in the expression of cytoprotective gene Hspa5, and Wfs1, a gene which when mutated causes juvenile-onset diabetes as part of Wolfram syndrome [574]. Furthermore, apoptosis was reduced in thapsigargin-treated MIN6 cells expressing Npas4 [446]. In summary, these in vitro studies suggested that transcriptional regulation by NPAS4 in response to β-cell stimulation and calcium influx are an adaptive mechanism to modulate β-cell function by limiting insulin transcription and secretion, as well as reducing palmitate-induced ER stress and enhancing β-cell survival. Further studies should investigate a role for NPAS4 in β-cells in vivo.

1.5 Modulation of insulin secretion through GPCRs and RGS proteins

To achieve optimal organismal glucose homeostasis, the β-cell features a wide array of GPCRs and counterregulatory RGS proteins [575-578]. As many GPCRs trigger a downstream increase in intracellular calcium levels, they contribute to the fine-tuning of insulin secretion and regulation of activity-dependent genes.

1.5.1 GPCR structure and signalling cascades

In general, GPCRs are characterized by their seven transmembrane-spanning α-helices, as well as three intra- and extracellular hydrophilic loops [579]. The ligand-sensing domain lies mainly at the extracellular N-terminus, while the intracellular C-terminal domain serves for downstream
signal transduction [579; 580]. Intracellularly, GPCRs are linked to heterotrimeric G-proteins made up of an α, β, and γ subunit [579; 580]. In its inactive state, the Gα subunit contains GDP and is bound to Gβγ subunits [579]. Upon ligand binding, GPCRs stimulate an exchange of GDP for GTP on Gα, inducing conformational changes of the intracellular receptor subunits which then activate signalling through downstream effectors [581]. This can occur without subunit dissociation through structural rearrangements [582; 583], or via dissociation of the Gα and Gβγ subunits [584-586]. Gβγ mainly stimulates ion (Ca²⁺, K⁺) channels such as GIRK, but may also act on PLC, AC, or PI3K [580; 587]. Further signalling diversity is achieved through Gα, which can be subdivided into four main classes. Gαs (for stimulatory) activates AC, leading to increases in cAMP and subsequent PKA activation [580] (Figure 1.2). Gαi/o-coupled (for inhibitory) receptors mainly inhibit Gαs signalling by blocking AC [580] (Figure 1.2); although they may also exert stimulatory actions through their Gβγ subunits. Members of the Gα12/13 subfamily directly interact with and often activate Rho GEFs, which in turn activate Rho GTPases to regulate downstream cytoskeletal dynamics [588; 589]. Gαq subfamily members activate PLC, which hydrolyzes PIP₂ into DAG and IP₃; while DAG activates PKC, IP₃ binds to the IP3R to cause ER calcium release into the cytoplasm [580] (Figure 1.2). Finally, intrinsic GTP hydrolysis activity of the Gα subunit converts GTP back to GDP, and if ligand binding from the GPCR is terminated, the G-protein subunits resume their inactive heterotrimeric confirmation at the receptor, completing the activation cycle [580] (Figure 1.2).

1.5.2 Regulation of insulin secretion by GPCRs

The β-cell is not an isolated cluster of cells with homotypic contacts – the islets is composed of five endocrine subtypes, is vascularized, innervated, and interspersed with connective tissue and immune cells [590-592]. All different cell types share varying degrees of contact, and signal to themselves and others [590-592]. Thus, the endocrine cells within the islets receive a multitude of
signals impacting on their function, which is often mediated via GPCR signalling. Expression of around 300 non-olfactory GPCRs has been detected in human β-cells [575]. They mediate a wide range of tasks, including hormone secretion [55]. Specifically, the depolarization-dependent triggering phase of insulin secretion is complemented by amplification or inhibition through various GPCRs [54; 55]. A large number of possible ligands modulating insulin secretion through GPCRs include peptides (glucagon, neuropeptide Y, ghrelin, cholecystokinin, kisspeptin), amino acids and derivatives (L-arginine, L-glutamate, γ-hydroxybutyric acid, γ-aminobutyric acid), fatty acids (palmitate, oleate, linoleate), steroids (estrogen, progesterone), amines (noradrenaline, serotonin, melatonin, dopamine), nucleotides (ADP, ATP, UDP, UTP), small organic molecules (acetylcholine, cannabinoids, α-ketoglutarate) and ions (H+, Mg2+, Zn2+, Ca2+) [54; 575]. Due to the vast number of different receptors and ligands, a complex picture of an integrative signalling network emerges.

1.5.2.1 Gαs-coupled GPCRs

Incretins are an important group of hormones promoting islet hormone release largely through GPCR signalling. They were originally defined as any gut hormone that stimulates or supplements pancreatic endocrine secretion under physiological conditions (i.e. the incretin effect) [593]. Well-established incretins include GIP [594] and GLP1 [595]; but other gut hormones such as gastrin [596] and cholecystokinin [597; 598] also qualify as incretins; all of them stimulate or augment insulin secretion and thereby contribute to the incretin effect [593; 599; 600]. The respective GPCRs for these incretins are widely expressed, including in islets [599; 601-605], and signal through both Gαs and Gαq subunits; although cholecystokinin type 1 and 2 receptors are mainly Gαq-coupled [606], whereas GIP and GLP1 receptors (GIPR, GLP1R) predominantly signal through Gαq [607; 608]. Due to their prominence in T2D pathophysiology and treatment [607; 609], GIPR and GLP1R are discussed as representatives of Gαq-coupled receptors.
Secretion of GIP and GLP1 from intestinal K- and L-cells, respectively, increases following a meal [599]; but both hormones are also expressed and secreted from pancreatic α-cells [610-612]. Rapid cleavage of systemic GLP1 by dipeptidylpeptidase-4 means that α-cell GLP1 is likely the dominant stimulus for β-cell GLP1R [613; 614]. Mechanistically, ligand binding to GIPR or GLP1R activates Gαs, which in turn stimulates AC to convert ATP to cAMP [607; 608]. Elevated cAMP levels activate PKA, which phosphorylates key kinases involved in the β-cell exocytosis machinery, including RAPGEF4 (known as EPAC2), thus promoting insulin secretion [607; 608].

The physiological roles of GIPR/GLP1R signalling are complex, and in the islet alone include stimulating insulin and somatostatin secretion, insulin transcription and β-cell proliferation, as well as suppressing glucagon secretion (GLP1 only; GIP stimulates it) and apoptosis [599; 609]. GIP and GLP1 also play a role in β-cell adaptation to increased insulin demand during pregnancy and obesity [615; 616], perhaps mediated by increases in intra-islet incretin expression and secretion, as is seen for GLP1 during non-diabetic obesity [617; 618]. However, the incretin effect is eventually reduced during T2D progression, contributing to β-cell failure [619].

Some knowledge about the role of GIPR and GLP1R in β-cells has been gathered using germline null mice. GLP1R null mice develop hyperglycemia and impaired glucose tolerance due to reduced circulating insulin, without changes in body weight, food intake, glucagon secretion, GSIS or insulin content and production [620-622]. GIPR null mice exhibit only slightly impaired glucose tolerance, but reduced insulin transcription and content [623]. A major limitation of these whole-body knockout mice is the concurrent loss of GIPR and GLP1R function in extra-pancreatic tissues; because their expression is widespread [609], their loss likely contributed to the glucose tolerance defects and other phenotypes in GLP1R and GIPR null mice. For comparison, β-cell specific GLP1R knockout mice do not develop glucose intolerance [624]. Furthermore, loss of one incretin receptor may be partially compensated for by the remaining
receptor: islets from GLPIR KO mice are more sensitive to GIP-stimulated cAMP production, and show increased circulating levels of GIP\cite{625}, whereas insulin responses to GLP1 and β-cell mass were increased in GIPR null mice\cite{623}. In conclusion, while unravelling the entire set of functions of GIPR and GLPIR in β-cells remains the subject of ongoing studies, they certainly amplify GSIS, because the combined effect of GIP and GLP1 makes up the majority of incretin-potentiated GSIS\cite{626}.

1.5.2.2 $\text{G}_{\alpha_i}$-coupled GPCRs

Examples for inhibitory $\text{G}_{\alpha_i}$-coupled GPCRs include the SSTRs. In the islet, somatostatin is secreted from δ-cells following glucose stimulation and acts on β-cells via inhibition of AC activity, reducing cAMP production and PKA activation, thus restricting excessive insulin release\cite{627; 628}. Double-knockout of Sstr1 and Sstr5 in mice results in loss of the somatostatin inhibitory effect on insulin secretion, and islets show increased basal and GSIS\cite{629}. Consequently, mice had improved glucose tolerance and hyperinsulinemia following a glucose bolus\cite{629}. In addition, islet cell hyperplasia was noted, with notable increases in α- and PP-cell number, suggesting a compensatory mechanism to protect from the dangerous effects of hypoglycemia\cite{629}. Notably, loss of either SSTR1 or SSTR5 results in different phenotypes, possibly because a concomitant decrease of Sstr5 expression is seen in Sstr1 null mice, whereas increased Sstr1 expression is seen in Sstr5 null mice\cite{630; 631}.

1.5.2.3 $\text{G}_{\alpha_q}$-coupled GPCRs

A large number of ligands activate $\text{G}_{\alpha_q}$-coupled GPCRs, which signal through IP$_3$ and PKC downstream of PLC activation, raising intracellular calcium levels and promoting exocytosis\cite{54}. The free fatty acid receptor 1 (FFAR1) is activated by FFAs such as oleate and palmitate\cite{244}. In contrast to the $\text{G}_{\alpha_i}$ standard of raising intracellular calcium levels, FFAR1 stimulates extracellular calcium influx via inhibition of the K$_{\text{ATP}}$ channel and activation of L-VDCC\cite{244}.
Some of its functions are a matter of debate \cite{244, 632}, but the consensus is that exposure to FFAs results in an amplification of second-phase GSIS, as this effect was lost in Ffar1 null mice \cite{633}. Ffar1 deficiency during obesity may have protective effects against insulin resistance, as the reduction in FFA-amplified insulin release ameliorates HFD-induced hyperinsulinemia, and thus protects from secondary consequences such as hyperglycemia, glucose intolerance, and increased hepatic glucose output \cite{633}. In contrast, mice overexpressing FFAR1 were prone to developing diabetes due to loss of this protective effect \cite{633}.

Neuronal control over insulin release is achieved via acetylcholine activation of the Gαq-coupled muscarinic acetylcholine receptor M3 (M3R) \cite{54}. Glucose homeostasis in mice with β-cell-specific loss of the M3R was impaired due to lower insulin release, whereas transgenic overexpression improved glucose tolerance due to an increase in insulin release \cite{634}. Mice overexpressing M3R were protected from the diabetogenic effects of HFD feeding \cite{634}.

Lastly, the β-cell insulin granule contains a mix of compounds including adenosine nucleotides, such as ADP and ATP, which are co-secreted with insulin \cite{376}. These can be sensed by the family of purinergic GPCRs, a family consisting of various members with different nucleotide affinities as well as subsequent outcomes on insulin secretion \cite{635}. Generally, ATP has been noted for its stimulating effect on insulin release \cite{248}, likely mediated through the P2 receptors (e.g. P2YRs) under glucose-stimulated conditions \cite{248, 635, 636}. There is, however, controversy about this assertion, as some noted an inhibitory effect of ATP, which could occur through different receptor subtypes or breakdown to adenosine \cite{635-637}. There are furthermore notable species-dependent differences in expression of purinergic receptor subtypes, such that unraveling a definitive role for purinergic receptors during insulin secretion remains difficult \cite{635}. Because various adenosine nucleotides are unavoidably co-secreted with insulin \cite{376}, interpreting agonist-evoked insulin secretion is a challenging task. Some insights are derived from studies in
mice, where the $\text{G}_\alpha_q$-coupled P2Y1R is activated by both ADP and ATP\textsuperscript{[635].} ADP possibly stimulates insulin release\textsuperscript{[638]}, whereas conflicting reports show both inhibition as well as stimulation by ATP at high glucose concentrations\textsuperscript{[639-641].} However, islets from $P2ry1$ KO mice exhibit reduced insulin secretion, suggesting the adenosine nucleotides do have a largely stimulatory effect through P2Y1R in mice\textsuperscript{[642].}

### 1.5.3 Modulation of GPCRs and insulin secretion by RGS proteins

As a testament to the importance of regulating insulin secretion, modulation by GPCRs can be further fine-tuned by RGS proteins\textsuperscript{[643].} There are 20 known canonical RGS proteins and 19 RGS-like proteins, divided into groups A-G, with the canonical RGS proteins in groups A-D\textsuperscript{[644].} RGS proteins contain a characteristic 120 amino acid $\alpha$-helical RGS domain with which they preferentially bind to active (GTP-bound) $\text{G}_\alpha$ subunits\textsuperscript{[580; 645]}, though they differ in their $\text{G}_\alpha$ subunit selectivity\textsuperscript{[644].} Canonically, RGS proteins facilitate $\text{G}_\alpha$ inactivation by enhancing their intrinsic GTPase activity (Figure 1.2), and are thus classified as GTPase-activating proteins (GAPs)\textsuperscript{[643; 645].} Binding and activation of GTPase activity in their respective target $\text{G}_\alpha$ ligand facilitates reaggregation of the heterotrimeric $\text{G}_\alpha$ complex and an abrogation of signalling\textsuperscript{[644].}

Several RGS proteins have been studied for their effect on insulin secretion\textsuperscript{[576; 577; 646].} These include RGS2, RGS4 and RGS16, which are among the most highly expressed RGS proteins in mouse islets\textsuperscript{[576].} Loss of $Rgs4$ leads to significant increases in insulin secretion in response to the muscarinic agonist OXO-M, suggesting it is a negative regulator of muscarinic insulin secretion\textsuperscript{[576].} Loss of $Rgs16$ led to the opposite phenotype, because RGS16 is a negative regulator of somatostatin release from $\delta$ cells, which normally inhibits insulin secretion\textsuperscript{[577].} $Rgs16$ overexpression further increased insulin secretion due to a lack of SST-dependent inhibition\textsuperscript{[577].} There are also indications that RGS16 modulates $\beta$-cell proliferation\textsuperscript{[577].} As these
studies highlight, not only is it important to modulate insulin secretion via GPCRs, but also to regulate GPCR signalling to achieve physiologically safe levels of insulin release.

### 1.5.4 The regulator of G-protein signalling 2 (RGS2)

Because *Npas4* overexpression causes a decrease in GLP1R-mediated insulin release\(^{[446]}\), a particular interest was placed in proteins that could potentially inhibit GPCR signalling in the \(\beta\)-cell, such as the RGS proteins. The small RGS domain protein RGS2 emerged as a main target of the transcription factor NPAS4. RGS2 is of particular interest in the \(\beta\)-cell because, like NPAS4, it is an activity-induced gene\(^{[643]}\), and thus well placed to mediate the effects of NPAS4 and adapt to the changing functional needs of \(\beta\)-cells during stimulatory activity.

Within the RGS protein family (group A-G), RGS2 is placed in the B/R4 family\(^{[644]}\). *Rgs2* mRNA was initially found in the eighth clone in a screen for \(G_0/G_1\) switch genes in activated human blood mononuclear cells, and hence initially named \(G_0/G_1\) switch gene 8 (\(G_0S8\))\(^{[647-649]}\). It was later renamed *RGS2* after amino acid similarities to yeast Sst2 and GPCR kinases (GRKs) were noted, leading to the establishment of a new family of RGS proteins sharing a highly conserved RGS domain of about 120 amino acids in length\(^{[650-654]}\). The C-terminal RGS domain of RGS2 extends from aa 83-199, whereas the N-terminal side of RGS2 contains regions important for membrane association\(^{[655]}\) and regulation of protein biosynthesis (Figure 1.6)\(^{[656]}\).

![Figure 1.6. Structure of RGS2.](image)

RGS2 is a 211 aa protein defined by its C-terminal regulator of G-protein signalling (RGS) domain. Other regions important for RGS2 function are a membrane association domain (MAD) regulating its subcellular localization, and a region involved in inhibition of protein synthesis (IPS). Numbers indicate aa positions.
In humans, the \textit{RGS2} gene is located on the forward strand of chromosome 1 (band 1q31.2) \cite{657} and contains 5 exons and 4 introns, generating a mature transcript of 1,354 bp \cite{649} consisting of a short 5'-UTR (31 bp), a 636 bp ORF, and a longer 3'-UTR (687 bp) \cite{658; 659}. The mouse gene is largely organized the same way \cite{660; 661}, located on the reverse strand of chromosome 1 (band 1F; 1 62.56 cM) \cite{662; 663}. While the ORF is the same size (636 bp) as in humans \cite{658}, the mouse 5'-UTR and 3'-UTR are longer (43 bp and 2,342 bp, respectively), resulting in a much larger transcript (3,021 bp) \cite{662}.

Human and mouse transcripts are both translated into a 211 aa protein with a size of 24 kDa \cite{664; 665}. This main isoform shares 96 % identity in mice and humans \cite{661}. However, there are several alternative ORFs \cite{666}; and three shorter splice variants exist in both species. Although none of the human alternative variants are protein coding \cite{658}, mice have one variant translated into a 130 aa protein, one transcript (which codes for a 71 aa peptide) undergoing nonsense-mediated decay, and one variant for which no protein is recorded \cite{662}.

\subsection{RGS2 expression and posttranslational modification}

\textit{RGS2} was initially found in activated mononuclear cells \cite{648}, but it is expressed more widely \cite{660}. Patients with acute leukemias show raised \textit{RGS2} mRNA levels \cite{657}. \textit{RGS2} is variably classified as an activity-dependent immediate early or delayed early gene \cite{667; 668}. Although calcium signalling alone is not sufficient to induce \textit{Rgs2} mRNA expression \cite{669; 670}, it is required for its full induction \cite{670}. Regulation of \textit{RGS2} promoter activity by binding of \textit{CREB1} and \textit{ETS1} transcription factors \cite{673} further supports a contribution of calcium signalling in \textit{RGS2} expression. \textit{RGS2} expression is mainly regulated by signalling pathways acting through PKA and PKC, and a wide range of stimuli inducing or suppressing \textit{RGS2} mRNA and protein expression are summarized in Table 1.1 \cite{643}. Depending on the organism, cell type and stimulus, \textit{RGS2} mRNA peaks between 30 min and 3 h after stimulation, before dropping markedly and reaching
pre-stimulation levels (Table 1.1) [643]. Its mRNA half-life is 29 min following lectin treatment of human lymphocytes, and 35 min in untreated cells [667].

**Table 1.1. Stimuli modulating RGS2 expression.**

Mechanisms in brackets indicate indirect or conclusive evidence, or assumptions/speculations made by the authors (NA = no mechanism provided). ▲ = upregulation; ▼ = downregulation; AGTR = angiotensin receptor; ADORA2A/B = adenosine A2A/B receptor; β-AR = β-adrenoreceptor; EGFR = epidermal growth factor receptor; FGF = fibroblast growth factor; hCG = human chorionic gonadotropin; mAChR = muscarinic acetylcholine receptor; NECA = 5’-(N-ethylcarboxamido)adenosine; PDGF = platelet-derived growth factor; PLD = Phospholipase D; PMA (TPA) = phorbol-12-myristat-13-acetate (12-O-tetradecanoylphorbol-13-acetate); PTGER = prostaglandin E receptors; PTH = parathyroid hormone; PTHR = PTH/PTHrP receptor; PTHrP = parathyroid hormone-related protein; PTK = protein tyrosine kinase; S1P = sphingosine-1-phosphate; Ref. = reference; SP1 = Sp1 transcription factor; TGF-β = transforming growth factor-β; TLR = toll-like receptor; TSHR = thyrotropin receptor; VSMCs = vascular smooth muscle cells. Modified from Kach et al. (2012) [643].

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<td>(cAMP)</td>
<td>T cells</td>
<td>[681]</td>
</tr>
<tr>
<td>PMA (TPA)</td>
<td>▲1-2 h</td>
<td>mRNA</td>
<td>PKC</td>
<td>VSMCs</td>
<td>[682]</td>
</tr>
<tr>
<td></td>
<td>▲0.5-6 h</td>
<td>mRNA</td>
<td>PKC</td>
<td>cardiomyocytes</td>
<td>[676; 683]</td>
</tr>
<tr>
<td></td>
<td>▲2 h</td>
<td>mRNA</td>
<td>PKC</td>
<td>osteoblasts</td>
<td>[674]</td>
</tr>
<tr>
<td></td>
<td>▲1-3 h</td>
<td>mRNA</td>
<td>PKC</td>
<td>SH-SY5Y cells</td>
<td>[669]</td>
</tr>
<tr>
<td>carbachol</td>
<td>▲0.5-4 h</td>
<td>mRNA,</td>
<td>(mAChR-Gαq/i, PKC)</td>
<td>1321N1 cells</td>
<td>[673]</td>
</tr>
<tr>
<td></td>
<td>▲0.5-4 h</td>
<td>mRNA</td>
<td>(mAChR-Gαq/i), PKC</td>
<td>SH-SY5Y cells</td>
<td>[669; 684]</td>
</tr>
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</table>


Table 1.1. Stimuli modulating RGS2 expression. (continued)

<table>
<thead>
<tr>
<th>Stimulus</th>
<th>Expression</th>
<th>Type</th>
<th>Mechanism</th>
<th>Tissue/Cells</th>
<th>Ref</th>
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<td>ADORA2A/B-Gαs</td>
<td>U373 MG cells astrocytes</td>
<td>[685]</td>
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<tr>
<td></td>
<td>▲1-5 h</td>
<td>mRNA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>▲0.5-3 h</td>
<td>protein</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ATP</td>
<td>▲2 h</td>
<td>mRNA</td>
<td>(Gαs, Gαq)</td>
<td>osteoblasts</td>
<td>[674]</td>
</tr>
<tr>
<td>ionomycin, concanavalin A</td>
<td>▲0.5-1.5 h</td>
<td>mRNA</td>
<td>(Ca2+)</td>
<td>mononuclear cells</td>
<td>[567]</td>
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<tr>
<td>cycloheximide</td>
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<td>mRNA</td>
<td>(stabilized mRNA-polysomes)</td>
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<td>[648]</td>
</tr>
<tr>
<td></td>
<td>▲15-45 min</td>
<td>mRNA</td>
<td></td>
<td></td>
<td>[667]</td>
</tr>
<tr>
<td>angiotensin II</td>
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<td>mRNA, protein</td>
<td>(AGTR-Gαq/i), PKC, iPLA2, PKA</td>
<td>VSMC</td>
<td>[682; 686-688]</td>
</tr>
<tr>
<td></td>
<td>▲1-12 h</td>
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<td>(AGTR-Gαq/i), Ca2+/CaM, CaMK, CaMK (Gαq; TGF-β)</td>
<td>H295R adrenal cells</td>
<td>[689]</td>
</tr>
<tr>
<td>angiotensin II (in vivo)</td>
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<td>(Gαq; TGF-β)</td>
<td>isolated cardiac fibroblasts</td>
<td>[690]</td>
</tr>
<tr>
<td></td>
<td>▼3-14 days</td>
<td>mRNA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>digoxin, ouabin</td>
<td>▲overnight</td>
<td>protein</td>
<td>Na+/K+ ATPase</td>
<td>VSMCs</td>
<td>[691]</td>
</tr>
<tr>
<td>digoxin (in vivo)</td>
<td>▲7 days</td>
<td>protein</td>
<td>Na+/K+ ATPase</td>
<td>heart, kidney</td>
<td>[691]</td>
</tr>
<tr>
<td>GIP</td>
<td>▲1 h</td>
<td>mRNA</td>
<td>NA</td>
<td>βTC3 cells</td>
<td>[692]</td>
</tr>
<tr>
<td>GLP1</td>
<td>▲45 min</td>
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<td>(Gαs) cAMP-PKA, Ca2+</td>
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<tr>
<td>SIP</td>
<td>▲1-2 h</td>
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<td>(Gαs)</td>
<td>VSMCs</td>
<td>[693]</td>
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<td>phenylephrine</td>
<td>▲0.5-3 h</td>
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<td>(Gαq1)</td>
<td>cardiomycocytes</td>
<td>[676; 683]</td>
</tr>
<tr>
<td>H2O2, peroxynitrite</td>
<td>▲1-4 h</td>
<td>mRNA, protein</td>
<td>(suppressed by PI3K, MAPK)</td>
<td>1321N1 cells</td>
<td>[694]</td>
</tr>
<tr>
<td>Heat shock</td>
<td>▲1-3 h</td>
<td>mRNA, protein</td>
<td>NA</td>
<td>1321N1 cells</td>
<td>[694]</td>
</tr>
<tr>
<td></td>
<td>▲0.5-2 h</td>
<td>mRNA</td>
<td>NA</td>
<td>SH-SY5Y cells</td>
<td>[695]</td>
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<tr>
<td>chemical and real ischemia/recovery</td>
<td>▲12-24 h</td>
<td>mRNA, protein</td>
<td>PKC</td>
<td>astrocytes</td>
<td>[696]</td>
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<tr>
<td>cell isolation, culture</td>
<td>▲0 min</td>
<td>mRNA</td>
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<tr>
<td></td>
<td>▼1 day</td>
<td>mRNA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>mechanical stress</td>
<td>▲6-24 h</td>
<td>mRNA, protein</td>
<td>cAMP</td>
<td>periodontal ligament cells</td>
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<tr>
<td></td>
<td>▼1-5 days</td>
<td>mRNA</td>
<td>NA</td>
<td>SH-SY5Y cells</td>
<td>[695]</td>
</tr>
<tr>
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<td>▲0.5 h - 9</td>
<td>mRNA</td>
<td>cAMP, SPI</td>
<td>3T3-L1 cells</td>
<td>[698; 699]</td>
</tr>
<tr>
<td>differentiation</td>
<td>▼1-5 days</td>
<td>mRNA</td>
<td>NA</td>
<td>SH-SY5Y cells</td>
<td>[695]</td>
</tr>
<tr>
<td>interleukin-2</td>
<td>▼2 h</td>
<td>mRNA</td>
<td>(reversed by cAMP)</td>
<td>T cells</td>
<td>[681]</td>
</tr>
<tr>
<td>lipopolysaccharides</td>
<td>▼1-24 h</td>
<td>mRNA</td>
<td>TLR1/2/4/6/9</td>
<td>macrophages</td>
<td>[700]</td>
</tr>
<tr>
<td></td>
<td>▼1-1.5 h</td>
<td>mRNA</td>
<td>TLR4, PKC-η, PLD</td>
<td>Raw264.7 cells</td>
<td>[701]</td>
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</tbody>
</table>
Subcellularly, RGS2 localizes to the plasma membrane and the nucleus [673; 714; 715] in dependence of PKC and PKA [716], possibly indicating activity-dependent changes in mode of action. The activity of RGS2 itself is regulated by various signals. Posttranslational modifications suppressing the inhibitory effect of RGS2 on Gαq include phosphorylation by PKC [717] and palmitoylation [718]. Furthermore, calcium signalling indirectly enhances RGS2 function. When Ca²⁺/CaM binds RGS2, it displaces or prevents binding of phosphatidylinositol 3,4,5-

Table 1.1. Stimuli modulating RGS2 expression. (continued)

<table>
<thead>
<tr>
<th>Stimulus</th>
<th>Expression</th>
<th>Type</th>
<th>Mechanism</th>
<th>Tissue/Cells</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>interferon β-1a</td>
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<td>mRNA</td>
<td>NA</td>
<td>leukocytes</td>
<td>[702]</td>
</tr>
<tr>
<td>hCG (in vivo)</td>
<td>▲2-12 h</td>
<td>mRNA</td>
<td>NA</td>
<td>ovary</td>
<td>[703]</td>
</tr>
<tr>
<td></td>
<td>▲12-39 h</td>
<td>mRNA</td>
<td>PTGER2/EGFR, PKA/MAPK, CREB1/ETS1</td>
<td>equine follicles</td>
<td>[671]</td>
</tr>
<tr>
<td></td>
<td>▲6-24 h</td>
<td>mRNA</td>
<td>(PTHR-Gαs), cAMP, PKA</td>
<td>bovine follicles</td>
<td></td>
</tr>
<tr>
<td>parathyroid hormones (PTH, PTHrP)</td>
<td>▲0.5-6 h</td>
<td>mRNA</td>
<td>(PTHR-Gαs), inhibited by vitamin D</td>
<td>osteoblasts</td>
<td>[674; 675; 704; 705]</td>
</tr>
<tr>
<td></td>
<td>▲1-3 h</td>
<td>mRNA</td>
<td></td>
<td>UMR106 cells</td>
<td>[675; 677]</td>
</tr>
<tr>
<td>PTH, prostaglandin E2 (in vivo)</td>
<td>▲1 h</td>
<td>mRNA</td>
<td>(PTHR/PTGER-Gαs), cAMP, PKA</td>
<td>bone</td>
<td>[704; 706]</td>
</tr>
<tr>
<td>prostaglandin E1/2</td>
<td>▲2 h</td>
<td>mRNA</td>
<td>(PTGER-Gαs)</td>
<td>T cells</td>
<td>[683]</td>
</tr>
<tr>
<td>thyrotropin</td>
<td>▲1-24 h</td>
<td>mRNA</td>
<td>(TSHR-Gαs)</td>
<td>thyroid epithelial cells</td>
<td>[707]</td>
</tr>
<tr>
<td>cholera toxin</td>
<td>▲1.5 h</td>
<td>mRNA</td>
<td>Gαs, cAMP, PKA</td>
<td>UMR106 cells</td>
<td>[677]</td>
</tr>
<tr>
<td>PDGF, FGF</td>
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<td>NA</td>
<td>VSMCs</td>
<td>[678; 708]</td>
</tr>
<tr>
<td>camptothecin, geldanamycin</td>
<td>▲1-4 h</td>
<td>mRNA</td>
<td>Cdk2</td>
<td>SH-SY5Y cells</td>
<td>[684]</td>
</tr>
<tr>
<td>amphetamine (in vivo)</td>
<td>▲1 h</td>
<td>mRNA</td>
<td>NA</td>
<td>brain caudate putamen</td>
<td>[709]</td>
</tr>
<tr>
<td>electroconvulsive seizures, electric impulses (in vivo)</td>
<td>▲0.5-4 h</td>
<td>mRNA</td>
<td>suppressed by dopaminergic receptors</td>
<td>brain</td>
<td>[668]</td>
</tr>
<tr>
<td>cardiac hypertrophy, constitutively active Gαq (in vivo)</td>
<td>▼0.5-14 months</td>
<td>mRNA, protein</td>
<td>Gαq</td>
<td>heart, cardiac myocytes</td>
<td>[710]</td>
</tr>
<tr>
<td>aortic constriction (in vivo)</td>
<td>▼2-7 days</td>
<td>mRNA</td>
<td>NA</td>
<td>heart</td>
<td>[710]</td>
</tr>
<tr>
<td>cardiac resynchronization (in vivo)</td>
<td>▲3 weeks</td>
<td>protein</td>
<td>NA</td>
<td>heart</td>
<td>[711; 712]</td>
</tr>
<tr>
<td>vascular hypertrophy (in vivo)</td>
<td>▼2 days</td>
<td>mRNA</td>
<td>angiotensin II-dependent</td>
<td>aorta (abdominal, thoracic)</td>
<td>[713]</td>
</tr>
</tbody>
</table>

Subcellularly, RGS2 localizes to the plasma membrane and the nucleus [673; 714; 715] in dependence of PKC and PKA [716], possibly indicating activity-dependent changes in mode of action. The activity of RGS2 itself is regulated by various signals. Posttranslational modifications suppressing the inhibitory effect of RGS2 on Gαq include phosphorylation by PKC [717] and palmitoylation [718]. Furthermore, calcium signalling indirectly enhances RGS2 function. When Ca²⁺/CaM binds RGS2, it displaces or prevents binding of phosphatidylinositol 3,4,5-
trisphosphate (PIP<sub>3</sub>), a phospholipid which reduces RGS2 GAP activity<sup>[719]</sup>. Protein half-life under basal conditions is 17.5 (±5.8) min, which can be increased to 77.9 (±43.4) and 54.4 (±26.1) min by treatment with Na<sup>+</sup>/K<sup>+</sup>-ATPase inhibitors ouabain and digoxin, respectively<sup>[691]</sup>. Proteasomal degradation of the RGS2 protein is mediated through a DDB1/CUL4B/FBXO44 E3 ligase complex<sup>[720]</sup>.

### 1.5.4.2 Function of RGS2

RGS2 has been studied in many cell types and animal models, and has numerous reported modes of action. It is unique for being relatively G<sub>α<sub>q</sub></sub>-selective GAP, whereas other group B RGS proteins also interact with G<sub>α<sub>i</sub></sub><sup>[721-723]</sup>. This characteristic is likely owed to its non-canonical protein structure, which makes G<sub>α<sub>i</sub></sub> binding inefficient<sup>[724; 725]</sup>. In consequence, RGS2 inhibits M1 and M2 muscarinic MAPK activation<sup>[668]</sup> and shows 5-fold higher efficiency in inhibiting IP<sub>3</sub> production than RGS4<sup>[714]</sup>. In ciliated airway epithelial cells, Rgs2 overexpression decreases ATP-stimulated intracellular calcium influx due to inhibition of G<sub>α<sub>q</sub></sub>-coupled purinergic signalling<sup>[726]</sup>. Vice versa, knockdown of Rgs2 enhanced ATP-induced calcium influx<sup>[726]</sup>. A role in inactivating G<sub>α<sub>i</sub></sub> signalling has been proposed in neurons, where synaptic G<sub>α<sub>i</sub></sub> limits calcium channel activity. Activity-induced Rgs2 expression reduced G<sub>α<sub>i</sub></sub> and thus, increased calcium concentration and enhanced presynaptic neurotransmitter release to modulate short-term synaptic plasticity<sup>[668; 727]</sup>. However, Chen et al. (1997)<sup>[660]</sup> found no interaction with G<sub>α<sub>i</sub></sub> or G<sub>α<sub>s</sub></sub>, as well as no or minor effects on G<sub>α<sub>i</sub></sub>-coupled signalling<sup>[651; 660]</sup>. Among non-canonical roles, RGS2 inhibits global protein synthesis<sup>[656]</sup>, modulates GIRK channel activity<sup>[728]</sup>, and directly binds AC to reduce cAMP production and indirectly limit G<sub>α<sub>s</sub></sub>-coupled receptor signalling<sup>[729]</sup>. Functionally, loss of RGS2 function causes cardiac and neurologic impairments<sup>[730; 731]</sup>. Rgs2 null mice develop cardiac hypertrophy with excessive vasoconstriction, with greater P2Y-dependent calcium influx in vascular smooth muscle cells<sup>[732]</sup>. Thus, RGS2 has protective effects...
in the heart [733; 734]. In the hippocampus, impaired synapse development caused decreased excitatory postsynaptic potentials, resulting in increased anxiety with decreased aggression in male mice [735]. Others observed neuroprotective effects mediated independent of RGS2 GAP activity, through binding to LRRK2, and inhibiting its kinase activity [736]. Supporting this finding, patients with Parkinson’s disease show reduced RGS2 expression [736]. Loss of RGS2 is associated with many neurological diseases in humans [737]. In addition, Rgs2 null mice display reduced T cell proliferation, and thus, impaired immunity [738].

A common denominator in the above studies is that the affected cell types (T cells, myocytes, neurons) depend on calcium influx for their function [738]. It is thus not surprising that loss of RGS2 disrupts calcium homeostasis in vascular smooth muscle cells [739]. Intracellular calcium in KO cells was lower at basal levels, but elevated more potently by thapsigargin treatment due to lower protein levels of PMCA4 and increased SERCA2 expression [739]. Another role in mediating calcium release was seen in mouse oocytes [740]. Lastly, exocrine pancreatic acinar cells from Rgs2 null mice showed increased carbachol-induced IP₃ generation and Ca²⁺ cyt oscillations, but compensated for this phenotype by increased calcium clearance [741]. Mechanistically, changes in IP3R and SERCA expression caused decreased ER calcium release and increased uptake in these mice [741].

In summary, RGS2 expression is largely cytoprotective, preventing cardiac hypertrophy and neurodegeneration. At least in neurons, myocytes and lymphocytes, RGS2 exerts its function through regulation of intracellular calcium levels. Given the prime importance of calcium signalling for β-cells function (section 1.3), RGS2 is predicted to affect insulin secretion.

1.5.4.3 The role of RGS2 in pancreatic islets

Fewer studies examined RGS2 in the pancreas or β-cells. In rat islets, and the conditionally transformed mouse pancreatic β-cell line βTC-tet [742], the incretin hormone GLP1 was sufficient
to stimulate $Rgs2$ expression in a cAMP-PKA signalling-dependent manner \cite{670} (Table 1.1). The effect was partially calcium-dependent; whereas GLP1-mediated $Rgs2$ induction was inhibited by the L-VDCC blocker nimodipine, calcium influx stimulated with the L-VDCC agonist BayK8644 was not sufficient for induction \cite{670}. Use of a RAPGEF4 activator, MAP2K1/2 (MEK1/2) inhibitor, or PI3K inhibitor did not affect $Rgs2$ expression \cite{670}. Interestingly, CaN inhibition via FK-506, as well as deficiency in the digestive hormone cholecystokinin, prevented pancreatic induction of $Rgs2$ mRNA via a fast-refeed scheme \cite{743; 744}. Further expression studies in islets and insulinoma cell lines confirmed that $Rgs2$ is modulated dynamically in response to $\beta$-cell stimuli in a dose- and time-dependent fashion, because $Rgs2$ was induced by KCl and forskolin in MIN6 cells, and glucose in murine islets \cite{446}. This effect correlated with regulation by the transcription factor NPAS4, which, when overexpressed, induced $Rgs2$ expression due to NPAS4 binding in an intronic and an enhancer region of $Rgs2$ \cite{446}. Furthermore, higher basal and lower forskolin-induced $Rgs2$ expression was seen in vivo in islets from HFD- and high-carbohydrate-fed mice, and in vitro following high glucose culture (3 days) of mouse and human islets \cite{393}.

In terms of function, some have proposed that RGS2 binds G$\alpha_s$ in $\beta$TC-tet cells \cite{692}; however, there has been no further confirmation of this interaction. Whereas prostaglandin E-mediated suppression of GSIS was not affected by adenoviral overexpression of $Rgs2$ (Ad-$Rgs2$) in INS1(832/13) cells, fold insulin secretion in high versus low glucose was lower in Ad-$Rgs2$ cells \cite{745}. In vivo, germline $Rgs2$ deficient mice have been used to study its role in $\beta$-cell survival and function \cite{646}. These mice initially showed enhanced second-phase insulin secretion following a glucose bolus, and islets were more responsive to both glucose and the GLP1R agonist exendin-4 \cite{646}. However, 25-week old mice showed a stark reduction in $\beta$-cell mass, circulating insulin, and body weight \cite{646}. This phenotype was accompanied by increased
susceptibility to hypoxic cell death following loss of RGS2\textsuperscript{[646]}. However, many questions about the role of RGS2 in β-cell function remain.

### 1.6 Research question

Much is known about β-cell function, including transcriptional regulation and calcium-dependent signals required for insulin secretion, and how pathophysiologic insults impinge on β-cells. However, we still lack a full picture of what constitutes a functional β-cell, and new discoveries regarding activity-dependent signals induced upon physiological β-cell stimulation continue to emerge. Because a hallmark of T2D is β-cell dysfunction, the development of new diabetes therapies requires that we understand the underlying biology of a functional β-cell. Emphases of this thesis include activity- and calcium-dependent transcriptional responses and regulation of insulin secretion via immediate early genes. I focused on the bHLH-PAS domain transcription factor, NPAS4, and the small GTPase-activating protein, RGS2, because previous work implicated these two factors in the regulation of β-cell function. Both 1) are activity-induced, 2) show cytoprotective effects, and 3) modulate insulin secretion. Thus, I hypothesized that in a dynamic physiological environment, activity-induced NPAS4 and RGS2 expression affords β-cells an important adaptive feedback response to optimize insulin secretion. Because the signals regulating their expression and the exact mechanism of their function in β-cells remain unknown, I sought to address these open questions through four main goals:

1) To determine the calcium-dependent signals that regulate NPAS4 expression and its cytoprotective function in β-cells.

2) To test whether NPAS4 regulates β-cell function \textit{in vivo} by using β-cell specific loss-of-function mouse models.
3) To identify the genome-wide DNA binding sites and motifs of NPAS4 and its interaction partner ARNT in β-cells.

4) To investigate whether RGS2 regulates β-cell function.
Chapter 2: Materials and Methods

2.1 Ethics

All animal work was approved by the University of British Columbia Animal Care Committee under protocols A13-0184, A14-0163, and A17-0158. Human islet work was approved by the BC Children’s Hospital Research Ethics Board under certificate # H09-00676.

2.2 Reagents

FK-506 (# 342500), Compound C (# 171264), PD0325901 (# 444968), KN-93 (# 422711), STO-609 (# 570250), and JNJ-42041935 (# 400093) were purchased from EMD Millipore (now MilliporeSigma; Burlington, MA, USA). AIP (# SCP0001), Akti-1/2 (# A6730), BIM-XI (# B4056), and rapamycin (# R8781) were from Sigma-Aldrich (Saint Louis, MO, USA). Carbachol (# 2810) and α,β-MeATP (# 3209) were from Tocris Bioscience (Bristol, UK). FCCP (# 15218), Oligomycin Complex (# 11341), and Rotenone (# 13995) were ordered from Cayman Chemical Company (Ann Arbor, MI, USA), antimycin A (# 2247) was from Biovision (San Francisco, CA, USA), tamoxifen (# T006000) was from Toronto Research Chemicals (Toronto, ON, Canada), and recombinant insulin (Novolin ge Toronto 10 mL, DIN 02024233) was from Novo Nordisk (Bagsværd, Denmark).

All oligonucleotides were ordered from Integrated DNA Technologies (Coralville, IA, USA). Cell culture ware, reagents and media were from HyClone as part of GE Healthcare Life Sciences (Marlborough, MA, USA), Life Technologies/Invitrogen/GIBCO (now Thermo Fisher Scientific; Waltham, MA, USA), or BD Falcon and Corning (both Corning, NY, USA). All other chemicals and reagents were from Thermo Fisher Scientific (Waltham, MA, USA) and its subsidiaries Thermo Scientific, Fisher Scientific, Applied Biosystems, Invitrogen, and Gibco; or MilliporeSigma (Saint Louis, MO, USA) and its subsidiaries EMD Millipore and Sigma-Aldrich.
2.3 Mouse strains and research diets

The wild-type and mutant mouse strains used in this thesis are listed in Table 2.1. All studies were performed on male mice up to 32 weeks of age, with all mutant mice kept on a B6 background (Table 2.1), and Cre recombinase alleles kept hemizygous. Genotypes of mutant animals were determined via PCR genotyping (primers see Table 2.1) on DNA isolated from ear punch biopsies.

Table 2.1. List of mouse strains.

<table>
<thead>
<tr>
<th>Strain designation (Nomenclature, MGI ID)</th>
<th>Mutation</th>
<th>Genotyping primers</th>
<th>Source</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>B6 (C57Bl/6J, MGI:3028467)</td>
<td>inbred wild-type strain</td>
<td>NA</td>
<td>Jackson Laboratory (Bar Harbor, ME, USA), stock number 000664</td>
<td>NA</td>
</tr>
<tr>
<td>CD-I (Crl:CD1(ICR), MGI:5649524)</td>
<td>outbred wild-type strain</td>
<td>NA</td>
<td>Charles River (Wilmington, MA, USA), strain code 022</td>
<td>NA</td>
</tr>
<tr>
<td>Ins1Cre (Ins1tm1.1(cre)Thor, MGI:5614359)</td>
<td>knock-in replacement (a Cre recombinase gene was inserted at the initiation codon of the insulin I exon 2, replacing the coding sequence)</td>
<td>LL655 GGAAGCAGAATTCAGATCTTG, LL656 GTCAACAGCAGCTTTTGTTGTC, LL657 GCTGGAAGATGGCGATTACG</td>
<td>MMRRC / Jorge Ferrer Laboratory</td>
<td>[746]</td>
</tr>
<tr>
<td>Npas4flox (Npas4tm2Meg, MGI:3828100)</td>
<td>targeted insertion (LoxP sites inserted upstream of exon 1 and downstream of exon 7)</td>
<td>LL41 CCCTGGCCCTTCTAATCAGAC, LL42 GCCATTTCTTCTTCTGCCTCC</td>
<td>Michael E. Greenberg Laboratory</td>
<td>[354]</td>
</tr>
<tr>
<td>Pdx1-CreER (Tg(Pdx1-cre/Esr1*)35.10Dam, MGI:2684321)</td>
<td>transgenic insertion at unknown location (insertion of a construct containing the Pdx1 promoter and a gene encoding a tamoxifen-inducible Cre recombinase/estrogen receptor fusion protein)</td>
<td>FCL15 CCCAGAATGCCAGATTACG, FCL255 CCCAGAATGCCAGATTACG, CGTGGAGATGCCGAGCCA</td>
<td>MMRRC / Douglas A. Melton Laboratory</td>
<td>[747]</td>
</tr>
</tbody>
</table>
Mice were housed on a 12-hour light-dark cycle in a humidity- and temperature-controlled room with ad libitum access to food and water. Upon weaning, animals were given a regular chow diet (CHW). At the outset of physiological measurements at 7 weeks of age, mice were either maintained on CHW or switched to HFD (Table 2.2). Up to 5 mice were housed per cage.

Table 2.2. List of rodent research diets.

<table>
<thead>
<tr>
<th>Diet</th>
<th>ID</th>
<th>Supplier</th>
<th>Energy source</th>
</tr>
</thead>
<tbody>
<tr>
<td>CHW</td>
<td>5053 - PicoLab Rodent Diet 20</td>
<td>LabDiet (Saint Louis, MO, USA)</td>
<td>Protein: 24.5 %</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Fat: 13.1 %</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Carbohydrates: 62.4 %</td>
</tr>
<tr>
<td>HFD</td>
<td>DI2331</td>
<td>Research Diets (New Brunswick, NJ, USA)</td>
<td>Protein: 17 %</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Fat: 58 %</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Carbohydrate: 25 %</td>
</tr>
</tbody>
</table>

For studies involving the tamoxifen-inducible Pdx1-CreER deleter strain, Npas4^{flox/flox} littermate were used as controls and Npas4^{flox/flox};Pdx1-CreER+ mice as KO. Tamoxifen was dissolved in corn oil at a concentration of 60 mg/mL (w/v). Dissolution of tamoxifen was aided by sonication for 8 cycles of 30 s using a cup horn sonicator (Misonix Sonicator S-4000; Fisher Scientific # 13101353) at 80 % power. At 6 weeks of age, mice were given three doses of 8 mg tamoxifen by oral gavage every other day over five days.

For β-cell specific Npas4 KO using the Ins1^{Cre} strain, various littermate controls (Npas4 floxed control: Npas4^{flox/flox}; hemizygous Cre control: Ins1^{WT/Cre+}; control for heterozygous loss of Npas4: Npas4^{flox/WT};Ins1^{WT/Cre+}) were used with Npas4^{flox/flox};Ins1^{WT/Cre+} KO animals.

2.4 Physiological measurements and tolerance tests in mice

Random fed body weights were taken weekly starting from 7 weeks of age. All blood samples were drawn after puncturing the saphenous vein with a 27½ gauge needle. Following either ad libitum feeding or 10 h of overnight fasting (23:00-09:00 PST), random fed and fasting blood
glucose levels were measured bi-weekly starting at 7 and 8 weeks of age, respectively, using a handheld glucometer (UltraMini Meter; OneTouch).

Pyruvate, glycerol, and oral glucose tolerance tests (PyrTT, GlyTT, and OGTT) were performed at 14, 18, or 24 weeks of age, respectively. Following ten-hour overnight fasting (23:00-09:00 PST), fasting blood glucose (0 min) was measured and mice were weighed. Mice were then given either 2 g/kg pyruvate (from 40 % w/v sodium pyruvate solution in sterile 1x PBS) or 2 g/kg glycerol (from 40 % w/v glycerol solution in sterile H₂O) via intraperitoneal injection, or 2 g/kg D-glucose (from 40 % w/v D-glucose solution in sterile H₂O) via oral gavage. Subsequent blood glucose levels were determined at 15 min intervals from 15-60 min (PyrTT), 15-120 min (GlyTT) or 15-90 min (OGTT). Area under the curve was calculated using Prism 6 (Graphpad).

Insulin tolerance tests were performed at 18 weeks of age. Following a two-hour fasting period from 09:00-11:00 PST, basal blood glucose (0 min) was measured and mice weighed. Mice were then given an intraperitoneal injection of 0.75 U/kg insulin (from 150 mU/mL insulin solution in sterile 1x PBS). Blood glucose levels were subsequently taken at 15 min intervals from 15-90 min post insulin injection. Blood glucose measurements were normalized to basal levels and area over the curve calculated with a baseline of 1.0 using Prism 6 (Graphpad).

2.5 MIN6 cell culture

The insulinoma cell line MIN6 [748; 749] was provided by Dr. Jun-Ichi Miyazaki (Osaka University, Japan). Cells were used between passages 26 to 36. MIN6 cells were maintained in DMEM/High Glucose (Hyclone # SH30003.03) medium supplemented with 10 % (v/v) FBS (Hyclone # SH30396.03), 2 mM L-glutamine (Hyclone # SH30034.01), and 1x penicillin-streptomycin solution (pen/strep; Hyclone # SV30010) (DMEM/HG). Cells were passaged once or twice a week and culture medium replenished every other day.
For experiments, cells were split into an appropriate culture vessel (24-, 12-, and 6-well plates, 10 and 15 cm dishes) and let to attach and recover overnight. Further details are stated with the respective procedure below.

2.6 Islet work

2.6.1 Mouse islet isolation

Islet isolations were based on methods reported by Lacy and Kostianovsky [750] and filtration modifications described by Salvalaggio et al. [751]. First, mice were anaesthetized with isoflurane and euthanized by cervical dislocation. Mice were placed on their dorsal side on a stereoscope stage, and the ventral side was disinfected with 70 % ethanol. A vertical median incision and median sternotomy were made to expose the abdominal and chest cavity, respectively, providing access to the common bile duct and pancreas. Curved hemostatic forceps were used to clamp the hepatopancreatic ampulla to block fluid from entering the duodenum or stomach. Depending on mouse age, size, and bile duct diameter, a 30- or 27½-gauge needle was used together with a syringe loaded with 2-4 mL of collagenase solution (Table 2.3). The needle was inserted into the common bile duct through the junction of the hepatic and cystic duct, and collagenase solution was perfused into the pancreas via the pancreatic duct. The distended pancreas was then excised, placed in a 50 mL tube on ice, and collagenase solution topped up to 5 mL.

Table 2.3. Solutions for islet isolation.

<table>
<thead>
<tr>
<th>Solution</th>
<th>Composition</th>
</tr>
</thead>
<tbody>
<tr>
<td>1x HBSS</td>
<td>137 mM NaCl, 5.4 mM KCl, 4.2 mM NaH₂PO₄, 4.1 mM KH₂PO₄, 10 mM Heps, 1 mM MgCl₂, 5 mM D-glucose, in sterile dH₂O</td>
</tr>
<tr>
<td>collagenase solution</td>
<td>700-900 U/mL collagenase type XI (Sigma-Aldrich # C7657), in sterile 1x HBSS</td>
</tr>
<tr>
<td>wash solution</td>
<td>1 mM CaCl₂, in sterile 1x HBSS</td>
</tr>
</tbody>
</table>

Next, tubes containing the perfused pancreata were placed in a 37 °C water bath for 14 min. The tubes were then manually agitated for 1-4 min, resulting in a homogeneous solution. Cold wash
solution (Table 2.3) was added, tubes inverted, the mixture centrifuged for 1 min at 1,100 rpm (Sorvall ST 16R; Thermo Fisher Scientific # 75004381), and supernatant carefully decanted. This step was repeated once, briefly agitating to resuspend the pellet in wash solution before centrifugation. Afterwards, the pellet was resuspended in 10 mL wash solution. Using a pipette, the mixture was applied onto a pre-wet 70 μm cell strainer, and rinsed once with 10 mL wash solution. The strainer was then inverted, and captured islets were released into a 10 cm petri dish by rinsing with 10 mL of RPMI 1640 (Hyclone # SH30096.01) medium supplemented with 10 % (v/v) FBS, 2 mM L-glutamine, and 1x pen/strep (RPMI). Islets were identified under a dissecting microscope (Olympus # SZX16) and manually transferred into clean 10 cm petri dishes containing RPMI medium. Depending on the application, islets were either used immediately or let to recover overnight in a humidified CO₂ incubator at 37 °C.

2.6.2 Human islet preparations

Following isolation and overnight culture at the Clinical Islet Laboratory at the University of Alberta (Edmonton, AB, Canada), human islet preparations were delivered by courier and transferred to petri dishes containing CMRL 1066 (Corning # 15-110-CVR) medium supplemented with 10 % (v/v) FBS, 2 mM L-glutamine, and 1x pen/strep (CMRL). Human islets were identified under a dissecting microscope (Olympus # SZX16), manually picked into clean 10 cm petri dishes containing CMRL medium, and used immediately or let to recover overnight in a humidified CO₂ incubator at 37 °C.

2.6.3 Islet dispersion

Mouse or human islets were transferred into tubes, briefly centrifuged at 300 x g, and RPMI or CMRL removed. Islets were washed with 1 mL of 1x PBS, centrifuged at 300 x g for 3 min, and PBS discarded. Dispersion was performed by incubation and occasional agitation in 200 μL 0.25 % trypsin/EDTA at 37 °C for up to 12 min. Trypsin was neutralized by adding 200 μL FBS
and 1 mL of 1x PBS. Cells were spun down at 300 x g for 3 min, supernatant was removed and the pellet resuspended in 500 μL RPMI (mouse) or CMRL (human). Cells were counted with a hemocytometer and seeded onto 24-well or 12-well plates at an appropriate density (0.1-0.6 x 10^6 cells/well).

2.7 Adenoviral work

2.7.1 Adenoviral construction

*Npas4*, EGFP, and *Glb1* adenoviruses (Ad-Npas4, Ad-EGFP, Ad-βGal) were constructed previously[^446; 752] using the AdEasy system[^753]. The *Rgs2* adenovirus (Ad-Rgs2) was constructed likewise. Briefly, a 1,341 bp fragment spanning the mouse *Rgs2* cDNA (5'-GCGGGAGTCTGAGAATG[...] TAAATAATGTCATTGAA-3'; *Rgs2* translation initiation codon underlined) was cloned into pCMV6-Kan/Neo (OriGene # PCMV6KN). The *Rgs2* open reading frame (ORF) was PCR-amplified using primers LL409 and LL410 (Table 2.4), and the resulting 659 bp product was ligated into the blunt end cloning vector pJET1.2/blunt (Thermo Fisher Scientific # K1232). Subsequently, the *Rgs2* ORF was released with NheI and HindIII, and ligated into compatible sites (XbaI + HindIII) in the pAC.CMV.pLpA shuttle vector. Using lipofectamine 2000 (Invitrogen # 11668027) according to the manufacturer’s instructions, pAC.CMV-Rgs2 and the adenoviral vector pJM17 were co-transfected into the adenoviral producer cell line HEK293T[^754; 755] at a molar ratio of 4:1. HEK293T cells in 6-well plates were incubated with transfection mix in 800 μL DMEM/HG for 6 h. Afterwards, DMEM/HG was topped up to 2 mL overnight, and medium was carefully replaced the next day. In co-transfected HEK293T cells, homologous recombination through adenovirus type 5 (Ad5) sequences in both vectors led to integration of the *Rgs2* expression cassette (CMV promoter, *Rgs2* ORF, polyA sequence) into the adenoviral expression vector. HEK293T cells were let to propagate adenovirus for 10 days, at which point they had lifted off the culture vessel. Crude adenoviral
lysates were harvested by pipetting and stored at -80 °C. Release of adenovirus was aided through breaking up cells via three rounds of freeze-thaw, after which the original crude Ad-Rgs2 was further propagated by transducing HEK293T cells in 15 cm dishes. RGS2 expression from crude lysate was confirmed via western blotting (not shown).

Table 2.4. Oligonucleotides and plasmids used during construction of Ad-Rgs2.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5’ &gt; 3’)</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>LL409</td>
<td>aagctagccgccATGCAAGTGCCATGTCCTGG</td>
<td>forward primer; capitalized = identical to Rgs2 ORF; underlined = NheI; italics = Kozak sequence</td>
</tr>
<tr>
<td>LL410</td>
<td>aaaagctCATGTAGCATGGGGCTCCGT</td>
<td>reverse primer; capitalized = identical to Rgs2 ORF; underlined = HindIII</td>
</tr>
</tbody>
</table>

2.7.2 Adenoviral purification

For production of high viral titres, five 15 cm dishes of HEK293T cells at 90-95 % confluence were transduced with crude Ad-Rgs2 in 20 mL DMEM/HG. After 1-3 days, crude lysate was collected by pipetting and stored at -80 °C prior to purification. Next, Ad-Rgs2 crude lysate was freeze-thawed three times, cellular debris spun down at 200 x g for 10 min, and supernatant filtered through a 0.2 μm bottle-top filter. The filtered lysate was treated with 10 U/mL benzonase (Sigma-Aldrich # E1014) for 30 min. Afterwards, an equal volume of 1x dilution buffer (20 mM Bicine, 0.6 M NaCl, pH 8.4) was added to the lysate. The dilute solution was drawn into a syringe and run through a Sartobind Membrane Adsorber Unit (Sigma-Aldrich/Sartorius # Q15F) at 20 mL/min. The filter unit was washed with 60 mL 1x wash buffer (10 mM Bicine, 0.4 M NaCl, pH 8.4), and filter-bound Ad-Rgs2 was eluted in 3 mL 1x elution buffer (10 mM Bicine, 0.61 M NaCl, pH 8.2). Glycerol was added to a final concentration of 7.5 % (v/v) for cryoprotection before storage at -80 °C. Adenoviral titre was determined via end-point dilution assay.
2.7.3 Adenoviral transduction

For all studies involving adenoviral transduction of MIN6 cells or islet, a multiplicity of infection (MOI) between 1:5 and 1:50 was used. In general, cells or islets were infected in a small volume of culture medium (DMEM/HG, RPMI or CMRL) for 2 h, followed by topping up medium and continued overnight transduction. Medium was replaced the next day and cells let to recover and express the adenoviral construct for another day before experimental treatments. Individual details are stated with the respective data.

2.8 MIN6 and islet cell stimulation

2.8.1 Seeding

MIN6 cells were seeded at a density of 0.2-0.3 x 10^6 cells/well into 24-well plates, 0.8 x 10^6 cells/well into 12-well plates, or 1.0-2.0 x 10^6 cells/well into 6-well plates, and let to attach overnight. At the end of the next day, cells were transferred to DMEM/Low Glucose (Hyclone # SH30002.02) medium supplemented with 10 % (v/v) FBS, 2 mM L-glutamine, and 1x pen/strep (DMEM/LG) overnight.

When needed, freshly cleaned islet preparations were dispersed using 0.25 % trypsin and seeded at an appropriate density into 24-well or 12-well plates to attach overnight. Whole mouse or human islets were generally let to recover in petri dishes containing RPMI or CMRL overnight, respectively. They were transferred to an appropriate culture vessel the next day.

2.8.2 Npas4 mRNA and protein expression studies

For analysis of Npas4 induction, MIN6 cells were seeded in 24-well plates at a density of 0.2 x 10^6 cells/well. Cells were pre-incubated with pharmacological inhibitors or vehicle (DMSO or dH2O) for 60 min (AIP, BIM-XI, KN-93, PD0325901, STO-609), 30 min (Compound C, FK-506) or 15 min (Akti-1/2, EGTA, rapamycin). Maximal Npas4 expression in MIN6 was
achieved by depolarization with 40 mM KCl in DMEM/HG for 2 h\textsuperscript{[446]}. The role of insulin signalling in \textit{Npas4} induction was assessed in MIN6 pre-incubated in serum-free KRBH (114 mM NaCl, 20 mM HEPES, 4.7 mM KCl, 1.2 mM KH\textsubscript{2}PO\textsubscript{4}, 2.5 mM CaCl\textsubscript{2}, 1.17 mM MgSO\textsubscript{4}, pH 7.4) supplemented with 2.8 mM glucose for 2 h. Subsequently, 20 nM of fast-acting human insulin (Novolin ge Toronto) or vehicle (1x PBS) was added and incubation continued for 2 h.

Whole mouse islets were transferred to 12-well plates and pre-incubated in KRBH supplemented with 2.8 mM glucose and 0.2 % (w/v) BSA (KRBH-2.8G) for 30 min. Vehicle (DMSO or dH\textsubscript{2}O) or inhibitor (10 \mu M Akti-1/2, 10 nM FK-506, 3 \mu M KN-93) was added and pre-incubation continued for 30 min. Subsequent stimulation was performed for 2 h in KRBH-2.8G or KRBH supplemented with 0.2 % (w/v) BSA and 25 mM glucose (KRBH-25G), in combination with vehicle or inhibitor, respectively.

For studies of \textit{Npas4} mRNA and protein stability, MIN6 cells were seeded in 24-well plates at a density of 0.3 x 10\textsuperscript{6} cells/well or 12-well plates at 0.8 x 10\textsuperscript{6} cells/well, and 6-well plates at 2.0 x 10\textsuperscript{6} cells/well, respectively. Cells were stimulated with 40 mM KCl in DMEM/HG for 2 h, followed by continued KCl exposure or transfer to DMEM/LG for 1-4 h, respectively. The transcriptional blocker Actinomycin D (5 \mu g/mL; Sigma-Aldrich # A1410) was used to study mRNA decay, and NPAS4 degradation was assessed using the proteasomal inhibitor MG132 (10 \mu M; Sigma-Aldrich # C2211). In MIN6 cells stimulated with 40 mM KCl in DMEM/HG for 2 h, the contribution of \textit{de novo} translation for maintaining NPAS4 expression was assessed during subsequent stimulation with or without KCl and the translational blocker cycloheximide (CHX; 1.5 \mu g/mL), respectively.
2.8.3  *Rgs2* mRNA and protein expression studies

Human islets were transferred to 12-well plates and pre-incubated in 1.5 mL KRBH-2.8G for 1 h. Subsequent 1-2 h stimulation was performed in 1.5 mL KRBH-2.8G, KRBH supplemented with 16 mM glucose and 0.2 % (w/v) BSA (KRBH-16G), or KRBH-2.8G with 10 μM forskolin.

To validate RGS2 expression in mutant *Rgs2* clonal lines, 1.0 x 10⁶ cells were seeded in a 6-well plate and treated as described (section 2.8.1). After overnight culture in DMEM/LG, cells were pre-incubated in plain KRBH for 1 h, followed by 1-2 h stimulation in KRBH-2.8G or KRBH-25G supplemented with 10 μM forskolin.

2.8.4  HIF1α protein and target gene expression studies

MIN6 cells, mouse and human islets were seeded into 24-well plates as described (section 2.8.1). To study HIF1α target gene expression, MIN6 cells or dispersed islets (section 2.6.3) were infected with Ad-Npas4 or Ad-EGFP at an MOI of 20:1 as described (section 2.7.3). Subsequently, cells were maintained in standard culture medium (DMEM/HG, RPMI or CMRL) and incubated at 37 °C in a humidified CO₂ incubator set to either 20 % or 1 % O₂ for 6 h (MIN6) or 24 h (islets).

As an alternative to hypoxia, HIF1α was stabilized with the PHD enzyme inhibitor JNJ-42041935 (JNJ-42) [756]. The inhibitor was validated in MIN6 cells (1.5 x 10⁶ cells/well in a 6-well plate) maintained in DMEM/HG supplemented with vehicle or 100 μM JNJ-42 for 2-6 h. HIF1α target gene expression in whole mouse islets was assessed after stimulation in KRBH-25G supplemented with 100 μM JNJ-42 for 2.5 h.

To study glucose-stimulated HIF1α stabilization, whole human islets were pre-incubated in KRBH-2.8G for 1 h before incubation with KRBH-2.8G or KRBH-25G in a 37 °C humidified CO₂ incubator set to 5 % O₂ for 4 h. Alternatively, HIF1α expression was measured following
incubation in KRBH-2.8G or KRBH-25G supplemented with vehicle or 100 \( \mu \text{M} \) JNJ-42 for 4 h at 1 % O\(_2\).

### 2.8.5 Sample collection

For gene expression studies (section 2.10), MIN6 or islet lysates were collected in 0.5-1 mL TRIzol (Life Technologies # 15596018).

For general protein work, NRSB-minus lysis buffer (Table 2.5) buffer was heated to 95 °C, cells rinsed with 1x PBS, and an appropriate volume (~100 \( \mu \)L per 200,000 MIN6 cells or 250 islets) hot NRSB-minus lysis buffer added. Lysate was transferred to a tube and heated for another 5 min. Samples were vortexed and DNA sheared by sonication (Misonix S-4000) at 80 % power for 4 min. Protein content was quantified by Pierce™ BCA Protein Assay Kit (Thermo Scientific # 23225) per manufacturer’s instructions, SDS loading dye added to 1x final concentration, and samples were stored at -20 °C until use.

#### Table 2.5. Sample buffers for CoIP and western blot.

<table>
<thead>
<tr>
<th>NRSB-minus lysis buffer</th>
<th>Basic NP-40 buffer</th>
<th>NP-40 lysis buffer</th>
<th>10x SDS loading dye</th>
</tr>
</thead>
<tbody>
<tr>
<td>62.5 mM Tris, pH 6.8</td>
<td>150 mM NaCl</td>
<td>1 mM Na(_3)VO(_4)</td>
<td>0.5 M Tris-base</td>
</tr>
<tr>
<td>1 mM Na(_3)VO(_4)</td>
<td>20 mM Tris, pH 7.4</td>
<td>50 mM NaF</td>
<td>25 % (v/v) SDS</td>
</tr>
<tr>
<td>1 mM NaF</td>
<td>1 mM EDTA</td>
<td>50 mM ( \beta )-glycerophosphate</td>
<td>40 % (v/v) glycerol</td>
</tr>
<tr>
<td>2 % (v/v) SDS</td>
<td>0.5 mM EGTA</td>
<td>1x protease inhibitor cocktail (Roche # 4693132001)</td>
<td>20 % (v/v) ( \beta )-mercaptoethanol</td>
</tr>
<tr>
<td>10 % (v/v) glycerol</td>
<td>5 % (v/v) glycerol</td>
<td>0.5 % (v/v) IGEPAL (Sigma-Aldrich # 18896)</td>
<td>0.002 % (w/v) bromophenol blue</td>
</tr>
<tr>
<td>in dH(_2)O</td>
<td>in dH(_2)O</td>
<td>prepared fresh for use</td>
<td>pH to 6.8</td>
</tr>
<tr>
<td>stored in the dark at RT</td>
<td>stored in the dark at 4 °C</td>
<td></td>
<td>stored at -20 °C</td>
</tr>
</tbody>
</table>

For co-immunoprecipitation (CoIP) studies, samples were collected in an appropriate volume (~100 \( \mu \)L per 300,000 MIN6 cells or 350 islets) of NP-40 lysis buffer (Table 2.5) as described below (section 2.11.1).
2.9 Insulin secretion assay and enzyme-linked immunosorbent assay (ELISA)

Pre-warmed KRHB solutions were used throughout the assay, and all incubations took place in a humidified CO\textsubscript{2} incubator at 37 °C.

For MIN6 cells, 0.2 x 10\textsuperscript{6} cells/well were seeded in 24-well plates. The next day, cells were infected with Ad-βGal or Ad-Rgs2 with an MOI of 5:1 as described (section 2.7.3). Following overnight culture in DMEM/LG, cells were pre-incubated in KRHB-2.8G for 1 h. Technical duplicates of Ad-βGal and Ad-Rgs2 infected cells were then stimulated in 500 μL KRHB supplemented with a range of glucose concentrations (2.8-25 mM) or 40 mM KCl in KRHB-2.8G for 2 h. Media were collected, centrifuged at 5,000 x g for 10 min, and supernatant taken for insulin ELISA. Remaining cells were trypsinized for counting on a hemocytometer, DNA extraction, or determination of protein content via BCA assay. Total insulin was extracted from some wells by overnight incubation with 500 μL Acid-EtOH solution (1 % v/v HCl and 70 % v/v EtOH in dH\textsubscript{2}O) at 4 °C. Supernatant was collected by centrifugation as above and samples were stored at -20 °C.

For mouse islets, technical duplicates of 40 islets were used per condition. Following one-hour pre-incubation in 1 mL KRHB-2.8G, islets were transferred to 24-well plates and incubated in 0.5 mL KRHB supplemented with a range of glucose concentrations (2-16 mM; 0.2 % w/v BSA), 40 mM KCl (in KRHB-2.8G), or exendin-4 (50 nM in KRHB-8G). Supernatant was collected by centrifugation and insulin content extracted by Acid-EtOH as described above. Samples were stored at -20 °C.

Insulin ELISA was performed using the Mouse Ultrasensitive Insulin ELISA (Alpco # 80-INSMSU-E01) or STELLUX® Chemi Rodent Insulin ELISA (Alpco # 80-INSMR-CH01) kits according to manufacturer’s instructions. Absorbance or chemiluminescence were read using SpectraMax 190 or SpectraMax L (Molecular Devices) microplate readers, respectively.
2.10 Gene expression analysis

2.10.1 RNA extraction

Samples stored at -80 °C were thawed and 100 µL chloroform added for each 500 µL TRIzol. Following thorough vortexing, samples were centrifuged for 10 min at 12,000 x g and 4 °C. The clear top layer was transferred into a tube with 300 µL isopropanol (for each 500 µL TRIzol). Samples were thoroughly vortexed, centrifuged for 15 min at 12,000 x g and 4 °C and supernatant was discarded. The nucleic acid pellet was washed twice by inverting with 1 mL ice-cold 70 % EtOH, centrifuging for 5 min at 12,000 x g, and discarding EtOH after each wash. Samples were briefly centrifuged once more, residual alcohol removed by pipetting, and nucleic acid pellets air-dried at 55 °C for 2-3 min. Subsequent DNase treatment was performed using the TURBO DNA-Free kit (Life Technologies # AM1907). For a total reaction volume of 25 µL, nucleic acid pellets were resuspended in 21.5 µL nuclease-free water, 2.5 µL TURBO DNase Buffer (1x) and 1 µL TURBO DNase Enzyme (50 U/mL), and incubated for 30 min at 37 °C. The DNase reaction was stopped with 5 µL of DNase Inactivation Reagent, samples vortexed thoroughly, and centrifuged at full speed (21,000 x g) for 3 min. The clear top layer was carefully transferred to a new PCR tube and RNA concentration determined using a Nanodrop 2000c spectrophotometer (Thermo Scientific # ND-2000C).

2.10.2 cDNA synthesis

Reverse transcription was performed using SuperScript III Reverse Transcriptase (Invitrogen # 18080044). For 25 µL total reaction volume, 0.5-2 µg RNA were combined with 0.4 µM oligo(dT) and 200 ng random hexamer primers in nuclease-free water up to 14.5 µL, and denatured in a thermocycler at 70 °C for 10 min. Samples were briefly cooled on ice and 10.5 µL master mix added to obtain final concentrations of 1x First-Strand Buffer, 10 mM DTT, 0.5 mM dNTPs, 1 U/µL RNaseOUT, and 2 U/µL Superscript III reverse transcriptase. After mixing the
samples gently, the following cDNA synthesis program run on a Mastercycler pro (Eppendorf # 95003001) thermocycler: 10 min at 15 °C, 10 min at 25 °C, 15 min at 37 °C, 45 min at 42 °C, 10 min at 50 °C, 5 min at 55 °C, 3 sec at 95 °C and 4 °C forever.

2.10.3 Quantitative real-time PCR (qPCR)

Quantitative real-time PCR (qPCR) was performed on a ViiA7 real-time PCR system (Applied Biosystems) in a 384-well plate format using fast run settings. Samples were run in triplicate using 40 ng of cDNA as template and PrimeTime primers/probes (IDT; see Table 2.6 and Table 2.7), all of which utilized FAM as reporter dye and ZEN/IBFQ as quenchers. Expression levels were quantified via the ΔΔCt method using GUSB as reference gene.

Table 2.6. Mouse qPCR primers.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Probe</th>
<th>Primer 1</th>
<th>Primer 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gusb</td>
<td>TCTAGCTGGAATGTTACTGCCTG</td>
<td>CACCCCTACCATCCTACG</td>
<td>ACTTTGCCACCTCCTACC</td>
</tr>
<tr>
<td>Ldha</td>
<td>AGCTCATCCGCAAGTTTTCAT</td>
<td>GCTCCCCAGAACAAGATTACAG</td>
<td>TCGCCCTGAGTTGTCTT</td>
</tr>
<tr>
<td>Npas4</td>
<td>GTTGGTTCCCTCCACTCTCCAT</td>
<td>GTCTCAACACATTCCCCTACGAAAG</td>
<td>GACAATATGCTACATTGAAAGC</td>
</tr>
<tr>
<td>Pdk1</td>
<td>TCTGTGTGAAGCTTCCGTCT</td>
<td>GACTGTGAGATGAGTGAACC</td>
<td>CAATCCGTTAACAAACCCAG</td>
</tr>
<tr>
<td>Rgs2</td>
<td>AGCGGGGAAGAAAGACGGGAACA</td>
<td>CTCGAAATGCAAGTCCTCAGATG</td>
<td>AAGTAGCTCAAACCCGACCTC</td>
</tr>
<tr>
<td>Scl16a3</td>
<td>CCCGTTGGTAGGAGGTAGATCTGGAATAA</td>
<td>CCTGTCATGCTTTGAGTGTTG</td>
<td>GGAAGGCTGGAAGTTGAGAAG</td>
</tr>
<tr>
<td>Sox9</td>
<td>AGGGTGTCCTTCTCGCTCTGCT</td>
<td>CAAGACTCTGGCAAGCTC</td>
<td>GGGCTGGTACCTTGAATCGG</td>
</tr>
<tr>
<td>Vegfa</td>
<td>TTCCGGTGAGGCTCGTGG</td>
<td>GCCAGCTTGGTAAACGAA</td>
<td>TGGTGACATGTAATGCTT</td>
</tr>
</tbody>
</table>
Table 2.7. Human qPCR primers.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Probe</th>
<th>Primer 1</th>
<th>Primer 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>GUSB</td>
<td>CAGATTCTAGGTGGAGACGCAGGC</td>
<td>AGGTGATGGGAAGAAGTG</td>
<td>AGGATTTGGTGAGGCAGTAC</td>
</tr>
<tr>
<td>LDHA</td>
<td>CCATTAGGTAACCGGAATCGGGCTG</td>
<td>CCGTTATTGGGAGCGGCTTG</td>
<td>TTTCCAATCCCATACAGG</td>
</tr>
<tr>
<td>NPAS4</td>
<td>ACGCTCTGTCACTAACTACCG</td>
<td>GTCTATCTACCTGGGC</td>
<td>CCTCTCTGCGATATATCT</td>
</tr>
<tr>
<td>RGS2</td>
<td>ACCAAAATCTCTGATTCACAGATATACAAG</td>
<td>CAGTTGTAAACGCAGCCAC</td>
<td>CCAAAGCTGTCCTCAAACG</td>
</tr>
<tr>
<td>SOX9</td>
<td>TCTGGAGACTTCTGAGACGCAGAGGA</td>
<td>ACTTGCAACAACGCAGAG</td>
<td>CTGCTTGGTAATCCGGGT</td>
</tr>
</tbody>
</table>

2.11 Co-immunoprecipitation

2.11.1 General procedure

Following treatment, MIN6 cells were washed twice with ice-cold PBS and lysed on ice using pre-cooled NP-40 lysis buffer (Table 2.5) for 1-3 min, and lysate collected by scraping and transferring into tubes. Islet were directly transferred into tubes containing NP-40 lysis buffer. Tubes were nutated at 4 °C for 10 min, insoluble material removed by centrifugation for 10 min at 5,000 x g and 4 °C, and supernatant transferred to a new tube. Protein concentration was determined via BCA assay and lysates either stored at -80 °C or used directly. For each lysate, 30 μg was set aside as “Input” fraction.

For co-immunoprecipitation, lysate with 100-300 μg total protein was transferred to a non-stick 1.5 mL tube (volumes were equalized with NP-40 lysis buffer), 1-2 μg primary antibody added, and the lysate-antibody solution nutated at 4 °C overnight. Dynabeads Protein G (Life Technologies # 10004D) were used at a concentration of 0.5-0.75 mg per μg of primary antibody. After three washes with basic NP-40 buffer and magnetic separation, beads were resuspended in basic NP-40 buffer (50 μL/sample), added to the lysate-antibody solution, and nutation continued for 4 h at 4 °C. Afterwards, bead-antibody-antigen complexes were
magnetically separated from the supernatant, washed three times with cold 0.02 % (v/v) Tween-PBS, transferred to a new tube, and resuspended in NP-40 lysis buffer (27-54 μL). SDS content was brought to 1-2 %, SDS loading dye (Table 2.5) added to 1x, and samples denatured at 95 °C for 5 min to elute “IP” fractions. Alternatively, IP fractions were eluted with hot NRSB-minus lysis buffer (Table 2.5). Samples were stored at -20 °C until use. For western blot, half the volume of each IP fraction (30 μL) was used.

### 2.11.2 Determining NPAS4 interaction partners

MIN6 cells cultured in DMEM/LG overnight were either 1) maintained in DMEM/LG (3 h), 2) stimulated with 40 mM KCl in DMEM/HG for 2 h, or 3) treated with 40 mM KCl in DMEM/HG for 2 h followed by a one-hour washout in DMEM/LG. Lysates were obtained as described in section 2.11.1, and 300 μg total protein were incubated with 1 μg anti-ARNT, anti-ARNT2, anti-ARNTL, or anti-NPAS4 antibodies (Table 2.8) at 4 °C overnight with gentle agitation. The next day, 0.5 mg Dynabeads Protein G were added to each lysate. The remaining procedure was as described in section 2.11.1, eluting IP fractions by boiling in NP-40 lysis buffer (Table 2.5) with 1 % (v/v) SDS and 1x SDS loading dye.

**Table 2.8. List of primary antibodies used for CoIP and immunoblot.**

<table>
<thead>
<tr>
<th>Target</th>
<th>Host</th>
<th>Supplier</th>
<th>Cat. #</th>
<th>For</th>
<th>Concentration</th>
</tr>
</thead>
</table>
| ARNT                    | Mouse| BD                  | 611079    | CoIP  | 1-2 μg:
|                         |      |                     |           | WB    | 1:1,000       |
| ARNT2 (B-5)             | Mouse| Santa Cruz          | sc-393613 | CoIP  | 1 μg:
|                         |      |                     |           | WB    | 1:1,000       |
| ARNTL (B-1)             | Mouse| Santa Cruz          | sc-365645 | CoIP  | 1 μg:
|                         |      |                     |           | WB    | 1:1,000       |
| β-ACTIN                 | Mouse| Sigma-Aldrich       | A5441     | WB    | 1:10,000      |
| GAPDH                   | Mouse| Sigma-Aldrich       | G8795     | WB    | 1:100,000     |
| CREB                    | Rabbit| EMD Millipore      | 17-600    | WB    | 1:1,000       |
| Cleaved Caspase-3 (Asp175) | Rabbit| Cell Signalling  | #9661     | WB    | 1:1,000       |
| ERK1/2                  | Mouse| R&D Systems        | MAB1576   | WB    | 1:1,000       |
### Table 2.8. List of primary antibodies used for CoIP and immunoblot. (continued)

<table>
<thead>
<tr>
<th>Target</th>
<th>Host</th>
<th>Supplier</th>
<th>Cat. #</th>
<th>For</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>HIF1α</td>
<td>Rabbit</td>
<td>Novus</td>
<td>NB100-479</td>
<td>WB</td>
<td>1:1,000</td>
</tr>
<tr>
<td>HIF1α (Human)</td>
<td>Mouse</td>
<td>BD</td>
<td>610958</td>
<td>WB</td>
<td>1:1,000</td>
</tr>
<tr>
<td>NFATC1</td>
<td>Rabbit</td>
<td>Bethyl Laboratories</td>
<td>A303-508A-T</td>
<td>WB</td>
<td>1:1,000</td>
</tr>
<tr>
<td>NPAS4</td>
<td>Rabbit</td>
<td>Sigma-Aldrich</td>
<td>HPA039255</td>
<td>CoIP</td>
<td>1 μg</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1:1,000</td>
</tr>
<tr>
<td>p70S6K</td>
<td>Mouse</td>
<td>R&amp;D Systems</td>
<td>MAB8962</td>
<td>WB</td>
<td>1:1,000</td>
</tr>
<tr>
<td>Phospho-AMPK Substrate Motif</td>
<td>Rabbit</td>
<td>Cell Signalling</td>
<td>#5759</td>
<td>WB</td>
<td>1:1,000</td>
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<tr>
<td>[LXRXX(pS/pT)]</td>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phospho-CREB (SI33)</td>
<td>Rabbit</td>
<td>R&amp;D Systems</td>
<td>AF2510</td>
<td>WB</td>
<td>1:1,000</td>
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<tr>
<td>Phospho-ERK1/2 (Thr202/Tyr204)</td>
<td>Mouse</td>
<td>Cell Signalling</td>
<td>#9106</td>
<td>WB</td>
<td>1:500</td>
</tr>
<tr>
<td>Phospho-NFATC1 (SI72)</td>
<td>Mouse</td>
<td>R&amp;D Systems</td>
<td>MAB5640</td>
<td>WB</td>
<td>1:500</td>
</tr>
<tr>
<td>Phospho-p70S6K (T421/S424)</td>
<td>Rabbit</td>
<td>R&amp;D Systems</td>
<td>AF8965</td>
<td>WB</td>
<td>1:1,000</td>
</tr>
<tr>
<td>Phospho-PKB Substrate Motif</td>
<td>Rabbit</td>
<td>Cell Signalling</td>
<td>#9614</td>
<td>WB</td>
<td>1:1,000</td>
</tr>
<tr>
<td>(RXXS*/T*)</td>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phospho-PKC Substrate Motif</td>
<td>Rabbit</td>
<td>Cell Signalling</td>
<td>#6967</td>
<td>WB</td>
<td>1:1,000</td>
</tr>
<tr>
<td>[(R/KXpSX(R/K)]</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RGS2</td>
<td>Chicken</td>
<td>Sigma-Aldrich</td>
<td>GW22245F</td>
<td>WB</td>
<td>1:1,000</td>
</tr>
<tr>
<td>Ubiquitin</td>
<td>Mouse</td>
<td>Enzo Life Sciences</td>
<td>BML-PW8810</td>
<td>CoIP</td>
<td>1 μg</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1:1,000</td>
</tr>
<tr>
<td>VHL</td>
<td>Mouse</td>
<td>BD</td>
<td>556347</td>
<td>CoIP</td>
<td>1 μg</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1:1,000</td>
</tr>
</tbody>
</table>

#### 2.11.3 Determining NPAS4 post-translational modification

MIN6 cells were stimulated as in section 2.11.2, except 10 μM of the proteasomal inhibitor MG132 were used throughout the assay to prevent degradation of ubiquitinated protein. Lysates were obtained as described in section 2.11.1, and 300 μg total protein were incubated with 1 μg anti-Ubiquitin antibody (Table 2.8) using gentle agitation at 4 °C overnight. The next day, 0.75 mg Dynabeads Protein G were added to each lysate. The remaining procedure was as described in section 2.11.1, eluting IP fractions by boiling in NP-40 lysis buffer (Table 2.5) with 1 % (v/v) SDS and 1x SDS loading dye.
2.11.4 Determining competition for ARNT binding between NPAS4 and HIF1α

MIN6 cells seeded in 6-well plates (1.5 x 10⁶ cells/well) were transduced with Ad-Npas4 or Ad-EGFP at an MOI of 20:1 (section 2.7.3). Following overnight culture in DMEM/LG, cells were incubated in DMEM/HG in a 37 °C humidified CO₂ incubator set to 1 % O₂ for 4 h. Afterwards, MIN6 cells were briefly rinsed with ice-cold 1x PBS and collected by scraping in NP-40 lysis buffer (Table 2.5) supplemented with 100 μM JNJ-42.

Mouse islets were pre-incubated in KRBH-2.8G for 1 h, followed by incubation in KRBH-25G and 20 % O₂ for 1 h, and finally KRBH-25G and 1 % O₂ for 2 h. Islets were transferred into tubes, rinsed with ice-cold PBS and lysed in pre-cooled NP-40 lysis buffer.

Both MIN6 cell and islet lysates were processed as described (section 2.11.1), except 100 μM JNJ-42 were maintained throughout the assay, and insoluble material removed by spinning at 8,000 x g for 3 min. Lysates were used for CoIP immediately after determining protein concentration via BCA assay. Lysate containing 300 μg or 100 μg total protein for MIN6 cells or islets, respectively, were incubated with 2 μg anti-ARNT antibody (Table 2.8), and nutated overnight at 4 °C. The remaining procedure was as described in section 2.11.1, eluting IP fractions by boiling in NRSB-minus lysis buffer (Table 2.5) with 1x SDS loading dye.

2.12 SDS-PAGE and Western Blotting

2.12.1 Sample preparation and Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Depending on the size of the main target protein, SDS-PAGE gels with 12 % (for 40-150 kDa proteins) or 15 % (for proteins < 40 kDa) running gel formulation were cast (Table 2.9) using the Mini-PROTEAN (Biorad # 1653308, 1653312, 1653365, 1653366, 1658051) system.
Table 2.9. SDS-PAGE gel formulations.

<table>
<thead>
<tr>
<th></th>
<th>Running Gel</th>
<th>Stacking Gel</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>15 %</td>
<td>12 %</td>
</tr>
<tr>
<td>1.5 M Tris, pH 8.8</td>
<td>3.75 mL</td>
<td>3.75 mL</td>
</tr>
<tr>
<td>dH₂O</td>
<td>1.021 mL</td>
<td>2.025 mL</td>
</tr>
<tr>
<td>30 % (w/v) Acrylamide:Bis-Acrylamide (150:1)</td>
<td>5.019 mL</td>
<td>4.015 mL</td>
</tr>
<tr>
<td>SDS (10 %)</td>
<td>100 μL</td>
<td>100 μL</td>
</tr>
<tr>
<td>TEMED</td>
<td>10 μL</td>
<td>10 μL</td>
</tr>
<tr>
<td>APS (10 %)</td>
<td>100 μL</td>
<td>100 μL</td>
</tr>
</tbody>
</table>

Gels were set up in a Mini-PROTEAN Tetra Cell (Biorad # 1658000) electrophoresis chamber in 1x Running buffer (Table 2.10). Samples (with 1x SDS loading dye) were denatured by heating at 95 °C for 5 min, vortexed briefly, and spun down. Generally, 30 μg of protein per sample were loaded into a well of the SDS-PAGE gel. Power was applied (Fisher Scientific # FB300-2) until protein standards (Precision Plus Dual Color, Biorad # 1610374; BLUeye Prestained, GeneDireX # PM007-0500) had sufficiently separated. SDS-PAGE gels were disassembled and immediately processed for western blotting.

Table 2.10. SDS-PAGE and Western blotting solutions.

<p>| | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>1x Running buffer</td>
<td>25 mM Tris, 192 mM glycine, 1 % (v/v) SDS, in dH₂O</td>
</tr>
<tr>
<td>1x Transfer buffer</td>
<td>25 mM Tris, 192 mM glycine, 20 % (v/v) MeOH, in dH₂O</td>
</tr>
<tr>
<td>1x TBS-T</td>
<td>150 mM NaCl, 50 mM Tris pH 7.5, 0.1 % (v/v) Tween-20, in dH₂O</td>
</tr>
<tr>
<td>Ponceau S solution</td>
<td>0.1 % (w/v) Ponceau S, 5 % (v/v) acetic acid, in dH₂O</td>
</tr>
<tr>
<td>Blocking buffer</td>
<td>5 % (w/v) non-fatty milk powder, in 1x TBS-T</td>
</tr>
</tbody>
</table>

2.12.2 Western Blotting

SDS-PAGE gels were briefly rinsed in 1x Transfer buffer (Table 2.10). PVDF membranes (Biorad # 1620177) were activated for 1 min in 100 % MeOH and washed in 1x Transfer buffer for 5 min. The blot was assembled as per manufacturer’s instruction, using either the XCell SureLock
Mini-Cell and XCell II Blot Module (Invitrogen # EI0002), or the Criterion Blotter (Biorad # 1704070) system, and protein transfer performed with ~65-75 V at 4 °C for 2 h. Afterwards, the blot was disassembled, PVDF membranes rinsed briefly with 1x TBS-T (Table 2.10) and transfer assessed by staining with Ponceau S solution (Table 2.10) for 1-5 min. Ponceau S was rinsed off with 1x TBS-T and membranes blocked with Blocking buffer (Table 2.10) at room temperature (RT) for 1 h. Subsequently, membranes were probed with primary antibodies (Table 2.8) in Blocking buffer, using gentle agitation at 4 °C overnight. The next day, blots were washed with TBS-T three times for 5-10 min before incubating with secondary antibodies (Table 2.11) in Blocking buffer for 1-2 h.

**Table 2.11. Secondary antibodies used for western blotting.**

<table>
<thead>
<tr>
<th>Target</th>
<th>Host</th>
<th>Conjugate</th>
<th>Supplier</th>
<th>Cat. #</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-Chicken IgY</td>
<td>Rabbit</td>
<td>HRP</td>
<td>Sigma-Aldrich</td>
<td>A9046</td>
<td>1:3,000 - 1:5,000</td>
</tr>
<tr>
<td>Mouse IgG, light chain specific</td>
<td>Goat</td>
<td>HRP</td>
<td>Jackson ImmunoResearch</td>
<td>115-035-174</td>
<td>1:3,000 - 1:10,000</td>
</tr>
<tr>
<td>Rabbit IgG, F(ab’)2 fragment specific</td>
<td>Goat</td>
<td>HRP</td>
<td>Jackson ImmunoResearch</td>
<td>111-035-047</td>
<td>1:3,000 - 1:5,000</td>
</tr>
</tbody>
</table>

Following secondary antibodies, membranes were washed three times for 30-60 min. Blots were incubated with Luminata Crescendo Western HRP substrate (EMD Millipore # WBLUR0500) for 3 min, blot luminescence captured on Bioflex MSI film (Mandel Scientific # MED-CLMS810) in a dark room, and films developed on an OPTIMAX film processor (PROTEC GmbH & Co. KG). For re-probing, membranes were rinsed in TBS-T and antibodies were stripped with Restore PLUS Western Blot Stripping Buffer (Thermo Fisher Scientific # 46430). Re-probing continued with the regular procedure from the blocking step as described above. Densitometric quantification of target band intensities was performed with ImageJ (NIH) and normalized to β-ACTIN or GAPDH.
2.13 FK-506 toxicity assay

Prior to infection, MIN6 cells were seeded at 1.0 x 10^6 cells/well into 6-well plates; islets were dispersed by 0.25 % trypsin digestion (section 2.6.3) and seeded into 12-well plates. All cells were infected with Ad-Npas4 or Ad-EGFP (section 2.7.3) at an MOI of 20:1 in 800 μL medium (MIN6) or 10:1 in 250 μL medium (islets) for 2 h. Afterwards, culture medium was topped up to 2 mL or 1 mL, respectively. The following day, medium was replenished and cells recovered for 6 h in DMEM/HG or RPMI prior to treatment. Cells were then transferred to DMEM/HG or RPMI containing vehicle (DMSO) or FK-506 (10 nM or 37 nM), and cultured for 24 h. Protein lysates were obtained in NP-40 lysis buffer (MIN6) or NRSB-minus lysis buffer (islets), and FK-506 cytotoxicity was assessed via cleaved caspase-3 western blot (Table 2.8; section 2.12).

2.14 Histology

2.14.1 Tissue fixation and sectioning

Mice were euthanized and abdominal and chest cavity exposed as described in section 2.6.1. For cardiac perfusion, an incision was made in the right atrium, and 5 mL 1x PBS followed by 5 mL 4 % PFA (in 1x PBS) were perfused through the left ventricle using a 27½ gauge needle. The pancreas was excised and fixed in 4 % PFA (in 1x PBS) at 4 °C overnight. Subsequently, the tissue was washed three times in 1x PBS and dehydrated through 24-hour incubations in 50 % and 70 % EtOH (in dH2O) at 4 °C, respectively. Afterwards, the pancreas was placed in histology cassettes and run through a dehydration series (30 min each of 2x 95 % EtOH, 3x 100 % EtOH, 2x Xylenes), followed by two times liquid paraffin (Paraplast X-TRA; Leica Biosystems # 39503002) at 65 °C in a temperature-controlled oven (1 h each). Finally, tissue was paraffin-embedded using the Histocentre 2 (Thermo Scientific Shandon) embedding station. Sections of 5 μm thickness were prepared using a Shandon Finesse microtome (Thermo Scientific Shandon), mounted onto glass slides, and dried at RT overnight.
2.14.2 Immunostaining

Pancreatic sections were de-paraffinized through a rehydration series (2x 5 min Xylenes, followed by 2 min each of 3x 100 % EtOH, 2x 95 % EtOH, 2x 70 % EtOH, 2x 50 % EtOH, 1x dH₂O). Subsequently, antigen retrieval of sections was performed in 95 °C hot citrate buffer (10 mM sodium citrate, 0.05 % v/v Tween-20, pH 6.0) for 20 min. Sections were let to cool at RT for 1 h, then washed twice with 1x PBS for 5 min. Sections were incubated in blocking buffer (5 % v/v horse serum in 1x PBS) for 1 h and incubated with primary antibodies in blocking buffer at 4 °C overnight. Primary antibodies (Error! Reference source not found.) were washed off by briefly rinsing slides in 1x PBS, and sections were incubated with secondary antibodies (Error! Reference source not found.) and the DNA dye TO-PRO-3 Iodide (0.1 μM; Life Technologies # T3604) in blocking buffer at RT for 1 h. Following another three 5 min washes with 1x PBS, sections were mounted with ProLong Diamond Antifade Mountant (Life Technologies # P36965).

Table 2.12. List of primary and secondary antibodies used for immunostaining.

<table>
<thead>
<tr>
<th>Target</th>
<th>Conjugate</th>
<th>Host</th>
<th>Supplier</th>
<th>Cat. #</th>
<th>Dilution</th>
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</thead>
<tbody>
<tr>
<td>RGS2</td>
<td>Unconjugated</td>
<td>Chicken</td>
<td>Sigma-Aldrich</td>
<td>GW22245F</td>
<td>1:200</td>
</tr>
<tr>
<td>INS</td>
<td>Unconjugated</td>
<td>Guinea Pig</td>
<td>DAKO</td>
<td>A0564</td>
<td>1:1,000</td>
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<tr>
<td>GCG</td>
<td>Unconjugated</td>
<td>Mouse</td>
<td>Sigma-Aldrich</td>
<td>G2654</td>
<td>1:2,000</td>
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<tr>
<td>SST</td>
<td>Unconjugated</td>
<td>Rat</td>
<td>EMD Millipore</td>
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<tr>
<td>Chicken IgY (IgG)</td>
<td>FITC</td>
<td>Donkey</td>
<td>Jackson ImmunoResearch</td>
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<tr>
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<td>Cy3</td>
<td>Donkey</td>
<td>Jackson ImmunoResearch</td>
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<tr>
<td>Mouse IgG</td>
<td>Cy3</td>
<td>Donkey</td>
<td>Jackson ImmunoResearch</td>
<td>715-166-150</td>
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</tr>
<tr>
<td>Anti-Rat IgG</td>
<td>Alexa Fluor 594</td>
<td>Donkey</td>
<td>Jackson ImmunoResearch</td>
<td>712-586-150</td>
<td>1:450</td>
</tr>
</tbody>
</table>

Images were taken with a Leica TCS SP8 confocal microscope system (Leica Microsystems) using HC PL APO 20x/0.75 IMM CORR CS2 (Leica Microsystems # 11506343) or HC PL APO 63x/1.40 Oil CS2 (Leica Microsystems # 11506350) objectives with oil immersion. Fluorophores were excited at 488 nm (FITC), 561 nm (Cy3, Alexa Fluor 594), or 633 nm (TO-PRO-3), and
emission was collected with photomultiplier tube detectors using collection gates set to 493-550 nm (FITC), 575-615 nm (Cy3, Alexa Fluor 594), or 640-700 nm (TO-PRO-3).

2.15 ChIP-Seq

2.15.1 DNA purification and Qubit fluorometric DNA quantification

Samples were brought to 200 µL with 1x PBS, 20 µL proteinase K (Roche # 3115879001) was added, and the mix incubated at RT for 2 min. Afterwards, 4 µL RNase A (Thermo Fisher Scientific # EN0531) and 1 mL of buffer PB (from QIAquick PCR Purification kit, Qiagen # 28104) were added, followed by incubation at RT for 20 min. Half volume of the solution was transferred onto a purification column (part of Qiagen # 28104) and centrifuged at 17,900 x g for 1 min. Flow-through was discarded and the step repeated with the other half. The column was washed with 750 µL buffer PE (part of Qiagen # 28104), centrifuged at 17,900 x g for 1 min, and, after discarding flow-through, centrifugation was repeated to dry the column. DNA was eluted into a new tube using 30 µL pre-warmed (55 °C) buffer EB (part of Qiagen # 28104), incubating 1 min, and centrifuging at 17,900 x g for 1 min. To maximise yield, this step was repeated with another 30 µL buffer EB.

DNA concentration was measured using the Qubit fluorometer (Life Technologies # Q32857) and Qubit dsDNA HS Assay kit (Life Technologies # Q32851) according to the manufacturer's instructions.

2.15.2 Bioanalyzer

To analyze DNA integrity and fragment sizes, the 2100 Bioanalyzer instrument (Agilent Technologies # G2939BA) and High Sensitivity DNA Analysis Kit (Agilent Technologies # 5067-4626) were used according to manufacturer’s instructions.
2.15.3 Chromatin immunoprecipitation (ChIP)

ChIP procedures were modified from Johnson et al. (2007) [757]. Per condition, two 15 cm dishes of MIN6 cells were seeded at a density of 1.0 x 10^7 cells. The next day, cells were transferred to DMEM/LG medium overnight. Treatment was performed in a 37 °C CO_2 incubator with three different experimental conditions: 1) DMEM/LG medium (basal; LG) for 2 h, 2) DMEM/HG medium with 40 mM KCl (stimulatory; KCL) for 2 h, or 3) DMEM/HG medium and 1 % O_2 (HIF1α-stabilizing; HYPO) for 4 h. Afterwards, EM-grade paraformaldehyde (Electron Microscopy Sciences # 15710) was added directly to stimulation media to a final concentration of 1 %, using gentle agitation at RT for 10 min. Cross-linking was stopped by adding glycine to a final concentration of 0.125 M and gentle agitation at RT for 5 min. Medium was removed and cross-linked cells washed thrice with an equal volume of cold (4 °C) 1x PBS. Residual 1x PBS was aspirated completely, cells scraped off in 8 mL cold (4 °C) Farnham lysis buffer (Table 2.13), transferred into 15 mL conical tubes, and pelleted by centrifugation at 2,000 rpm and 4 °C for 5 min. Supernatant was removed, cell pellets snap-frozen in liquid nitrogen and stored at -80 °C.

Table 2.13. ChIP solutions.
All buffers were filtered with a 0.2 - 0.45 μm filter unit; all percentages are v/v.

<table>
<thead>
<tr>
<th>Farnham lysis buffer</th>
<th>RIPA buffer</th>
<th>PBS/BSA</th>
<th>LiCl IP wash buffer</th>
<th>1x TE</th>
<th>IP elution buffer</th>
</tr>
</thead>
<tbody>
<tr>
<td>5 mM PIPES, pH 8.0</td>
<td>0.1 % SDS</td>
<td>5 mg/mL BSA (fraction V)</td>
<td>100 mM Tris, pH 7.5</td>
<td>10 mM Tris-HCl, pH 7.5</td>
<td>1 % SDS</td>
</tr>
<tr>
<td>85 mM KCl</td>
<td>0.5 % sodium deoxycholate</td>
<td>500 mM LiCl</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.5 % IGEPAL</td>
<td>1 % IGEPAL</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>protease inhibitor cocktail (Roche # 5056489001); add just before use</td>
<td>protease inhibitor cocktail (Roche # 5056489001); add just before use</td>
<td>protease inhibitor cocktail (Roche # 5056489001); add just before use</td>
<td>1 % sodium deoxycholate</td>
<td>0.1 mM Na_2EDTA</td>
<td>0.1 M NaHCO_3</td>
</tr>
<tr>
<td>in dH_2O</td>
<td>in 1x PBS</td>
<td>in 1x PBS</td>
<td>in dH_2O</td>
<td>in dH_2O</td>
<td>in dH_2O</td>
</tr>
<tr>
<td>stored at 4 °C for 4-6 weeks</td>
<td>stored at 4 °C for 4-6 weeks</td>
<td>prepared fresh, kept at 4 °C</td>
<td>prepared fresh, kept at 4 °C</td>
<td>prepared fresh, kept at 4 °C</td>
<td>prepared fresh, kept at RT</td>
</tr>
</tbody>
</table>
Samples were thawed and kept on ice prior to sonication. Initially, pellets were resuspended in 1 mL Farnham lysis buffer by gentle pipetting and rotation at 4 °C for 10 min. Crude nuclear fractions were obtained by centrifugation at 2,000 rpm at 4 °C for 5 min, discarding the supernatant and resuspending via pipetting in 1 mL RIPA buffer (Table 2.13). Exactly 1 mL of crude nuclei was transferred into a milliTUBE (Covaris # 520135), and the tube inserted into the M220 Holder XTU Insert (Covaris # 500422). To obtain genomic fragments of 150-300 bp in size, sonication was performed with a M220 focused-ultrasonicator (Covaris # PN 500295) using 20 % duty cycle, 75 W peak incident power, and 200 cycles per burst, at 4 °C for 20 min. Sonicated lysate was transferred to a 1.5 mL microcentrifuge tube and insoluble material was removed by centrifugation at 20,000 x g for 10 min at 4 °C. Supernatant was transferred to a new tube and kept on ice while quality checks were performed. Briefly, DNA concentration was quantified using an aliquot of 10-20 μL sonicated chromatin, as described in section 2.15.1. DNA fragment sizes of sonicated lysate were assessed by running 1 μg lysate on a 1 % agarose gel containing RedSafe Nucleic Acid Staining Solution (FroggaBio # 21141).

In the meantime, primary antibody (Mouse anti-ARNT, Novus # NB100-124; Rabbit anti-NPAS4, Sigma-Aldrich # HPA039255) was coupled to Dynabeads (M-280 Sheep anti-Mouse IgG, Life Technologies # 11201D; M-280 Sheep anti-Rabbit IgG, Life Technologies # 11203D). For each lysate, 200 μL Dynabeads were prepared. Initially, beads were added into a tube on ice containing 1 mL cold (4 °C) PBS/BSA (Table 2.13). Beads were mixed by inverting, magnetically separated from supernatant, and resuspended in 1 mL cold PBS/BSA. This step was repeated three times, resuspending the beads in 200 μL PBS/BSA after the final wash. Next, beads were split into two tubes of 100 μL each, adding 4 μg of primary antibody (anti-ARNT or anti-NPAS4) into one aliquot (for ChIP), and an equal volume PBS/BSA into the other (for bead control). Both tubes were gently mixed on a rotator platform at 4 °C for 2-4 h. Afterwards, supernatant was removed on a magnetic rack, and uncoupled/antibody-coupled beads washed three times.
with 1 mL PBS/BSA. After the last wash, uncoupled/antibody-coupled beads were resuspended in 100 μL PBS/BSA each.

From each chromatin sample, an aliquot of 1.25-2.5 μg chromatin (5 %) was adjusted to 50 μL with RIPA buffer and kept as input, whereas two tubes with 25-50 μg chromatin each were adjusted to 400 μL with RIPA buffer. To the latter two samples, 100 μL of either uncoupled (bead control fraction) or antibody-coupled (ChIP fraction) Dynabeads were added, and incubation continued on a rotator at 4 °C overnight; input samples were rotated alongside. The next day, beads containing immuno-bound chromatin (chromatin-beads) were magnetically separated from supernatant. The chromatin-beads were washed five times with 1 mL LiCl wash buffer (Table 2.13) on a rotator at 4 °C for 3 min each. In between washes, chromatin-beads were separated magnetically and supernatant discarded. A final wash was performed with 1 mL TE buffer (Table 2.13) on a rotator at 4 °C for 1 min. Chromatin-beads were magnetically separated from supernatant and resuspended in 200 μL IP elution buffer at RT, whereas 150 μL elution buffer was added to input fractions. Samples were briefly mixed by vortexing, and immuno-bound chromatin eluted from beads by heating and shaking every 10 min at 65 °C for 1 h. Input samples were processed alongside. Afterwards, samples were centrifuged at 14,000 x g for 3 min, and eluate containing immunoprecipitated chromatin collected on a magnetic rack, disposing of the beads. Cross-linking was reversed by incubating ChIP eluate and inputs at 65 °C overnight, with agitation every 10 min. The next day, ChIP eluate was purified and DNA concentration measured as described in section 2.15.1. Initial assessment of DNA enrichment by ChIP was performed via qPCR, using Fast SYBR Green Master Mix (Applied Biosystems # 4385612) as per manufacturer’s instructions with oligonucleotides outlined in Table 2.14.
Table 2.14. Oligonucleotides used for ChIP SYBR Green qPCR.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Region</th>
<th>Forward primer (5’ &gt; 3’)</th>
<th>Reverse primer (5’ &gt; 3’)</th>
<th>Purpose</th>
</tr>
</thead>
<tbody>
<tr>
<td>FoxP1</td>
<td>enhancer</td>
<td>Primer LL711: GTCGTTGGGGGAAGTCCAT</td>
<td>Primer LL712: TGTGTACACAGTGGTCGCTC</td>
<td>potential ARNT binding site</td>
</tr>
<tr>
<td>Ldha</td>
<td>promoter</td>
<td>Primer LL713: CACGATGTCCCTGCAAGAGT</td>
<td>Primer LL714: TGGTGCTTGCCAGGAGTTGT</td>
<td>potential ARNT binding site</td>
</tr>
<tr>
<td>Lepr</td>
<td>promoter</td>
<td>TAGAATGGAGCACTAGGTTG</td>
<td>CTCTTATAACTGCCCCAGTG</td>
<td>negative control</td>
</tr>
</tbody>
</table>

2.15.4 Library construction

Library construction was performed with the DNA SMART ChIPSeq 48 A kit (Takara Bio USA # 634866) or the ThruPLEX DNA-seq 48S kit (Takara Bio USA/Rubicon Genomics # R400427) according to the manufacturer’s instructions. DNA integrity, concentration and size following library construction were assessed using Qubit (without prior DNA purification; section 2.15.1), Bioanalyzer (section 2.15.2), and KAPA Library Quantification kit for Illumina platforms (KAPA Biosystems/Roche # KK4824 – 07960140001) according to the manufacturer’s instructions.

2.15.5 Sequencing

Ultra-high throughput sequencing was performed on a NextSeq 500 platform (Illumina # SY-415-1001) using either the NextSeq 500/550 Mid Output v2 (150 cycles) kit (Illumina # FC-404-2001) or NextSeq 500/550 High Output v2 (75 cycles) kit (Illumina # FC-404-2005) according to the manufacturer’s instructions.

2.15.6 Sequencing data processing and analysis

Sequencing results were concatenated in the .fastq file format and aligned to the mouse genome (mm10) using the BWA MEM algorithm:

```bash
bwa mem -M -t 32 mm10 input_read1.fastq.gz input_read2.fastq.gz > output.sam
```
Single-read data:

\texttt{bwa mem -M -t 32 mm10 input\_read1.fastq.gz > output.sam}

Next, the aligned sequencing file was converted into .bam format using Samtools:

\texttt{samtools view -b -o output.bam input.sam}

The resulting .bam files were sorted by name to fix read pairing information and flags using Samtools:

\texttt{a. samtools sort -o output.nSrt.bam -O bam -n -T \( /tmp/tmp.nSrt \) @ 32 input.bam}

\texttt{b. samtools fixmate -O bam input.nSrt.bam output.fixed.bam}

Subsequently, .fixed.bam files were re-sorted by coordinates through Samtools and duplicates removed with Picard:

\texttt{a. samtools sort -o output.sorted.bam -O bam -T \( /tmp/tmp.sorted \) @ 32 input.fixed.bam}

\texttt{b. java -Xmx16g -jar picard.jar MarkDuplicates ASSUME\_SORTED=true REMOVE\_DUPLICATES=true INPUT=input.sorted.bam OUTPUT=output.srtRmDup.bam METRICS\_FILE=srtRmDup.metrics.out}

The resulting srtRmDup.bam files were merged by experimental conditions (replicates merged into one .bam file), and the resulting files indexed and alignment statistics calculated using Samtools:

\texttt{a. samtools merge -f output.RmDupMerged.bam input\_replicate\_1.srtRmDup.bam input\_replicate\_2.srtRmDup.bam [...]}

\texttt{b. samtools index -b input.RmDupMerged.bam}
c. `samtools flagstat input.RmDupMerged.bam > output.stats.out`

Next, peaks enriched in IP fractions over Input (Control) fractions were called via MACS2:

`macs2 callpeak -t input_IP.RmDupMerged.bam -c input_Control.RmDupMerged.bam -g mm -n Example_Name -B -q 0.01 -m 4 40 --bw 400 --outdir /Output_Folder/`

MACS2 produced .bed files of called peaks, which were used to identify enriched DNA binding motifs and to annotate peaks using HOMER2:

a. `findMotifsGenome.pl input_summits.bed mm10 /Outout_Folder/ -size 50 -mask -len 6,7,8,9,10,11,12 -p 16 -S 20`

b. `annotatePeaks.pl input_summits.bed mm10 -size 1000 -gsize 2150570000 -annStats input_summits.bed > output_peaks.annotated.txt`

Bedtools was used to determine overlap between ChIP:ARNT and ChIP:NPAS4 peaks:

`bedtools intersect -wo -a /summits_ARNT.bed -b /summits_NPAS4.bed > overlap.out`

Further data analysis was performed in IGV, Prism 7, and Excel.

### 2.16 Calcium imaging

To assess a role for RGS2 in regulating calcium influx, MIN6 cells were seeded at 1 x 10^6 cells/well onto glass cover slips (25 mm; VWR # 16004-310) in a 6-well plate and let to attach in 2 mL DMEM/HG. The next day, transduction with Ad-Rgs2 and Ad-βGal was performed as described (section 2.7.3) using an MOI of 50:1. After overnight culture in DMEM/LG, MIN6 cells were incubated with 1 mL serum-free KRBH-2.8G containing 5 μM of the Ca^{2+} indicator Fura-2-AM [758] (Invitrogen # F1221) for 30 min at 37 °C. Cover slips were then transferred to a 2 mL perifusion chamber and washed with KRBH-2.8G at a flow rate of 2.5 mL/min for another 30 min. Ca^{2+}_{cyt} levels were monitored in cells perifused with reagents in KRBH in the following
order: 2.8 mM glucose for 10 min, 25 mM glucose for 20 min, 2.8 mM glucose for 20 min, and 2.8 mM glucose with 40 mM KCl for 5 min. Widefield fluorescent images were taken with a DMI6000 inverted microscope (Leica Microsystems) using a HC PL FLUOTAR 10x/0.30 DRY (Leica Microsystems # 11506505) objective and digital camera (Leica Microsystems # DFC365 FX). Briefly, this involved alternating excitation of intracellular Fura-2 at 340 nm (calcium-bound Fura-2) and 380 nm (calcium-free Fura-2) in 10 s intervals. The respective emission (F_{340} and F_{380}) was acquired using a 409 nm dichroic mirror and 502-538 nm emission filter. Ca^{2+}_{cyt} levels were expressed as the F_{340} / F_{380} ratio, and area under the curve (AUC) was determined using Prism 6.

*Rgs2* mutant cell lines were seeded at 1 x 10^6 cells/well onto 25 mm glass cover slips in a 6-well plate and let to attach in 2 mL DMEM/HG. After overnight culture in DMEM/LG, cells were treated identically to the above procedure.

To exclude an effect of KN-93 on calcium influx, MIN6 cells seeded as above were pre-incubated in serum-free KRBH-2.8G containing 5 μM Fura-2-AM and either 3 μM KN-93 or vehicle (dH2O) for 30 min, after which they were transferred to a perifusion chamber and washed with KRBH-2.8G ± KN-93 for another 20 min. F_{340} and F_{380} emission recordings started at this point, and baseline calcium levels in KRBH-2.8G ± KN-93 were measured for 10 min. Afterwards, cells were perifused with KRBH-2.8G supplemented with 40 mM KCl ± KN-93 for 5 min, after which perifusion was stopped. Imaging continued for another 10 min to observe the decline from peak Ca^{2+}_{cyt} levels to a steady tonic plateau.

### 2.17 Metabolic flux assay (Seahorse)

XFe96 Spheroid Microplates (Agilent Technologies/Seahorse Biosciences # 102959-100) were coated with 100 μg/mL poly-D lysine (Sigma-Aldrich # P0899) for 1 h at RT. Excess poly-D-lysine was removed and microplates dried for 30 min at 37 °C. Islet medium (100 μL
RPMI) was added to the microplate and 10-12 N4 PKO or control islets loaded per well. The next day, 100 μM JNJ-42 or vehicle (DMSO) was added. Following a 24-hour incubation period, islets were washed with Seahorse base medium (Sigma-Aldrich # D5030; 2 mM glutamine, 2.8 mM glucose, pH 7.4). Islets were then incubated in base medium (containing 100 μM JNJ-42 or vehicle, respectively) for 90 min in a non-CO₂ incubator at 37 °C. The microplate was subsequently transferred into a Seahorse XFe96 Analyzer to measure oxygen levels and calculate oxygen consumption rate (OCR). Basal OCR was recorded in base medium for three measurement cycles (1 min mix, 1 min wait, 8 min measure) before injecting assay compounds, and measurements taken sequentially with 25 mM glucose (24 cycles), 4 μM oligomycin (3 cycles), 2 μM FCCP / 10 mM pyruvate (3 cycles), and 4 μM antimycin A / 4 μM rotenone (3 cycles). OCR was normalized to baseline and AUC calculated using the Seahorse XF Cell Mito Stress Test Summary Report.

To study an effect of RGS2 on oxygen consumption, wild-type (B6) islets cells were dispersed, seeded onto a Seahorse XF96 microplate at 20,000 cells/well, and transduced with Ad-βGal (control) or Ad-Rgs2 at an MOI of 10:1. After 1 day of recovery, cells were pre-incubated in Seahorse base medium in a non-CO₂ incubator for 1 h. After transfer to the Seahorse metabolic analyzer, OCR was measured in response to gradually increasing glucose concentrations (2.8-25 mM). Basal OCR was recorded in base medium for 5 measurement cycles (1 min mix, 3 min measure) before injecting assay compounds, and measurements taken sequentially with 5.5 mM, 11 mM, 16 mM, and 25 mM glucose (3 cycles each of 3 min mix, 3 min measure). OCR was normalized to baseline and AUC calculated using Prism 7.

2.18 CRISPR-Cas9 gene editing

The general strategy was described by Krentz et al. (2014) [759], including construction of the CRISPR-Cas9 vector pCCC. The sgRNAs for targeting exon 1 of mouse Rgs2 (Table 2.15) were
designed based on an algorithm by Doench et al.\textsuperscript{[760;761]} and cloned into the BbsI site of pCCC according to Ran et al. (2013).\textsuperscript{[762]} For the donor plasmid, a 498 bp \textit{Rgs2} homology region with a mutation replacing an alternative translation initiation codon in \textit{Rgs2} exon 1 with stop codons (5'-ATGAAGCGGA-3' > 5'-TAGATAGCTAGGTAC-3'; start and stop codons underlined) was cloned into pJET1.2/blunt, generating pJET1.2/Rgs2-STOP.

**Table 2.15. Sequences of sgRNAs and oligonucleotides for cloning into the pCCC vector.**
Underlined uppercase letters represent sgRNA sequence, bold uppercase letters highlight the PAM sequence, and lowercase letters denote BbsI-compatible overhangs for cloning into the pCCC BbsI site.

<table>
<thead>
<tr>
<th>sgRNA (score)</th>
<th>sgRNA (and \textit{Rgs2} genomic sequence (5' &gt; 3'))</th>
<th>Oligonucleotide sequences</th>
</tr>
</thead>
<tbody>
<tr>
<td>sg720/21 (0.6016)</td>
<td>GTACCATGGGACAAGAGTGC CTGGATACCCTGGACAAGAGTGCAGGCAA</td>
<td>LL720 5'-caccGTACCATGGGACAAGAGTGC-3' 3'-CATGGGATCCGGTCTTCAGGcaa-5'</td>
</tr>
<tr>
<td>sg722/23 (0.6468)</td>
<td>ATGGGACAAGAGTGCAGGCAA ACCATGGGACAAGAGTGCAGGCAAAGGGCCC</td>
<td>LL722 5'-caccGATGGGACAAGAGTGCAGGCAA-3' LL723 3'-CTACCTCGTTCTCAGGTTGTTcaa-5'</td>
</tr>
</tbody>
</table>

Low-passage MIN6 cells (≤ p27) were used for genome editing and seeded at a density of 1 x 10^6 cells/well in a 6-well plate. The next day, cells were transfected using lipofectamine 3000. First, 6.25 μL lipofectamine 3000 and DNA (2.5 μg pJET1.2/Rgs2-STOP with 0.625 μg of either pCCC-sg720/21 or pCCC-sg720/21) combined with 6.25 μL P3000 reagent were separately diluted in 125 μL DMEM/High Glucose without supplements. Both solutions were combined at RT for 15-20 min before adding onto MIN6 cells in 1 mL DMEM/High Glucose without supplements. After 6 h, medium was brought to 2 mL full culture medium (DMEM/HG) and transfection continued overnight. Medium was replaced the next day, and cells were FACS-sorted 24 h later. GFP-positive cells were diluted to single-cell density in 15 cm dishes and allowed to form clonal populations. DMEM/HG was changed every 4-7 days. After 4-6 weeks, clones were manually picked into 96-well plates containing 30 μL of 0.05 % trypsin, digested at 37 °C for 10 min, and trypsinization stopped by topping up with 150 μL DMEM/HG. Medium was changed every 2-3 days until cells were confluent. Replica plates were prepared by passaging with trypsin. One plate was used for genotyping by extracting DNA with 35 μL...
QuickExtract (Epicentre # QE09050); briefly, cells were lysed 10 min at 37 °C, lysate transferred to a 96-well PCR plate, and incubated for 10 min at 65 °C and 5 min at 95 °C before storage at -20 °C. The remaining 96-well plate was used to grow up clones via serial passaging, and liquid nitrogen stocks were prepared. Initial genotyping of Rgs2 clones was done via PCR using primers LL703/LL705 (Table 2.16), generating a product of 346 bp only in clones carrying the Rgs2-STOP mutation. Results were confirmed via KpnI digest of a PCR product across the mutated site using primers LL515/LL514 (Table 2.16). The wild-type 568 bp product was not digested by KpnI, whereas two fragments of 397 bp and 176 bp were generated by KpnI from the 573 bp fragment in mutant Rgs2-STOP clones. Finally, LL515/LL514 PCR products of select clones were sent for standard Sanger sequencing.

Table 2.16. CRISPR-Cas9 genotyping oligonucleotides.

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence (5' &gt; 3')</th>
</tr>
</thead>
<tbody>
<tr>
<td>LL515</td>
<td>GAAGTG CCTACAAATCTCTCTGT</td>
</tr>
<tr>
<td>LL514</td>
<td>GGTAGC TTGCTAGTG GGTG TAT</td>
</tr>
<tr>
<td>LL703</td>
<td>GCGGAGAAATAGATAGCTGGTAC</td>
</tr>
<tr>
<td>LL705</td>
<td>AGCTGATAACGTCCACCG</td>
</tr>
</tbody>
</table>

2.19 Statistics

Statistical tests were performed in Prism 6 and 7 (GraphPad, San Diego, CA, USA). Statistical tests are indicated in figure legends. Generally, two groups were compared using Student’s t-test (parametric) or Mann-Whitney U test (nonparametric), whereas three or more groups were compared using one- or two-way ANOVA (parametric) or Kruskal-Wallis test (non-parametric), with an appropriate multiple comparisons test, respectively. Significance is denoted with * = p ≤ 0.05, ** = p ≤ 0.01, or *** = p ≤ 0.001. All data is presented as mean ± SEM.
Chapter 3: *In vitro* regulation of NPAS4 expression in β-cells

3.1 Background

To control systemic glycemia, β-cells must sense and respond to changes in ambient glucose with the release of precise doses of the glucose-lowering hormone insulin \[77; 763; 764\]. This process involves β-cell glucose uptake, metabolism through oxidative phosphorylation to generate ATP, and depolarization via closure of the ATP-sensitive potassium (K\(_{ATP}\)) channel \[77; 763; 764\]. Subsequent opening of the voltage-gated L-VDCC results in influx of calcium into the cytosol and insulin exocytosis \[77; 763; 764\]. Precise spatiotemporal regulation of calcium levels in different subcellular compartments is a crucial part of this process in mature, functional β-cells \[77; 763; 764\]. Calcium is a broadly-acting second messenger controlling many processes, which in β-cells includes signalling pathways (Figure 1.3, Figure 3.1) enhancing insulin secretion, β-cell function and survival \[77; 264; 382; 391; 418; 422; 446; 765-769\]. The first step in the signalling cascades involves binding of free calcium, either directly or via the sensor protein CaM (Ca\(^{2+}/CaM\)), which then activates the CaMK, CaN, MAPK and PI3K-PKB pathways \[1; 770\]. Most calcium signalling pathways modulate the activity of downstream transcription factors, such as members of the CREB, FOXO, NFAT and MEF2 families (Figure 1.3, Figure 3.1) \[1; 770\]. Under normal physiological conditions, adaptive gene expression changes mediated by activation of these transcription factors are beneficial for β-cells. For example, activation of CREB promotes insulin- \[^{423}\] and incretin-dependent \[^{392; 602}\] survival, NFAT stimulates insulin production \[^{382}\], secretion \[^{398}\], and β-cell survival \[^{397; 399}\], and FOXO1 maintains β-cells in a mature state \[^{771}\]. However, chronic activation of these pathways has deleterious effects. During T2D progression, hyperglycemia and increased fatty acid levels tend to increase β-cell activity and intracellular calcium influx \[^{772; 773}\], which when becoming chronic leads to β-cell dysfunction, dedifferentiation, ER stress and apoptosis \[^{202; 394; 410; 411; 415; 434; 437; 438; 774; 775}\]. This correlates with
widespread gene expression changes in T2D islets \cite{296; 481}. At the same time, detrimental effects of excessive calcium signalling in T2D patients can be reversed by temporarily blocking β-cell activity with diazoxide, promoting “β-cell rest” \cite{439; 440}. Together, these studies highlight the importance of maintaining an appropriate balance of calcium signalling, and warrant further investigation of the calcium-dependent mechanisms underlying transcriptional changes and the switch from β-cell maintenance to dysfunction.

Figure 3.1. Simplified schematic of calcium-dependent signalling pathways targeted by pharmacological inhibitors.

Glucose-induced depolarization and ligand-receptor interaction lead to a rise in cytosolic calcium (Ca^{2+}_{cyt}) levels, via calcium influx through the L-type voltage-dependent calcium channel (L-VDCC) and ER calcium release. The rise in Ca^{2+}_{cyt} leads to activation of kinase or phosphatase activity, which in turn results in the recruitment of transcription factors to regulatory elements and changes in gene transcription. Inhibitors in red.
The bHLH-PAS domain transcription factor NPAS4 was previously classified as an immediate early gene in neurons and β-cells \[^{354;446}\]. Its expression in mouse islets and MIN6 cells was induced substantially by glucose or membrane depolarization, respectively, and depended on an increase in intracellular calcium levels \[^{446}\]. Functionally, it prevents β-cell cytotoxic insults by reducing ER stress and apoptosis \[^{446}\], perhaps due reductions in insulin production \[^{446;776}\]. Although the prior study by Sabatini et al. (2013) \[^{446}\] clarified much of the role NPAS4 plays in β-cells \textit{in vitro}, the calcium-dependent pathways that regulate its expression remained unknown. Consequently, studies in this chapter were designed to reveal the calcium-dependent pathways controlling NPAS4 expression as well as protein stability.

### 3.2 NPAS4 expression depends on CaMK, PKB and CaN signalling in β-cells

In MIN6 cells, high glucose with KCl, but not high glucose alone, potently induced \textit{Npas4} mRNA expression, with an average 45-fold induction over basal levels within a two-hour period (Figure 3.2) \[^{446}\]. The divalent ion chelator EGTA completely prevented upregulation of \textit{Npas4} (Figure 3.2). These results are consistent with the previously published activity- and calcium-dependent induction of \textit{Npas4} in β-cells \[^{446}\].
Figure 3.2. Npas4 induction is calcium-dependent.
MIN6 cells were incubated in basal medium alone (white bars; Basal) or plus 40 mM KCl (black bars; KCl) for 2 h before treatment with 5 mM of the calcium chelator EGTA (n = 23). Significance was determined using a one-way ANOVA with Dunnett's multiple comparisons test. *** p ≤ 0.001.

To delineate which calcium signalling pathways control NPAS4 expression, I chose a pharmacological approach using inhibitors of calcium-dependent kinases and phosphatases. Initially, I verified inhibitor effectiveness via immunoblot in response to membrane depolarization in MIN6 cells. Bands corresponding to proteins containing PKC, AMPK and PKB consensus phospho-motifs were reduced when using the PKC inhibitor BIM-XI (Figure 3.3 A), AMPK inhibitor Compound C or CaMKK inhibitor STO-609 (Figure 3.3 D&E), and PKB inhibitor Akti-1/2, respectively (Figure 3.3 F). KCl-induced phosphorylation of MAPK3/1 was abolished with the MAP2K1/2 inhibitor PD0325901 (Figure 3.3 B), while the CaMK inhibitor KN-93 reduced phospho-CREB, and the MTOR inhibitor rapamycin reduced phospho-P70S6K (Figure 3.3 C). A more specific peptide inhibitor of CaMKII, AIP, only modestly decreased phospho-CREB expression (Figure 3.3 C). KCl treatment slightly reduced phosphorylation of NFATC1, and the inhibitor of the phosphatase CaN, FK-506, prevented this trend (Figure 3.3 H). Together, these results demonstrate that the chosen treatment (high glucose + KCl) stimulates calcium signalling pathways in MIN6 cells, and that inhibitors worked efficiently (except AIP) at the concentrations used.
**Figure 3.3. Positive controls for pharmacological inhibitors using immunoblot for relevant phospho-proteins in MIN6 β-cells.**

MIN6 cells were stimulated with either basal medium (Basal) or 40 mM KCl in high glucose medium (KCl) with control (water/DMSO) or the indicated inhibitor. (A) 100 nM BIM-XI reduced phosphorylation of several proteins bearing the PKC substrate motif (R/K)XpSX(R/K). (B) 1 μM PD0325901 inhibited Thr202/Tyr204 phosphorylation of MAPK3/1. (C) 3 μM KN-93 reduced Ser133 phosphorylation of CREB, while 4 μM AIP did not. (D&E) 1 μM Compound C and 3 μM STO-609 reduced phosphorylation of several proteins bearing the AMPK substrate motif LXRXX(pS/pT). (F) 10 μM Akti-1/2 reduced phosphorylation of proteins bearing the PKB substrate motif RXX(pS/pT). (G) 25 nM rapamycin (Rapa) inhibited Thr421/Ser424 phosphorylation of P70S6K. (H) 100 nM FK-506 modestly reduced dephosphorylation of NFATC1 at Ser172.
3.2.1 Regulation at the RNA level

Using the inhibitors described above, *Npas4* mRNA induction was measured and compared to high-glucose/KCl-induced expression. KN-93 was the most potent suppressor of *Npas4* expression, completely abrogating *Npas4* induction (Figure 3.4B). Importantly, this was not due to defects in calcium influx (Figure 3.4A), which was impaired by KN-93 in other studies [765; 777]. Because KN-93 may affect multiple members of the CaMK family [297], a role for CaMKII was examined with the specific CaMKII inhibitor AIP [778]. At the RNA level, AIP significantly reduced *Npas4* induction by KCl, albeit at lower levels, suggesting that the inhibition of *Npas4* induction achieved with KN-93 is at least partially dependent on CaMKII (Figure 3.4C). Because AIP only moderately inhibited phospho-CREB (Figure 3.3C), it is possible that greater reduction of *Npas4* induction could be achieved using a more potent CaMKII inhibitor.

Figure 3.4. *Npas4* mRNA expression in MIN6 β-cells relies on the CaMK, PKB and CaN signalling pathways.
(A) MIN6 cells on glass cover slips were pre-incubated with 5 μM Fura-2-AM plus KN-93 or vehicle (water) in KRBH containing 2.8 mM glucose (KRBH-2.8G) for 30 min, after which they were transferred to a perfusion chamber and washed with KRBH-2.8G ± KN-93 for another 20 min before starting recordings (n = 4). (B-F) MIN6 cells were kept in basal medium, either alone (white bars; Basal) or plus 40 mM KCl (black bars; KCl) for 2 h in the presence of inhibitors or vehicle control. (B) Npas4 induction was completely prevented with the CaMK inhibitor KN-93 at either 3 μM or 30 μM (n = 3). (C) Using 4 μM of the CaMKII-specific peptide inhibitor AIP partially reduced Npas4 induction (n = 7). (D) Treatment with 10 μM of Akti-1/2 partially reduces Npas4 mRNA induction, while treatment with 25 nM of the MTOR inhibitor rapamycin (Rapa) had no effect (n = 3). (E) Treatment with 20 nM human insulin in low glucose KRBH did not induce Npas4 expression (n = 3). (F) The CaN inhibitor FK-506 (tacrolimus) significantly reduced Npas4 induction at doses of 10 nM and 100 nM (n = 5). Significance was determined using an unpaired two-tailed Student’s t test (A, C, E) or a one-way ANOVA with Dunnett’s multiple comparisons test (B, D, F). * p ≤ 0.05; ** p ≤ 0.01.

The main role for the β-cell is to secrete insulin, but it can also sense insulin itself via the insulin receptor and insulin-like growth factor I receptor, enabling autocrine-paracrine effects on β-cells [779]. Insulin signalling through its receptors involves activation and translocation of PKB to the plasma membrane (via PI3K-generated PIP3, PDPK1, MTOR; Figure 1.3) [366; 779]. Upon activation, PKB phosphorylates various targets, including MTOR [366; 779]. The best described role of autocrine insulin signalling is in the maintenance of β-cell mass [780-782], but it also increases cytosolic calcium levels [237; 783] and insulin secretion [784; 785], and modulates gene expression [786; 787]. Some elements of autocrine insulin signalling, particularly those regarding insulin secretion, are debated [788-791], perhaps due to dose-dependent effects [788; 792; 793]. The role of insulin receptor signalling for Npas4 expression was thus assessed using the PKB inhibitor Akti-1/2, and the MTOR inhibitor rapamycin. Modestly reduced Npas4 induction is observed with high doses of Akti-1/2, whereas rapamycin fails to inhibit Npas4 induction (Figure 3.4 D).

Furthermore, although insulin signalling induces transcription of other genes in β-cells [794], application of physiologic doses of insulin alone was not sufficient for the induction of Npas4 in the absence of other stimuli (such as serum, glucose) (Figure 3.4 E).

Another important signalling hub in β-cells is through the phosphatase CaN, via dephosphorylation and nuclear translocation of the NFAT transcription factors. The CaN inhibitor FK-506, which is used clinically as an immunosuppressant, reduced Npas4 induction
significantly at doses as low as 10 nM, indicating that CaN signalling is an important pathway through which *Npas4* transcription is activated (Figure 3.4 F).

Finally, pharmacological inhibition of CaMKK, AMPK, PKC or MAP2K1/2 did not affect *Npas4* induction (Figure 3.5 A-D). There was, however, a trend towards slightly decreased *Npas4* mRNA levels following AMPK and MAP2K1/2 inhibition (Figure 3.5 B&D).

![Figure 3.5](image.png)

**Figure 3.5. Pharmacological inhibition of several calcium signalling pathways did not affect *Npas4* mRNA induction.**

MIN6 cells were kept in either basal medium alone (white bars; Basal) or plus 40 mM KCl (black bars; KCl) for 2 h in the presence of inhibitors or vehicle control. (A-D) 3 μM and 30 μM of the CaMKK inhibitor STO-609 (A; *n* = 3-4); 1 μM and 10 μM AMPK inhibitor Compound C (B; *n* = 3-4); 10 nM and 100 nM of PKC inhibitor BIM-X1 (C; *n* = 3); or 1 μM and 10 μM of MAP2K1/2 (MEK1/2) inhibitor PD0325901 (D; *n* = 5) did not significantly reduce induction of *Npas4* expression. Significance was determined using a one-way ANOVA with Dunnett’s multiple comparisons test.
In summary, the above findings reveal that CaMKII, PKB and CaN signalling pathways are key elements in the regulation of *Npas4* transcription in β-cells.

### 3.2.2 Regulation at the protein level

To determine whether NPAS4 protein levels were affected in the same way as mRNA by these inhibitors, I used a similar experimental approach as noted above, followed by immunoblotting. As expected, KN-93 completely abrogated NPAS4 induction in both MIN6 cells and islets (Figure 3.6 A-D), likely due to its pronounced inhibition of *Npas4* transcription (Figure 3.4 B). Strikingly, while inhibition of PKB only resulted in a modest reduction in *Npas4* transcript (Figure 3.4 D), application of inhibitor resulted in a marked decrease at the protein level, strongly implicating effects on protein translation or stability (Figure 3.6 E-H). The effects were independent of MTOR signalling, since rapamycin had no effect (Figure 3.6 E&F). The CaN inhibitor FK-506 reduced NPAS4 expression by an even greater magnitude than at the transcriptional level (Figure 3.4 F) in both MIN6 and islets, raising the possibility that CaN has additional effects on NPAS4 translation or stability (Figure 3.6 I-L).
Figure 3.6. NPAS4 protein expression in β-cells depends on the CaMK, PKB and CaN signalling pathways.
MIN6 cells (A&B; E&F; I&J) were kept in either basal medium alone (Basal) or were stimulated with 40 mM KCl (KCl) for 2 h in the presence of inhibitors or vehicle control. Mouse islets (C&D; G&H; K&L) were treated in KRBH containing 2.8 mM (2.8G) or 25 mM (25G) glucose for 2 h in the presence of inhibitors or vehicle control. (A-D) Under stimulatory conditions with KCl in MIN6 (A&B; n = 4) or high glucose in islets (C&D; n = 3), NPAS4 protein was not induced when CaMKs were inhibited with 3 μM KN-93. (E-H) 10 μM of the PKB inhibitor, Akti-1/2, prevented NPAS4 induction in response to KCl in MIN6 (E&F; n = 3) and high glucose in islets (G&H; n = 3), while MTOR inhibition with 25 nM rapamycin (Rapa) treatment did not. ND = not detectable. (I-L) A dose of 10 nM FK-506 significantly reduced the induction of NPAS4 protein levels following KCl treatment in MIN6 cells (I&J; n = 3) and high glucose in islets (K&L; n = 4). Significance was determined using a two-tailed Student’s t test with Welch’s correction. * p ≤ 0.05; ** p ≤ 0.01; *** p ≤ 0.001.

As expected, most inhibitors that did not significantly affect Npas4 transcripts (Figure 3.5) did not alter its protein levels (Figure 3.7). One notable exception was the AMPK inhibitor Compound C, which markedly decreased NPAS4 protein induction (Figure 3.7 I&J) despite not affecting transcription (Figure 3.5 B); this was similar to observations made with PKB inhibition. Furthermore, despite a small effect on Npas4 mRNA induction (Figure 3.4 C), AIP failed to significantly decrease NPAS4 protein, possibly due to low effectiveness, suggesting that modest reductions in transcription can be compensated by either increased translation or reduced protein degradation (Figure 3.7 G&H). Finally, it was noted that the MAP2K1/2 inhibitor, PD0325901, caused a notable mobility shift of NPAS4 from ~120 kDa to ~110 kDa (Figure 3.7 A&B), implicating NPAS4 phosphorylation by MAPKs.
Figure 3.7. MAP2K1/2, CaMKK, PKC and CaMKII signalling pathways do not affect NPAS4 protein expression.
MIN6 cells were kept in either basal medium alone (Basal) or with 40 mM KCl (KCl) for 2 h in the presence of inhibitors or vehicle control. (A-H) No decrease in NPAS4 protein could be observed using inhibitors of MAP2K1/2 (A&B; PD0325901 = 1 μM; n = 4), CaMKK (C&D; STO-609 = 3 μM; n = 4), PKC (E&F; BIM-XI = 100 nM; n = 4) or CaMKII (G&H; AIP = 4 μM; n = 4). (I&J) 10 μM of the AMPK inhibitor Compound C caused a decrease in NPAS4 protein induction (n = 3). * = mobility shift of the NPAS4 band when using the MAP2K1/2 inhibitor in (A). Significance was determined using a two-tailed Student’s t test with Welch’s correction. * p ≤ 0.05.

In summary, NPAS4 is highly regulated at the protein level, as CaN, CaMK, PKB and AMPK signalling affect NPAS4 translation or protein stability, while MAPK signalling causes post-translational modifications of NPAS4.

### 3.3 Cellular activity dynamically regulates NPAS4 expression in MIN6 β-cells

The dynamics of Npas4 induction in response to cellular activity were studied in collaboration with Dr. Paul V. Sabatini [2; 3]. Npas4 mRNA declined soon after an initial two-hour stimulus, and in non-stimulatory medium reached basal levels after 4 h (Figure 3.8 A). However, continued depolarization with KCl exerted a stabilizing effect (half-life: 55.7 ± 12.3 min) over transfer into non-stimulatory basal medium (half-life: 24.8 ± 2.5 min), and Npas4 mRNA levels remained significantly elevated 4 h after the initial KCl stimulus (Figure 3.8 A). This period past the initial two-hour stimulus coincided with a reduced responsiveness to re-stimulation by a second KCl stimulus (Figure 3.9). Furthermore, Npas4 is dependent on de novo transcription, as Npas4 mRNA levels declined quickly in both KCl medium with the transcriptional inhibitor actinomycin D (half-life: 28.1 ± 3.1 min) and basal medium with actinomycin D (half-life: 12.6 ± 4.1 min) (Figure 3.8 B). In both conditions, similar Npas4 expression close to basal levels was seen 2 h after the initial stimulus (Figure 3.8 B).
Figure 3.8. Cellular activity dynamically regulates Npas4 expression.
MIN6 cells were kept in basal medium (Basal) or stimulated with 40 mM KCl (KCl) for 2 h. (A&B) Afterwards, cells were further stimulated with KCl (+ KCl) or transferred to basal medium (+ Basal) in the absence (A) or presence (B) of the transcriptional blocker actinomycin D (+ ActD; 5 μg/mL). Npas4 mRNA levels gradually declined during both + KCl and + Basal treatments (A), with mRNA half-life ($t_{1/2}$) of 55.7 (±12.3) min and 24.8 (±2.5) min, respectively. With addition of ActD (B), Npas4 expression returned to basal levels more rapidly under both + KCl and + Basal conditions. KCl treatment had a stabilizing effect with a $t_{1/2}$ of 28.1 (±3.1) min, versus 12.6 (±4.1) min under Basal treatment. No Npas4 induction was seen when ActD was added from the start of the experiment, together with KCl (6 h KCl + ActD). $t_{1/2}$ was determined via nonlinear regression for one phase exponential decay ($n = 4$-$5$). (C-H) Over the course of 4 h, continued exposure to KCl (C&D; $n = 5$) or transfer to basal medium (E&F; $n = 5$) led to a gradual decline in NPAS4 protein levels. MIN6 cells in stimulatory KCl medium treated with the translational inhibitor cycloheximide (CHX) for 1 or 2 h exhibited a rapid decline in NPAS4 levels as compared to without CHX (G&H; $n = 3$). Significance was determined using a two-tailed Student’s t test with Welch’s correction (A, B) or a one-way ANOVA with Dunnett’s multiple comparisons test (D, F, H). * $p \leq 0.05$; ** $p \leq 0.01$.

![MIN6](image)

**Figure 3.9.** Npas4 expression in β-cells remains refractory for several hours following depolarization.

MIN6 cells were stimulated for 2 h with 40 mM KCl (KCl) and transferred to basal medium for 1, 2, 3, or 5 h before re-stimulating with 40 mM KCl for 2 h. MIN6 cells remained refractory to KCl restimulation for up to 5 h before Npas4 induction comparable to the initial KCl treatment was observed ($n = 4$). Significance was determined using a one-way ANOVA with Dunnett’s multiple comparisons test. * $p \leq 0.05$; ** $p \leq 0.01$.

Similar to the above effects, NPAS4 protein levels in basal medium declined almost linearly by 50 % every hour (Figure 3.8 E&F). Continued KCl exposure reduced the impact on NPAS4 decline, since protein levels were only reduced by half after 4 h (Figure 3.8 C&D). However, this effect was not dependent on protein stability, but driven by translation, as NPAS4 levels in the presence of the translational blocker CHX were reduced almost to baseline despite the presence of KCl (Figure 3.8 G&H).
In summary, \textit{Npas4} mRNA has a short half life, which is regulated by continued \textit{de novo} transcription in the presence of a stimulus, even though \(\beta\)-cells enter a refractory period of up to 5 h during which only a fraction of the initial \textit{Npas4} induction is achieved. Likewise, NPAS4 protein has a short half-life in \(\beta\)-cells and is continuously degraded even under stimulatory conditions.

### 3.4 NPAS4 is degraded via the ubiquitin-proteasome pathway

The previous experiments established a rapid decay in NPAS protein once translation is stopped; however, the degradation pathway that is involved is not known. Cells recycle damaged or surplus proteins mainly via a process involving ubiquitination and proteasomal degradation, which is especially important in \(\beta\)-cells to prevent the induction of ER stress by uncleared protein aggregates, such as misfolded insulin \cite{795}. Using the proteasomal inhibitor MG132, continual degradation of NPAS4 was either reversed or attenuated upon continued stimulation in KCl or transfer to basal medium, respectively (Figure 3.10 A-D). Of note, a trend towards accumulation of NPAS4 in KCl-stimulated and MG132-treated cells suggests that reduced proteasomal turnover, which is seen during \(\beta\)-cell activity \cite{796}, may contribute to the stabilization of NPAS4. To corroborate findings that NPAS4 degradation was ubiquitin-dependent, I performed CoIP (Figure 3.10 E). Indeed, when ubiquitin was immunoprecipitated, NPAS4 protein could be detected, and seemed to increase when transferred to non-stimulatory medium (Figure 3.10 E).
Figure 3.10. Npas4 is degraded via the ubiquitin-proteasome pathway.
MIN6 cells were kept in either basal medium alone (Basal) or with 40 mM KCl (2 h KCl) for 2 h in the presence of 10 μM of the proteasomal inhibitor MG132 or vehicle control. (A-D) MIN6 cells were subjected to either stimulation in KCl medium (+ KCl; A&B) or basal medium washout (+ Basal; C&D). Blocking the proteasome with MG132 prevented the gradual degradation of NPAS4 protein after 3 h and 6 h of KCl treatment (A&B; condition 3 and 4 + MG132). Addition of MG132 also prevented NPAS4 degradation following 1 h in basal medium, but NPAS4 levels modestly declined after 3 h basal washout (C&D; condition 3 and 4 + MG132). (E) MIN6 cells were cultured in basal medium (1), treated for 2 h with 40 mM KCl (2) or KCl followed by a one-hour washout in basal medium (3), all in the presence of 10 μM MG132. Lysates were immunoprecipitated (IP) with an ubiquitin-specific antibody (IP α Ub). NPAS4 immunoreactivity was observed in KCl-treated (2', 3') but not unstimulated (1') IP fractions (n = 3). Significance was determined using a one-way ANOVA with Bonferroni’s multiple comparison test. * p ≤ 0.05; *** p ≤ 0.001.

After determining that NPAS4 was subject to ubiquitin modification and degradation through a proteasomal pathway, I asked which enzyme is responsible for ubiquitinating NPAS4. Another bHLH-PAS domain transcription factor, HIF1α, is subject to ubiquitin-dependent proteasomal degradation. The protein which carries out this modification, VHL, is an enzyme with E3 ubiquitin ligase activity as part of a multi-protein complex. Thus, I hypothesized that VHL might interact with and ubiquitinate NPAS4, and carried out MIN6 cell stimulation and CoIP as described (Figure 3.10) to test this. Without the presence of the proteasomal inhibitor MG132 (compare Figure 3.10), NPAS4 levels had noticeably decreased in the low glucose washout condition (LG W.O., Figure 3.11) compared to stimulation with high glucose and KCl (HG/KCl, Figure 3.11), suggesting that NPAS4 was ligated with ubiquitin and degraded as shown before (Figure 3.10). However, while VHL is detected in the input, it is not seen in the IP fractions following NPAS4 IP (Figure 3.11); indicating that there likely is no interaction between the two.
MIN6 cells were cultured in basal medium (LG), stimulated for 2 h with 40 mM KCl and high glucose (HG/KCl), or stimulated by HG/KCl followed by 1 h in LG medium (LG W.O.). Cells lysates were immunoprecipitated (IP) with an NPAS4-specific antibody (IP: NPAS4), followed by immunoblotting. VHL was detected in the input, but not in IP fractions. As a positive control, NPAS4 and ARNT (see Chapter 4) were co-immunoprecipitated in KCl-treated (HG/KCl, LG W.O.), but not unstimulated (LG), IP fractions. Representative blot of n = 3.

In summary, these results demonstrate that NPAS4 is post-translationally modified with ubiquitin and targeted for proteasomal degradation upon termination of stimulatory conditions in β-cells. This process is not mediated by VHL as is the case for HIF1α. It appears that rapid and efficient degradation of NPAS4 may be physiologically relevant in re-adjusting to a non-stimulatory state, such as is the case following the lowering of blood glucose by insulin.

3.5 NPAS4 prevents FK-506 induced cytotoxic insult to β-cells

CaN signalling is important for β-cell survival. It has been shown that systemic administration of the immunosuppressant tacrolimus (FK-506) in solid organ transplantation is associated with impairments in glucose homeostasis, β-cell toxicity and development of new onset diabetes [397;
In vitro and in vivo experiments have shown that FK-506 causes apoptosis and early graft failure of human islet transplants in mice [397; 399]. In support of these findings, apoptotic β-cell death was detected in pancreatic biopsies of patients receiving FK-506 [419]. Since induction of Npas4 mRNA and protein expression in β-cells is inhibited by FK-506 (Figure 3.4 F; Figure 3.6 I-L), I hypothesized that the toxic effects of FK-506 are, in part, due to reductions in Npas4 expression. Thus, restoring Npas4 expression would protect against FK-506-initiated apoptosis.

To test this hypothesis, I assessed the survival of FK-506-treated cells in the presence or absence of Npas4 expression. Npas4 was adenovirally overexpressed in MIN6 and dispersed islet cells to bypass the inhibitory action of FK-506 on activity-dependent Npas4 induction, with EGFP-transduced cells serving as controls. Cleaved caspase 3 was measured as a marker for apoptosis. As expected, treatment with FK-506 induced apoptosis, as a 3-fold increase in cleaved caspase 3 protein was detected in EGFP-transduced MIN6 cells following 24-hour treatment with either 10 nM or 37 nM FK-506 (Figure 3.12 A&B). In contrast, Npas4 overexpression completely prevented the FK-506-mediated upregulation of cleaved caspase 3 (Figure 3.12 A&B). A similar trend was observed in dispersed murine islet cells, albeit this did not reach statistical significance (Figure 3.12 C&D).
Figure 3.12. FK-506 induced β-cell death is prevented by overexpression of Npas4.

MIN6 cells or dispersed mouse islet cells were infected with control virus (Ad-EGFP) or Ad-Npas4 overnight at a multiplicity of infection of 20:1 or 10:1, respectively. Following 24-hour treatment with vehicle (DMSO) or clinically relevant doses of FK-506 (10 nM or 37 nM), expression of the apoptosis marker cleaved caspase-3 was assessed. (A&B) Ad-EGFP infected MIN6 had significantly increased levels of cleaved caspase-3 after treatment with 10 nM and 37 nM FK-506, whereas Ad-Npas4 transduced MIN6 did not (n = 5). (C&D) Following 24-hour treatment with vehicle or 37 nM FK-506, dispersed islet cells infected with Ad-EGFP showed increased cleaved caspase-3 expression, while Npas4 transduction prevented it (n = 5). Significance was determined using a one-way ANOVA with Bonferroni’s multiple comparison test. *** p ≤ 0.001.

Together, these results indicated that NPAS4 reduces FK-506-induced apoptosis, and that toxic effects of FK-506 on β-cells during immunosuppressive regimen may in part be due to reductions in Npas4 expression.
3.6 Discussion

Although a previous study provided first evidence for a cytoprotective role of NPAS4 in β-cells \(^{[446]}\), the exact calcium-dependent signalling pathways controlling NPAS4 expression and stability had not been fully defined. In this chapter, I provide experimental data showing that induction of \(Npas4\) mRNA depends on CaMKII, PKB, and CaN signalling. Notably, while NPAS4 protein levels are affected by these same pathways, inhibition of PKB and CaN signalling reduced NPAS4 protein to a greater degree than mRNA. In addition, inhibition of AMPK signalling did not affect \(Npas4\) mRNA, but led to significant reductions in NPAS4 protein. This suggested that calcium signalling controlled all aspects of \(Npas4\) expression including transcription, translation, and protein stability, with diverse regulation especially at the protein level. \(Npas4\) mRNA and protein levels are tightly regulated, rapidly induced upon depolarization and rapidly depleted once β-cell activity is abolished. NPAS4 post-translational modifications included ubiquitin for proteasomal degradation, as well as potential phosphorylation events via MAPKs. Based on a previous study in pheochromocytoma cells \(^{[550]}\), MAPK-dependent phosphorylation of the NPAS4 C-terminal TAD increases its transcriptional activity; an effect also seen with other bHLH-PAS transcription factors, such as HIF1α \(^{[798]}\). Alternatively, phosphorylation by MAPKs could contribute to nuclear localization, as is the case for HIF1α \(^{[799]}\). Altogether, the signalling pathways controlling NPAS4 expression play an important role for β-cell survival, as exemplified via studies showing that the toxic effects of the CaN (and NPAS4) inhibitor FK-506 can be prevented via restoration of \(Npas4\) expression.

While the dynamics of \(Npas4\) mRNA expression have been studied, questions remain about post-transcriptional mechanisms controlling mRNA stability. Other groups studying \(Npas4\) in neuronal cells have identified several microRNAs which negatively regulate \(Npas4\) mRNA \(^{[549]}\). Specifically, it was suggested that miR-224 and miR-203 target the 3’-UTR of the \(Npas4\) mRNA,
thus causing transcript degradation \cite{549}. Whether the same mechanism is active in β-cells remains an open question, but rapid Npas4 mRNA decay in β-cells supports the notion that this process is facilitated, possibly via miRNAs.

Furthermore, β-cell activity exerts a mild stabilizing effect on Npas4 mRNA, extending its half-life by 2.2-fold independent of de novo transcription. Another study has made similar observations for preproinsulin mRNA, where culture with elevated glucose levels increased mRNA half-life by 2.6-fold \cite{385}. The glucose and cAMP-dependent effects were reportedly mediated via binding of polypyrimidine tract binding protein to the 3'-UTR of preproinsulin, leading to its stabilization \cite{800}. Whether this effect could be due to prevention of miRNA binding to the 3'-UTR is unknown. Further studies have to be conducted to establish whether a similar mechanism extends Npas4 mRNA half-life during β-cell stimulation.

Regarding protein stability, I found that inhibition of the proteasome largely reduces NPAS4 degradation. Together with experiments showing that NPAS4 is ubiquitinated, this established that ubiquitin-dependent proteasomal degradation is the main pathway for NPAS4 clearance, ensuring rapid degradation following removal of a stimulus. Thus, NPAS4 is confined to periods of β-cell activity and thus, is a likely mediator of adaptive changes during this time. Interestingly, another study has noted the downregulation of genes from the ubiquitin-proteasome degradation pathway during β-cell activity \cite{796}. The significance of these changes is unknown; however, it is interesting to speculate that reductions in the proteasome are what underlies the reduced degradation of NPAS4 seen with KCl. A possible advantage of temporarily reduced proteasomal activity to β-cells would be enhanced cytoprotective effects through increased and prolonged NPAS4 expression \cite{446}. However, any limitation in proteasome activity should be limited in time, because chronic proteasome inhibition and buildup of ubiquitinated
protein has detrimental effects, such as ER stress and apoptosis; especially during heightened β-cell stress, such as glucose exposure [801-803].

When it comes to determining which enzyme is responsible for the ubiquitination of NPAS4, a possible candidate was VHL. VHL is part of a ubiquitin E3 ligase complex and is involved in the degradation of the PAS domain transcription factor, HIF1α [511]. However, I was unable to detect an interaction between NPAS4 and VHL, suggesting that ubiquitination is mediated by another enzyme. I cannot rule out that an interaction between NPAS4 and VHL was missed due to insufficient assay sensitivity, as binding is likely transient and short-lived, and followed by immediate proteasomal clearance. Future experiments using a proteasomal inhibitor (e.g. MG132) should eliminate this problem. Moreover, while proteasomal degradation seemed to be the main pathway of NPAS4 clearance, a decline of NPAS4 levels in non-stimulatory medium despite the presence of MG132 indicated that alternative degradation pathways may clear NPAS4. Potential candidates are lysosomal cathepsins or calpain-mediated decay [804; 805].

Signalling pathways controlling NPAS4 expression have been studied in other cell types, particularly the neuronal lineage. Neurons and pancreatic β-cells share many functional similarities due to shared gene expression profiles, thus similarities in NPAS4 regulation were likely. I observed a trending, but not significant, decrease in β-cell Npas4 mRNA levels following inhibition of MAPK signalling, which is similar to modestly decreased hippocampal Npas4 induction [550]. This suggested that MAPKs are not a primary pathway for the regulation of Npas4 transcription in β-cells, but perhaps contribute by cross-signalling to other pathways. Interestingly, the same study noted that the C-terminal domain of NPAS4 was phosphorylated by MAPK, enhancing its transcriptional activity [550]. We observed a mobility shift when MAPK signalling was inhibited, suggesting NPAS4 is phosphorylated via this pathway. While this has not been tested in β-cells, it is tempting to speculate that the same mode of action is operational
both in neurons and β-cells. Further studies are necessary to follow up on this observation, and to establish whether there is any functional and physiological consequence of MAPK signalling for NPAS4 activity. In addition, PKC signalling neither affected Npas4 expression in β-cells nor during hippocampal Npas4 induction\textsuperscript{[550]}, ruling out a role for this pathway in regulating NPAS4 expression.

The CaMK inhibitor KN-93 caused stark reductions in Npas4 mRNA and protein. Importantly, while KN-93 is reported to reduce calcium influx by blocking L-VDCC subunits α-1C and α-1D (CACNA1C and CACNA1D)\textsuperscript{[777]}, no defects in depolarization-induced calcium influx were observed in KN-93-treated MIN6 cells. Neuronal NPAS4 expression has been associated with CaMK activity\textsuperscript{[806]}, particularly via the CaMKIV isoform\textsuperscript{[567]}, but specific pharmacological inhibitors of CaMKIV were not available at the time these studies were conducted. Since KN-93 may inhibit all CaMK isoforms\textsuperscript{[297]}, a role for CaMKIV in the induction of NPAS4 in β-cells cannot be ruled out completely. However, it should be noted that the CaMK isoforms CaMKI and CaMKIV are activated via phosphorylation by CaMKKα/β, and I did not observe a change in β-cell NPAS4 levels in response to the CaMKK inhibitor STO-609. This suggested that effects of KN-93 are mainly driven via inhibition of CaMKII, supported by the observation that the more specific CaMKII inhibitor AIP also decreased Npas4 mRNA levels. The reason for the less potent reduction in Npas4 induction could be low cell permeability; which may also be the reason why NPAS4 protein levels were unaffected by AIP, as only modestly reduced mRNA levels could still allow for the translation of similar amounts of protein.

CaMKK signalling may also lead to activation of AMPK\textsuperscript{[807]}. The roles of AMPK are diverse and not completely understood in β-cells\textsuperscript{[369; 769]}, but depolarization-induced calcium influx can activate this kinase in β-cells\textsuperscript{[808]}. In agreement with not observing an effect after CaMKK inhibition, an effect on Npas4 mRNA was also not seen with the AMPK inhibitor Compound C.
In contrast, Compound C caused significant reductions in NPAS4 protein, potentially suggesting an effect on Npas4 translation or protein turnover. The opposing roles for AMPK signalling on transcription and translation need to be interpreted cautiously, because this inhibitor has documented off-target effects, such as DYRK family protein kinases \(^{809}\). Among these, DYRK1A phosphorylates PKB, and thus DYRK1A inhibition is predicted to partly mimic the effects of Akti-1/2 with reduced NPAS4 induction. Moreover, although the CaMKK inhibitor STO-609 is more specific, one of its potential off-targets is AMPK \(^{809}\). Since STO-609 can inhibit AMPK, but no effect on NPAS4 is seen, I conclude that the inhibitory effect of Compound C on NPAS4 is not due to inhibition of AMPK, but rather through unknown off-target effects.

Exposing human islets to repeated bouts of high glucose exposure, interspersed by low glucose breaks, increased levels of active CaMKII \(^{311}\). In response to a subsequent stimulus, these islets showed enhanced GSIS when compared to non-pretreated or KN-93-treated islets \(^{311}\). This concept was termed “metabolic memory”, and involved expression changes of several key stimulus-secretion coupling proteins involved in glucose sensing, insulin release, and production \(^{311}\). In this chapter, I made two observations that argue in favor of an involvement of NPAS4 in “metabolic memory”. First, NPAS4 is expressed following glucose exposure in islets, and the CaMKII inhibitors KN-93 and AIP reduce the induction of Npas4 mRNA and/or protein in MIN6 cells and dispersed murine islets. Second, a refractory period meant that breaks in between stimuli were required for the induction of similar amounts of Npas4 mRNA. These observations suggest that intermittent high glucose stimuli, such as following a meal, would increase levels of active CaMKII and in turn NPAS4 expression; whereas in between meals, NPAS4 expression is blocked. As increased insulin secretion was observed in the original publication \(^{311}\), I propose that NPAS4 may be contributing to the phenomenon of “metabolic memory” by enhancing β-cell function specifically during times of increased demand (Chapter 4).
Many growth factors, such as insulin, signal through their receptors to phosphorylate and activate PKB and its downstream targets. NPAS4 expression is also induced by growth factors, for example in a PKB-dependent manner following NGF exposure of neuronal cells. In MIN6 cells and islets in vitro, β-cell-secreted insulin is likely one of the most abundant growth factors in the culture medium. Autocrine-paracrine insulin signalling in β-cells is physiologically relevant and dynamic, because β-cells express insulin receptors, and exposing islets or insulinoma cells to insulin elicits calcium signals, increases insulin secretion, and modulates gene expression; although this is not without controversy. Because the same signals (membrane depolarization, calcium influx) that trigger insulin exocytosis and autocrine-paracrine insulin signalling also activate NPAS4, the role of PKB in NPAS4 expression was examined. Insulin exposure in the absence of glucose did not induce Npas4 mRNA, suggesting that insulin signalling alone is not sufficient to trigger the rise in cytosolic calcium levels required for Npas4 induction. Instead, I speculate that PKB activity may be able to amplify Npas4 expression via additional calcium mobilization following membrane depolarization-induced opening of the L-VDCC. This would help explain the modest inhibitory effects of Akti-1/2 on depolarization-induced Npas4 mRNA levels. At the protein level, however, Akti-1/2 reduced NPAS4 induction by almost 80%, suggesting translation or protein stability are critically dependent on PKB. Indeed, PKB affects cap-dependent translation initiation through MTORC1, yet I observed no inhibitory effect on NPAS4 with the MTOR inhibitor rapamycin, suggesting that inhibition is not due to effects on MTORC1-dependent translation. There may be other, MTOR-independent pathways through which PKB affects translation, for example those reported for HIF1α. Moreover, direct or indirect modulation of protein stability through phosphorylation of NPAS4 or an interaction partner cannot be excluded, and remain to be tested. Whichever the mechanism, it is likely that some of the physiological outcomes of PKB activation are mediated through increased or prolonged
expression of NPAS4. Constitutively active PKB reduces β-cell ER stress and apoptosis \[819; 820\], a phenotype similar to Npas4 overexpression \[446\], thus supporting the idea that pro-survival effects of PKB are in part mediated through NPAS4.

The phosphatase CaN is another calcium-dependent signalling pathway important for β-cell function and survival \[^1\]. For example, β-cell specific inactivation of some downstream effectors of CaN, the NFAT transcription factors, resulted in an early diabetic phenotype including severe hyperglycemia, glucose intolerance and hypoinsulinemia due to defects in β-cell proliferation and gene expression \[382\]. In support of these mouse studies, administration of the CaN inhibitor, tacrolimus (FK-506), as an immunosuppressant in solid organ transplantation, has cytotoxic effects including graft failure, β-cell apoptosis and new onset diabetes \[397; 420; 797; 821-826\]. In this chapter, I showed that Npas4 mRNA and protein expression depends on CaN signalling and is inhibited by FK-506 at therapeutic doses used for immunosuppression. In addition, Npas4 overexpression prevented β-cell toxicity of FK-506 measured via upregulation of cleaved caspase 3. Of note, these experiments were performed in normal culture medium with average glucose levels, and β-cell activity and NPAS4 induction are possibly limited. Thus, the toxic effects of FK-506 could be initiated independent of NPAS4, but exacerbated in the absence of NPAS4 induction. In other words, NPAS4 is not necessary but sufficient to prevent FK-506-induced β-cell toxicity. Together, this suggested that cytoprotective effects of CaN are in part mediated through NPAS4 expression. In this context, β-cell survival has been demonstrated to depend on CaN-NFAT \[^422\] and CaMKIV-CREB \[^316; 423; 424\] signalling; and a common downstream target is IRS2 \[^317; 827\], which regulates the activity of pro-survival insulin signalling through PKB. NPAS4 has been implicated in reducing β-cell ER stress \[^446\], and here its regulation by NFAT, CaMK and PKB, as well as pro-survival effects have been demonstrated. I thus propose that NPAS4 acts as a β-cell survival co-incidence sensor, which is activated by pro-survival signalling pathways to promote β-cell survival. Whether this is solely through reduction of ER stress,
induction of IRS2 and other pro-survival genes, may be tested in genome-wide sequencing of chromatin immunoprecipitation samples (Chapter 5).

Lastly, even though they are often described as discrete entities, it is important to realize that calcium signalling pathways do not exist in isolation, and different pathways often intersect. As an example, crosstalk between the CaN and PI3K/PKB signalling pathway was implicated in the regulation of islet survival [422]. This synergy is of note, since inhibition of both pathways affected NPAS4 expression, providing further support for the notion that pro-survival signals of both pathways may converge in part on NPAS4.

In summary, my studies identified calcium-dependent signalling pathways and post-translational modifications involved in NPAS4 expression and stability, including CaN, PKB, and CaMK pathways. Further work is needed to study transcription factors modulating NPAS4 expression downstream of these calcium signalling pathways, i.e. CREB, NFAT, and FOXO1. NPAS4 expression is rapidly induced following depolarization, and degraded when stimulation was removed, consistent with a role as an adaptive immediate early gene during cellular activity. Furthermore, my studies provide a first glimpse at post-translational modifications of NPAS4 in β-cells (i.e. ubiquitination, possibly phosphorylation), and warrant further research to uncover which modifications regulate NPAS4 stability, localization, and transcriptional activity in β-cells. Finally, the finding that NPAS4 prevents FK-506-induced apoptotic signals corroborates the role of NPAS4 in β-cell survival downstream of calcium signalling. However, unanswered questions remain about whether NPAS4 affects β-cell function, if it has a role in β-cells in vivo, and whether it is involved in, or dysregulated by, T2D.
Chapter 4: NPAS4 modulates the hypoxic response and β-cell maintenance

4.1 Background

The islets of Langerhans are richly vascularized structures; it is estimated that they receive 10-15% of total pancreatic blood flow while only constituting 1-2% of total pancreatic mass [525]. This highlights the heightened energy needs of these groups of cells; pancreatic β-cells, for example, have substantial energy needs for insulin biosynthesis, trafficking, and exocytosis, including active ion fluxes to restore membrane potential following depolarization. To satisfy these energy demands, β-cells rely on the conversion of glucose into ATP through glycolysis and oxidative phosphorylation; other substrates may feed into these pathways as well [76]. Under aerobic conditions, β-cells derive only 2% of ATP yield from glucose through glycolysis, whereas the remaining 98% are derived from the TCA cycle [404]. β-cells have extensive energy demands to maintain action potentials and insulin exocytosis – it is estimated that β-cells consume 40-50% of ATP supply for insulin production and secretion, and about 20% for ion transport [404]. Thus, mechanisms exist to optimize glucose utilization via mitochondrial metabolism and thus ATP generation during periods of increased need, such as when blood glucose levels rise and insulin is actively secreted.

One group proposes a mechanism, aptly named “calcium/metabolic coupling process” [403], by which glucose metabolism and calcium influx specifically through L-VDCCs, but not from intracellular stores, is required for triggering insulin secretion [403; 406; 828]. This calcium-dependent process correlates with enhanced oxygen consumption rate (OCR) possibly driven by ATP usage [403; 406; 828], and is defective in diabetic β-cells [829]. The factor underlying the calcium/metabolic coupling process is not known, but may involve CaM-dependent
proteins \[^{403}\]. Notably, expression of the transcription factor NPAS4 following β-cell stimulation depends on CaM-dependent kinases downstream of calcium influx (Chapter 3).

A by-product of mitochondrial activity is the generation of ROS, such as hydrogen peroxide and superoxide \[^{530}\]. β-cells are sensitive to oxidative damage induced by these oxygen radicals \[^{535}\]. Surprisingly, even though β-cell activity results in amplified ROS formation, β-cells only express very little antioxidant defense genes. One reason for this apparent contradiction is that ROS also serve important signalling functions, such as modulating insulin secretion. On the other hand, they also modulate gene expression through stabilization of the bHLH-PAS domain transcription factor, HIF1α \[^{512, 514}\]. Declining oxygen levels during periods of high glucose and high metabolic demand may also contribute to stabilization of HIF1α \[^{527, 528}\]. HIF1α stabilization and heterodimerization with its partner factor, ARNT \[^{480}\], leads to transcriptional changes favouring anaerobic glycolysis over oxidative phosphorylation \[^{830}\], thus reducing ATP yield while preventing ROS generation. Whilst this represents an important and ancient adaptation to react to acute oxidative and hypoxic stress, chronic induction may be undesirable in β-cells, which are repeatedly exposed to high glucose, ROS formation, and whose performance is intrinsically linked to the ability to generate ATP. Therefore, I hypothesize that β-cells have evolved a homeostatic mechanism which dampens the effects of metabolic HIF1α stabilization.

Another bHLH-PAS transcription factor found in metabolically active β-cells is NPAS4 \[^{446, 464}\]. The bHLH-PAS family binds DNA as obligate heterodimers, and like HIF1α, NPAS4 interacts with ARNT, as was shown in neuronal cells \[^{539, 571}\]. A competitive nature between different bHLH-PAS transcription factors, including NPAS4 \[^{539}\], has been reported previously \[^{831-833}\], and some studies suggest this involves competition for ARNT \[^{832, 834, 835}\]. In the previous chapter, it was demonstrated that calcium-dependent NPAS4 expression partly depends on CaMKII. Consequently, this chapter addresses the hypothesis that NPAS4 is an essential
calcium-induced metabolic coupling protein and key mediator of the “calcium/metabolic coupling process”, specifically through counteracting HIF1α activity by competing for ARNT binding. In addition to *in vitro* experiments testing this hypothesis, the physiological consequences of loss of NPAS4 are studied in a β-cell specific KO mouse model.

4.2 NPAS4 dampens HIF1α activity by competing for ARNT binding

To test the bHLH-PAS heterodimerization competition hypothesis, I first used CoIP to test whether NPAS4 interacts with Class II factors in pancreatic β-cells. Because NPAS4 interacted with Class II partner factors (ARNT, ARNT2, and traces of ARNTL) in neuronal cells [571], I performed CoIPs using antibodies against NPAS4, ARNT, ARNT2, and ARNTL. N.B. that interactions with Class II factor ARNTL2 were not tested, as ARNTL2 transcript was not detected in human pancreas [836] and it did not heterodimerize with NPAS4 in other cells [539; 571].

I used MIN6 cells because they are β-like cells and because they can be readily grown in culture. As shown before (Chapter 3), depolarization with glucose and KCl (HG/KCl) maximized NPAS4 induction, and low glucose washout (LG W.O.) led to declining NPAS4 levels, whereas ARNT expression remained unchanged (Figure 4.1). Using this experimental system, I observed robust interaction of ARNT and NPAS4 when both proteins were present, including during maximal NPAS4 induction (HG/KCl) or when NPAS levels declined (LG W.O.), and in both IPs against ARNT or NPAS4, respectively (Figure 4.1 A&B). The predicted molecular weight of ARNT2 is 79 kDa, and a weak band corresponding to this size is seen in MIN6 cell input and IP samples (Figure 4.1 C). A faint band corresponding to the major 110 kDa NPAS4 isoform was detected following ARNT2 IP (Figure 4.1 C). Although ARNTL was robustly expressed in β-cells, I did not observe a direct NPAS4:ARNTL interaction (Figure 4.1 D).
MIN6 cells were maintained in low glucose (LG) medium, stimulated with 25 mM glucose and 40 mM KCl (HG/KCl) for 2 h to induce NPAS4 expression, or incubated with HG/KCl medium for 2 h followed by low glucose washout (LG W.O.) for 1 h. (A&B) Robust NPAS4:ARNT interaction was seen when either ARNT (A) or NPAS4 (B) were immunoprecipitated. (C) A faint NPAS4:ARNT2 interaction was observed when ARNT2 was immunoprecipitated. (D) NPAS4 was not detected following ARNTL immunoprecipitation. Representative blots of n = 3. Black arrowheads denote the band corresponding to the specified target.

These experiments demonstrated that observations about NPAS4 interactions made in neuronal cells \[571\] are largely applicable to β-cells; confirming that NPAS4 heterodimerized with general partner factors of the ARNT1/2 subgroup, but not the circadian ARNTL1/2 factors.

Furthermore, using an available single-cell RNA-Seq (scRNA-Seq) resource for human islets \[296\], ARNT2 expression was more prevalent in α- and PP-cells, which agreed with low protein expression in MIN6 cells (Figure 4.1 C). In view of this observation, and because both

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**Figure 4.1. NPAS4 interacts with Class II bHLH-PAS proteins in insulin-producing pancreatic β-cells.**
NPAS4 and ARNT were robustly expressed as well as strongly co-enriched in IP fractions, it followed that the main interaction partner for NPAS4 in β-cells is ARNT.

After confirming that NPAS4 interacted with the same Class II factor as HIF1α⁴⁸⁰, further studies were designed to answer whether competitive binding for ARNT between HIF1α and NPAS4 existed. Previous work by Dr. Paul V. Sabatini showed that stabilization of HIF1α signalling interfered with NPAS4 induction [³; ⁴]; and thus, Npas4 was adenovirally overexpressed in MIN6 cells before stabilization of HIF1α through short-term culture under hypoxic conditions. In this manner, I sought to approximately mimic a hypothetical physiologic scenario in which a large glucose bolus first induced NPAS4 before causing stabilization of HIF1α. As predicted, when ARNT was immunoprecipitated from MIN6 cells treated this way, HIF1α was co-immunoprecipitated (Figure 4.2 A&B). Notably, Ad-Npas4 infection itself caused an upregulation of ARNT levels (Figure 4.2 A&C). An increase in ARNT expression was also observed in certain cell lines [⁴⁸⁷; ⁴⁸⁸] and human islets [⁴⁸⁰] in response to hypoxic stimuli. This suggested that modulation of the hypoxic response via NPAS4 affects ARNT expression in MIN6 cells; although effects driven by adenoviral overexpression need to be interpreted cautiously. Furthermore, the resulting increase in ARNT in both input as well as immunoprecipitated fraction of Ad-Npas4 cells (Figure 4.2 A&C) could provide HIF1α with more ARNT to interact, potentially masking competition with NPAS4. Nonetheless, Ad-Npas4 transduced cells showed markedly reduced HIF1α co-immunoprecipitated with ARNT when compared to controls (Figure 4.2 A&B). This indicated that NPAS4 indeed titrated ARNT away from HIF1α.

To complement these studies with an animal model for loss of NPAS4, Npas4floxflox;Pdx1-CreER+ KO (N4 PKO) mice were selected. A benefit of these KO mice was that they were previously characterized as sensitive to metabolic stress, because HFD feeding induced hyperglycemia, glucose intolerance and impairments in insulin secretion due to loss of Npas4 [⁴]. Thus, islets
obtained from HFD fed N4 PKO and control mice were subjected to glucose stimulation to induce NPAS4 (in controls), followed by hypoxic incubation to stabilize HIF1α. When ARNT was immunoprecipitated from islets so treated, I saw a trend towards increased HIF1α levels in N4 PKO islets compared to controls (Figure 4.2D). Together, these results supported my hypothesis that NPAS4 suppressed HIF1α signalling in islets by binding and occupying the obligate partner factor ARNT, thereby effectively reducing functional HIF1α:ARNT heterodimers.

**Figure 4.2. NPAS4 overexpression and KO modulate HIF1α:ARNT interaction.**

(A-C) MIN6 cells were transduced with control adenovirus (Ad-EGFP) or adenovirus driving Npas4 expression (Ad-Npas4). Following 4 h in 1% O2 to stabilize HIF1α, ARNT was immunoprecipitated and immunoblot for HIF1α and NPAS4 performed. HIF1α CoIP with ARNT was significantly reduced in Ad-Npas4 transduced cells (A&B). In addition, Ad-Npas4 cells expressed more ARNT (n = 4). (D) After 9 weeks of HFD feeding, islets from control and Npas4 knockout (N4 PKO; Npas4<sup>lox/lox</sup>;Pdx1<sup>-CreER</sup>+) mice were isolated, stimulated with 25 mM glucose at 20% O2 for 1 h before transfer to 1% O2 for 2 h. ARNT IP reveals a trending increase in HIF1α:ARNT binding in HFD fed N4 PKO islets. Representative blot for n = 2. IP: Ms = IgG isotype control specific for an irrelevant target. Black arrowheads denote the band corresponding to the specified target. Significance was determined using an unpaired two-tailed Student’s t test (B, C). * p ≤ 0.05.

If NPAS4 indeed competed with HIF1α for ARNT binding, induction of NPAS4 was predicted to reduce HIF1α signalling. In collaboration with Dr. Paul V. Sabatini [3], the expression of HIF1α target genes was assessed following adenoviral Npas4 overexpression. Under standard culture conditions at atmospheric oxygen levels (~20%), basal HIF1α target gene (Ldha, Slc16a3, Pdk1, and Vegfa) [521] expression was similar between control (Ad-EGFP) and Npas4 overexpressing
(Ad-Npas4) MIN6 cells (Figure 4.3 A-D). However, when HIF1α was stabilized using hypoxic culture conditions (1 % O₂ for 6 h), induction of HIF1α target transcripts was markedly lower in Ad-Npas4 MIN6 cells (Figure 4.3 A-D). To confirm these findings in primary cells, wild-type mouse islets were isolated and transduced with Ad-EGFP or Ad-Npas4, and cultured in 1 % O₂ for 24 h. Under these conditions, the hypoxia-mediated upregulation of \textit{Ldha}, \textit{Slc16a3} and \textit{Sox9} expression was significantly impaired in Ad-Npas4 islets (Figure 4.3 E-G). A trending decrease was also noted for \textit{Vegfa} (Figure 4.3 H). Perhaps, \textit{Vegfa} induction in islets relied on HIF2α, which is not highly expressed \cite{483; 484}, or HIF-independent factors \cite{837}, and thus was not sufficient to induce significant differences. Using the same experimental design, transcript levels of HIF1α targets \textit{LDHA} and \textit{SOX9} were decreased in Ad-Npas4 human islets, in both 20 % O₂ and 1 % O₂ conditions. These results indicated that NPAS4 is a potent suppressor of HIF1α signalling in pancreatic β-cells.

Figure 4.3. \textit{Npas4} overexpression blocks induction of HIF1α target genes in β-cells.
MIN6 cells or islets were transduced with adenovirus driving the expression of *Npas4* (Ad-Npas4) or EGFP (Ad-EGFP) as a control, and cultured with atmospheric oxygen levels (20 % O₂) or hypoxia (1 % O₂). (A-D) Following six-hour culture, hypoxia-induced expression of HIF1α target genes *Ldha, Slc16a3, Pdk1* and *Vegfa* was significantly reduced in Ad-Npas4 MIN6 (n = 3). (E-H) Wild-type (B6) mouse islets infected with Ad-Npas4 and cultured in 1 % O₂ for 24 h expressed significantly lower levels of HIF1α targets *Ldha, Slc16a3* and *Sox9*, including less *Sox9* in 20 % O₂ (n = 4). (I&J) Expression of *LDHA* and *SOX9* was decreased in human islets transduced with Ad-Npas4 and cultured in 1 % O₂ for 24 h (n = 5). Significance was determined using an unpaired two-tailed Student’s t test (A-D) with Welch’s correction (E-J). * p ≤ 0.05, ** p ≤ 0.01, *** p ≤ 0.001. A version of this figure was previously published (Sabatini, 2017).

Stabilization of HIF1α leads to a metabolic switch from oxidative phosphorylation to anaerobic glycolysis. The induction of HIF1α during metabolic challenge, such as high glucose exposure (Figure 4.4 A, B&D), ultimately limits β-cell function when efficient glucose metabolism to ATP is needed for insulin secretion. The physiological consequence of NPAS4 versus HIF1α competition may therefore be short-term suppression of HIF1α signalling to optimize metabolism and insulin secretion, to ensure glucose is efficiently removed from circulation. If this hypothesis were correct, I expected that loss of *Npas4* in β-cells would lead to increased HIF1α signalling and reduced metabolic activity. N4 PKO islets were tested for an increase in HIF1α signalling via HIF1α target gene expression in the presence of the PHD enzyme inhibitor JNJ-42041935 (JNJ-42) [756], which increased HIF1α levels by blocking its degradation (Figure 4.4 C&D). As predicted, N4 PKO islets expressed higher levels of HIF1α target genes *Ldha* and *Slc16a3* (Figure 4.4 E&F).
Figure 4.4. HIF1α stabilization by glucose and JNJ-42041935 in islets.

(A&B) High glucose (25 mM) enhanced HIF1α expression in human islets cultured for 4 h at physiologic oxygen concentration (5% O2) (n = 3). (C) The PHD enzyme inhibitor JNJ-42041935 (JNJ-42; 100 μM) stabilized HIF1α expression in MIN6 cells following culture for 2, 4 and 6 h. Representative blots of n = 3. (D) High glucose (25 mM) and JNJ-42 (100 μM) further increased hypoxia-mediated HIF1α expression after four-hour culture of human islets at 1% O2. Representative blots of n = 2. (E&F) Co-culture with 25 mM glucose and 100 μM JNJ-42 for 2.5 led to higher expression of HIF1α target genes Ldha and Slc16a3 in N4 PKO islets compared to controls (n = 4). Significance was determined using an unpaired two-tailed Student’s t test (E&F). * p ≤ 0.05.

Because ATP production requires O2 as an electron acceptor, oxygen consumption rate (OCR) can be measured to assess whether loss of Npas4 also causes a decrease in metabolic activity.
Thus, OCR was measured in control or N4 PKO islets exposed to high glucose in the presence or absence of JNJ-42. Control islets exhibited a stable rise in OCR in response to glucose without JNJ-42, which was maintained over time (Figure 4.5 A&B). Intriguingly, N4 PKO islets showed reduced glucose-stimulated OCR compared to controls (Figure 4.5 A&B). Addition of JNJ-42 and thus HIF1α stabilization lowered glucose-stimulated OCR in control islets, but did not further decrease OCR in N4 PKO islets, which were significantly different from controls (Figure 4.5 C&D). This suggested that HIF1α activity in N4 PKO islets was already elevated, such that further increasing HIF1α levels with JNJ-42 (Figure 4.4 C&D) did not cause an additional decrease in OCR.

**Figure 4.5.** Loss of *Npas4* impairs respiratory metabolism in pancreatic β-cells.
Following 2 weeks HFD feeding (9 weeks of age), islets were obtained from control and N4 PKO mice. (A&B) Stimulation of oxygen consumption by high glucose (25 mM) exposure was significantly diminished in islets from vehicle (DMSO) treated N4 PKO mice compared to controls. Maximal mitochondrial respiratory capacity, assessed by FCCP exposure, was also reduced (n = 8). (C&D) In comparison, these parameters were not different between control and N4 PKO islets when treated with JNJ-42 to stabilize HIF1α and thus dampen oxygen consumption. Non-mitochondrial respiration, which was determined through Ant/Rot injection, was slightly elevated in JNJ-42 treated N4 PKO islets (n = 8).

OCR = oxygen consumption rate; AUC = area under curve; 2.8G/25G = 2.8 mM/25 mM glucose; Olm = 4 μM oligomycin; FCCP = 2 μM carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone; Ant/Rot = 4 μM antimycin A/rotenone. Significance was determined using multiple unpaired t tests with false discovery rate calculated via two-stage linear step-up procedure of Benjamini, Krieger and Yekutieli, with q = 5 % (A, C); or ordinary unpaired two-tailed Student’s t test (B&D). * p ≤ 0.05, ** p ≤ 0.01.

Together, these studies functionally demonstrated the opposing nature of HIF1α and NPAS4 in β-cells. Whereas chronic elevation in HIF1α reduced metabolic activity, activity-dependent induction of NPAS4 competed with HIF1α for ARNT binding and thus suppressed HIF1α signalling. This ensured that β-cell energy metabolism was optimized during periods of elevated energy demand, such as rising blood glucose levels following a meal, whereas loss of Npas4 predisposed to perturbations in islet metabolic activity. In support of this view, islets from T2D donors had higher basal levels of NPAS4, but failed to induce NPAS4 in response to glucose [4]. This finding indicated that dysregulated NPAS4 expression could be a contributing factor in the islet pathology of T2D. To investigate the role of NPAS4 in diabetes progression in vivo, β-cell specific Npas4 KO mice were generated.

4.3 Specific deletion of Npas4 from mouse β-cells causes mild glucose intolerance

Dr. Paul V. Sabatini previously used the Pdx1-CreER strain to show that HFD-fed N4 PKO mice develop fasting hyperglycemia, glucose intolerance, impaired GSIS, and β-cell dedifferentiation [3; 4]. However, recombination was also observed in the arcuate nucleus of the brain, an area that controls feeding behaviour, and increased food intake could explain increased hepatic gluconeogenesis and insulin resistance in the N4 PKO mice [3; 4]. To investigate a β-cell autonomous role for NPAS4 in vivo, I crossed the β-cell specific Ins1Cre knock-in
replacement strain \cite{746} to mice carrying the \textit{Npas4}^{\text{flox}} allele \cite{354}. \textit{Npas4}^{\text{flox}/\text{flox};\text{Ins1}^{\text{WT}/\text{Cre}+}} (N4 IKO) mice exhibited recombination in \(\beta\)-cells, but not in the arcuate nucleus \cite{3; 4}, causing a drastic loss of \textit{Npas4} expression in the islet (Figure 4.6 A). In agreement, body mass composition, food or water intake of N4 IKO mice matched that of controls \cite{3; 4}, suggesting that recombination was limited to \(\beta\)-cells, and thus, eliminating any behavioral changes due to neuronal recombination previously seen in the N4 PKO mouse.

Chronically elevated HIF1\(\alpha\) signalling is detrimental to \(\beta\)-cell function, identity and viability \cite{497; 519-521; 838}. Following loss of \textit{Npas4}, an increase in HIF1\(\alpha\) signalling would likely manifest during conditions promoting HIF1\(\alpha\) stabilization. Feeding a HFD increases HIF1\(\alpha\) levels in other tissues \cite{839; 840}; at the same time, increasing obesity, insulin resistance and lipotoxicity promote \(\beta\)-cell oxidative and ER stress. The diabetogenic setting of HFD feeding was thus predicted to exacerbate loss of \textit{Npas4}, and chosen to tease out the \(\beta\)-cell-specific role of \textit{Npas4} in N4 IKO mice \textit{in vivo}.

From the age of 7 weeks, N4 IKO and control mice were kept on either standard chow diet (CHW) or moved to HFD and monitored regularly. Several control genotypes were used, namely \textit{Npas4} floxed controls (\textit{Npas4}^{\text{flox}/\text{flox}}), hemizygous Cre controls (\textit{Ins1}^{\text{WT}/\text{Cre}+}), and controls for heterozygous loss of \textit{Npas4} (\textit{Npas4}^{\text{flox}/\text{WT};\text{Ins1}^{\text{WT}/\text{Cre}+}}). Throughout the monitoring period, no differences in body weights between control and N4 IKO became apparent, except for larger weight gain in the HFD fed cohorts compared to CHW (Figure 4.6 B&C).
Figure 4.6. No differences in body weight gain in N4 IKO mice.

(A) Compared to floxed controls (Npas4<sup>flox/flox</sup>), islets from Npas4 knockout (N4 IKO; Npas4<sup>flox/flox</sup>;Ins1<sup>WT/Cre+</sup>) mice showed a marked reduction of Npas4 transcript following two-hour culture in basal (2.8 mM glucose) and stimulated (25 mM glucose) conditions (n = 7). (B&C) Body weights in CHW and HFD feeding regimen were similar in N4 IKO and control (Npas4<sup>flox/flox</sup>, Ins1<sup>WT/Cre+</sup>, and Npas4<sup>flox/WT</sup>;Ins1<sup>WT/Cre+</sup>) mice (n = 4-51). Open symbols = chow diet (CHW), solid symbols = high fat diet (HFD); colours represent genotypes as indicated. Significance was determined using an unpaired two-tailed Student's t test with Welch's correction (A) or a two-way ANOVA with Holm-Sidak's multiple comparisons test (B, C). * p ≤ 0.05, *** p ≤ 0.001.
The development of hyperglycemia was monitored through bi-weekly measurement of fed (random) and fasted blood glucose levels. While random glycemic levels were largely the same between all genotypes, a few CHW and HFD fed controls had elevated glycemic levels at several timepoints (Figure 4.7 A&B), most commonly in CHW fed Cre controls. The significance of this observation is unknown, but could indicate an effect of the knock-in replacement of one Ins1 allele. Nevertheless, fasting glycemia was essentially the same across all genotypes and ages (Figure 4.7 C&D).

Figure 4.7. Normal random and fasting glycemia in N4 IKO mice.

CHW or HFD feeding started at 7 weeks of age. (A&B) Random fed blood glucose levels are largely comparable between control and N4 IKO mice. Some CHW and HFD fed controls (Ins1WT/Cre+ and Npas4fox/WT;Ins1WT/Cre+) showed elevated random glycemia at a few time points when compared to either N4 IKO or other controls (n = 4-35). (C&D) With two exceptions, fasting glycemia was similar in CHW and HFD fed N4 IKO and control mice at all ages (n = 4-51). Significance was determined using a two-way ANOVA with Holm-Sidak's multiple comparisons test. * p ≤ 0.05, ** p ≤ 0.01, *** p ≤ 0.001.
To preclude the possibility that underlying differences in insulin resistance or gluconeogenesis masked any phenotype, mice were subjected to insulin, pyruvate, and glycerol tolerance tests (ITT, PyrTT, GlyTT). This was done at an age when N4 PKO had started to exhibit increased gluconeogenic output and insulin resistance\[3; 4\]. Relative to controls, N4 IKO mice did not exhibit increased glucose excursions in response to gluconeogenic substrates (Figure 4.8 A-F), nor did they develop insulin resistance (Figure 4.8 G-I). While these results suggested that β-cell specific loss of Npas4 does not worsen hyperglycemia and insulin tolerance in the absence of recombination in the brain, defects in glucose tolerance might still be appreciable.

Figure 4.8. N4 IKO mice do not show intolerance to gluconeogenic substrates or insulin.
(A–C) During a 2 g/kg pyruvate tolerance test following 7 weeks of CHW or HFD diet feeding, control and N4 IKO mice showed largely similar glucose excursions. CHW fed Cre controls had significantly elevated glycemia in response to pyruvate, with a similar trend observed for HFD fed Cre controls (n = 4–16).

(D–F) Following 11 weeks of CHW or HFD diet feeding, no significant differences in glycemic levels of control and N4 IKO mice were observed in response to a 2 g/kg glycerol bolus (n = 3–18). (G–I) No differences in insulin sensitivity of control and N4 IKO mice were noted during a 0.75 U/kg insulin tolerance test following 11 weeks of diet feeding (n = 4–16). AUC = area under the curve; AU = arbitrary unit. Significance was determined using a two-way ANOVA (A&B, D&E, G&H) or one-way ANOVA (C, F, I), with Holm-Sidak’s multiple comparison test, respectively. * p ≤ 0.05, ** p ≤ 0.01, *** p ≤ 0.001.

To assess the response to a hyperglycemic challenge, oral glucose tolerance tests were performed at an age at which N4 PKO mice had previously shown signs of overt glucose intolerance [3; 4].

Because dual loss of Npas4 in the hypothalamus and islets of N4 PKO likely led to a synergistic defect, impairments in glucose homeostasis of N4 IKO mice, due to specific loss of Npas4 only in islets, were likely to be milder and delayed. As with N4 PKO mice, CHW fed N4 IKO mice exhibited no differences in glucose tolerance (Figure 4.9 A&C). At an age when N4 PKO mice had shown overtly impaired glucose homeostasis (after 17 weeks of HFD diet feeding, at 24 weeks of age), glycemic control in HFD fed N4 IKO mice was only slightly worsened at the 60 min and 90 min time point, respectively (Figure 4.9 B&C).

![Figure 4.9](image-url)  
**Figure 4.9.** HFD-fed N4 IKO mice exhibit mild glucose intolerance.

(A–C) Following 17 weeks of diet feeding, a 2 g/kg oral glucose tolerance test was performed with the first post-glucose bolus measurement taken at 15 min. Male CHW fed control and N4 IKO mice had identical glycemia, but HFD fed N4 IKO mice exhibited slightly elevated blood glucose at 60 min and 90 min (n = 3–24). Significance was determined using a two-way ANOVA (A&B) or one-way ANOVA (C), with Holm-Sidak’s multiple comparisons test, respectively. * p ≤ 0.05.
In summary, these findings confirmed that loss of \textit{Npas4} expression specifically in β-cells (N4 IKO) eliminated most of the phenotypes associated with dual loss of \textit{Npas4} in hypothalamus and islets (N4 PKO). Consequently, a comparatively much milder and delayed worsening of glucose tolerance was the only defect seen thus far. Complimentary work using AAV-Cre-mediated postnatal \textit{Npas4} ablation confirmed that further defects were not masked by compensatory mechanisms due to prenatal loss of \textit{Npas4} in the N4 IKO mouse \citep{3;4}. Nevertheless, glucose tolerance tests revealed that loss of β-cell \textit{Npas4} expression predisposes β-cells to late impairments in response to prolonged diabetogenic stress. This was in agreement with \textit{in vitro} work showing reduced β-cell metabolic activity in \textit{Npas4} KO islets; suggesting that increased HIF1α signalling in HFD fed N4 IKO animals was a likely cause of mild defects in glucose clearance. In future studies, it will be informative to investigate discrete contributions of brain-specific \textit{Npas4} KO.

\section*{4.4 Discussion}

Studies in this chapter outline a mechanism limiting unchecked hypoxic signalling, which is required in pancreatic β-cells for appropriate glucose homeostasis to occur. By restricting transcriptional changes invoked by HIF1α which would favour glycolysis, NPAS4 optimises β-cell metabolism for oxidative phosphorylation. Loss of \textit{Npas4} leads to reduced oxidative phosphorylation and glucose intolerance in mice.

All aerobic cells have to strike a balance between energy demand and oxygen supply. In the case of β-cells, the generation of ATP is a major determinant of their function \citep{76}, rendering them exquisitely reliant on the processing of metabolites via oxidative phosphorylation and thus, oxygen. High metabolic activity generates ROS as by-products \citep{530}, and since β-cells produce low antioxidant defense \citep{522;841}, they accumulate oxidative damage \citep{535}. Aside from the danger of oxidative damage to cellular components, ROS may also play a role in inducing hypoxic
Previous studies demonstrated that ROS may induce HIF1α via inhibition of PHD enzymes, thus preventing HIF1α hydroxylation. This setting may be more physiologically relevant under aerobic conditions, when PHD enzyme activity would normally still prevent HIF1α stabilization. This would explain why high glucose exposure, which increases ROS production amongst others, leads to increased levels of HIF1α in human islets in the above experiments. An alternative explanation, in the context of whole organisms, is that in cases of chronic hyperglycemia such as diabetes, blood oxygen supply is depleted due to glucose metabolism, thus creating a relatively hypoxic environment. Hypoxic adducts have been found in sections of hyperglycemic mice and in islets exposed to high glucose, supporting this more traditional view. However, tissue oxygen levels in whole organisms remain notoriously difficult to measure reliably in vivo.

One of the hallmarks of diabetes is hyperglycemia, leading to the prediction of heightened ROS levels in the diabetic islet. Prolonged ROS exposure over time may result in increased HIF1α levels and activity. This would skew metabolism from an OXPHOS state to anaerobic glycolysis, impairing ATP yield and β-cell function. In support of this view, mitochondrial metabolism improved when diabetic animals were treated to reduce ROS levels. Aside from ROS and hypoxia, other metabolites also promote stabilization of HIF1α, likewise through inhibition of PHD enzymes. One such example is pyruvate, an intermediate in complete glucose oxidation; increasing pyruvate levels may act in a feed-forward fashion to promote glycolysis, leading to further pyruvate buildup. In the context of diabetes, chronic hyperglycemia would thus perpetuate the metabolic impairment of β-cells, including perturbed NPAS4 expression; and if the underlying β-cell dysfunction were not rectified, it would lead to gradual and progressive worsening of hyperglycemia and glucose tolerance over time.
It has been suggested that HIF1α is increased in T2D β-cells [296; 435; 481; 515; 528]. Studies in collaboration with Dr. Paul V. Sabatini showed that loss of Npas4 results in OXPHOS defects, impaired insulin secretion and dedifferentiation, leading to impaired glucose homeostasis and development of diabetes. This was associated with increased expression of HIF1α target genes. Notably, NPAS4 was dysregulated in islets of T2D donors [3; 4]. Furthermore, conditions promoting HIF1α stabilization, such as JNJ-42041935 or hypoxia, prevented the induction of NPAS4 in mouse and human islets [3; 4]. This suggests that part of the metabolic dysfunction in T2D which is associated with increased HIF1α activity may result as a consequence of inadequate NPAS4 levels.

In contradiction to this, some have observed a decrease in HIF1α in some non-endocrine tissues during diabetic hyperglycemia [844-846]. Those findings highlight how different tissues may regulate HIF1α in a different fashion due to unique metabolic activities and possibly in an insulin-dependent manner. It thus makes sense that a mechanism to counteract HIF1α, in the form of NPAS4, exists in β-cells, where HIF1α increases in the diabetic milieu. In further support of the importance of proper regulation of bHLH-PAS domain transcription factors, one study has seen reduced expression of the class II factor ARNT in T2D donor islets [478]. ARNT KO mice also became diabetic after 2-3 months [478], a time frame which is comparable with Npas4 KO mice. Since ARNT interacts with most Class I bHLH-PAS factors, including NPAS4, it can be speculated that loss of NPAS4 activity partly contributes to the metabolic impairment seen in these donors and animals. It emphasizes the importance of appropriate levels of bHLH-PAS transcription factors.

Because HIF1α limits energy supply, its stabilization is detrimental to β-cell function in the long run. Mouse models support this notion, since β-cell specific VHL KO mice, which cannot hydroxylate and degrade HIF1α, become diabetic due to diminished insulin release and β-cell
dedifferentiation. Notably, β-cell Hif1α KO also resulted in a diabetic phenotype in mice, suggesting that an appropriate balance of HIF1α signalling is important. Lastly, defective calcium signalling in β-cells of mice with an activating mutation in the KATP channel subunit Kcnj11 leads to glycogen accumulation, perhaps due to increased HIF1α-driven expression of genes promoting glucogenesis. Together, this supports the idea that NPAS4 is the calcium-dependent nutrient-induced signal in healthy β-cells, which counteracts the effects of physiological stabilization of HIF1α during phases of metabolic activity.

Some diabetes therapies are aimed at driving insulin exocytosis; however, this may drive β-cell exhaustion and failure in the long run. Promoting β-cell rest with diazoxide may be beneficial especially in the initial stages of treatment, together with exogenous insulin, to reduce the underlying hyperglycemia first. This may give β-cells time to re-differentiate and mature. A way in which such therapies work may be 1) reduced β-cell metabolic activity, due to the lowering of blood glucose via other routes, 2) followed by a concurrent decrease in ROS, 3) reducing HIF1α activity, 4) and thus, promoting a more mature, functional β-cell phenotype. In these functional cells, calcium-dependent NPAS4 expression may be restored, further ensuring a check on HIF1α activity and differentiation status. It will be informative to examine whether stabilization of NPAS4, via small molecules, may in itself be potent to drive re-differentiation and restore β-cell function in diabetic animals.
Chapter 5: Characterization of the gene regulatory network controlled by NPAS4 in mouse β-cells

5.1 Background

Female Arnt\textsuperscript{floxflox} RIP-Cre mice develop glucose intolerance and reduced glucose- and arginine-stimulated insulin secretion, whereas males only showed mild glucose intolerance \[478\].

Alternatively, another study found no \textit{in vivo} defect in glucose homeostasis on a normal chow diet in either sex, using the same mouse strains \[850\]. Of note, RIP-Cre controls had slightly worsened glucose homeostasis of their own, possibly due to recombination in the hypothalamus \[850\]. Interestingly however, islets isolated from Arnt KO mice exhibited impaired GSIS \textit{in vitro} \[850\]. This was due to a lower glucose-stimulated increase in the NADPH/NADP\textsuperscript{+} ratio, which in turn reduced glucose-dependent increases in the ATP:ADP ratio and Ca\textsuperscript{2+} cyt influx \[850\]. This study thus suggests that the underlying defect in Arnt KO mice is of a metabolic nature. Notably, the Arnt KO phenotype is reminiscent of defects seen in the Npas4 KO mouse that is described in Chapter 4.

In the previous two chapters, the calcium-dependent pathways leading to NPAS4 expression in β-cells, its interaction with ARNT, and competition with HIF1α have been investigated. Metabolic defects were seen in Npas4 KO islets \textit{in vitro}, due to increased HIF1α target gene expression and reduced oxidative metabolism (Chapter 4). While phenotypes are not identical to the Arnt KO mouse, ARNT has many interaction partners, and defects in the knockout may only be partially attributable to loss of NPAS4 activity. Thus, NPAS4 and HIF1α together with ARNT regulate β-cell metabolism in an opposing manner, yet the exact transcriptional networks which are under their respective control have not been elucidated in full in β-cells. To begin to unravel the impact of NPAS4 expression in β-cells, the genetic targets of these transcription factors were probed in an unbiased genome-wide screen via NPAS4 and ARNT chromatin
immunoprecipitation combined with ultra-high throughput sequencing (ChIP-Seq). I hypothesized that NPAS4:ARNT and HIF1α:ARNT bind to enhancers and promoters to regulate metabolic genes under different physiologic conditions, and thus inversely modulate β-cell function. Due to their hypothesized similar consensus binding motif, binding of a subset of identical targets is predicted.

5.2 NPAS4 and ARNT binding motif analysis

To delineate the gene regulatory network under control of NPAS4, ARNT, and HIF1α in β-cells, ChIP-Seq was performed in MIN6 cells cultured under basal (low glucose; LG), stimulatory (high glucose and KCl; KCL), or HIF1α-stabilizing (high glucose and 1 % O2; HYPO) conditions (Figure 5.1). Protocols were optimized for obtaining small (200-350 bp) DNA fragments (Figure 5.1 A) suitable for ChIP-Seq, and ChIP was performed using antibodies against ARNT (ChIP:ARNT) or NPAS4 (ChIP:NPAS4). Prior to ChIP-Seq library construction and next generation sequencing, I ascertained ChIP enrichment. For instance, sequences predicted to be bound by HIF1α:ARNT, such as the forkhead box P1 (Foxp1) enhancer or Ldha promoter, were enriched in ChIP:ARNT following high glucose exposure under hypoxia (HYPO) (Figure 5.1 B&C). Because NPAS4 is absent under basal conditions (Chapter 3), and not induced significantly during hypoxia even with stimulatory conditions [3; 4], no enrichment was seen in LG and HYPO ChIP:NPAS4 samples. In contrast, there was a trend towards enrichment of NPAS4 at the Foxp1 enhancer site following induction by depolarization (KCL; Figure 5.1 B). Thus, I commenced library construction using input and IP fractions. I validated that samples showed suitable DNA fragment sizes (250-600 bp) following size selection (Figure 5.1 D), and subsequently sequenced libraries. The resulting reads were mapped to the mouse genome as described (section 2.15.6), and peaks called based on sequence enrichment in IP over input.
Figure 5.1. ChIP validation.

(A) Following sonication, input fractions from basal (LG), stimulatory (KCL), or HIF1α-stabilizing (HYPO) condition showed enrichment of small (200-300 bp) DNA fragments suitable for ChIP-Seq.

(B&C) Following ChIP, ARNT and NPAS4 binding at the Foxp1 enhancer and Ldha promoter was assessed via qPCR (n = 5).

(D) Following library construction and size selection, Bioanalyzer analysis confirmed uniform DNA fragment sizes, with the majority 250-600 bp in size, and a maximum of 1,500 bp. The black dotted line indicates removal of fragments smaller than 200 bp by single size selection. Green and purple lines represent lower and upper size marker, respectively. Significance was determined using a one-way ANOVA with Dunnett’s multiple comparisons test. * p ≤ 0.05; *** p ≤ 0.001.

As a first measure to evaluate the ChIP-Seq results, enriched sequences were analyzed for known DNA binding motifs. Because both HIF1α and NPAS4 interact with ARNT [539; 851], they both recognize the core Class II PAS bHLH motif 5’-GTG-3’ [516; 539; 852], but preceding base preferences differ subtly. In the absence of NPAS4 (LG and HYPO), HIF1α:ARNT was expected
to be the dominant transcription factor and thus the hypoxia response element 5’-TACGTG-3’ should be found in ChIP:ARNT, but not ChIP:NPAS4 samples. In contrast, due to competition for ARNT binding between NPAS4 and HIF1α under stimulatory conditions (KCL), the similar but more flexible NPAS4 consensus motif of 5’-NCGTG-3’ might be expected.

As predicted, there was no significant enrichment of sequences in LG ChIP:NPAS4 samples (not shown; n = 4), confirming the absence of NPAS4 under basal conditions. In contrast, sequences were enriched for HIF1α:ARNT motifs (5’-TACGTG-3’; Table 5.1) in LG ChIP:ARNT samples, confirming an earlier assessment in Chapter 4 that HIF1α transcriptional activity is enhanced in the absence of NPAS4. This may also be partially attributable to the Warburg effect seen in tumour cells, reflecting the insulinoma origin of MIN6 cells. Some other motifs enriched in LG ChIP:ARNT included homeobox motifs (5’-(T/C)AATTA-3’), such as that of key β-cell transcription factor NKX6.1 (5’-TAAT-3’), suggesting key targets of NKX6.1 might be co-regulated by HIF1α:ARNT.
NPAS4:ARNT participates in regulating transcription by binding close to sites with AP-1 (bZIP) motifs. This motif is regulated by cellular activity. The second most enriched known motif in KCL ChIP:NPAS4 samples was 5′-TGA(G/C)TCA-3′ (Table 5.2). This bZIP motif is recognized by proteins of the heterodimeric activator protein 1 (AP-1) complex (JUN, FOS, ATF families), which is regulated by cellular activity. The second most enriched motif 5′-(A/T)CTAC-3′ belonged to the forkhead transcription factor family (e.g. FOXA2). Although these observations were partly unexpected, it raises the possibility that NPAS4:ARNT participates in regulating transcription by binding close to sites with AP-1 and FOX motifs.

Table 5.1. Known motifs in low glucose (LG) ChIP:ARNT samples.
Identification and ranking (#) of known DNA binding motifs based on significant (p) enrichment of sequences in LG ChIP:ARNT fractions (Target Seq) over LG input fractions (Background Seq). n = 4.

<table>
<thead>
<tr>
<th>#</th>
<th>Motif</th>
<th>Name</th>
<th>p</th>
<th>Target Seq [%]</th>
<th>Background Seq [%]</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td><img src="image1.png" alt="Motif 1" /></td>
<td>SpiB (ETS)</td>
<td>10^{-15}</td>
<td>232 (0.87)</td>
<td>103.9 (0.49)</td>
</tr>
<tr>
<td>2</td>
<td><img src="image2.png" alt="Motif 2" /></td>
<td>Nkx6.1 (Homeobox)</td>
<td>10^{-9}</td>
<td>2175 (8.12)</td>
<td>1524.9 (7.12)</td>
</tr>
<tr>
<td>3</td>
<td><img src="image3.png" alt="Motif 3" /></td>
<td>HIF1α (bHLH)</td>
<td>10^{-9}</td>
<td>111 (0.41)</td>
<td>47.4 (0.22)</td>
</tr>
<tr>
<td>4</td>
<td><img src="image4.png" alt="Motif 4" /></td>
<td>Rfx5 (HTH)</td>
<td>10^{-8}</td>
<td>154 (0.58)</td>
<td>74.1 (0.35)</td>
</tr>
<tr>
<td>5</td>
<td><img src="image5.png" alt="Motif 5" /></td>
<td>Nrf2 (bZIP)</td>
<td>10^{-7}</td>
<td>22 (0.08)</td>
<td>4.8 (0.02)</td>
</tr>
<tr>
<td>6</td>
<td><img src="image6.png" alt="Motif 6" /></td>
<td>HIF2α (bHLH)</td>
<td>10^{-6}</td>
<td>143 (0.53)</td>
<td>74.4 (0.35)</td>
</tr>
<tr>
<td>7</td>
<td><img src="image7.png" alt="Motif 7" /></td>
<td>Lhx2 (Homeobox)</td>
<td>10^{-4}</td>
<td>770 (2.88)</td>
<td>528.5 (2.47)</td>
</tr>
<tr>
<td>8</td>
<td><img src="image8.png" alt="Motif 8" /></td>
<td>RARγ (NR)</td>
<td>10^{-4}</td>
<td>14 (0.05)</td>
<td>3.9 (0.02)</td>
</tr>
<tr>
<td>9</td>
<td><img src="image9.png" alt="Motif 9" /></td>
<td>Lhx3 (Homeobox)</td>
<td>10^{-4}</td>
<td>1264 (4.72)</td>
<td>907.3 (4.24)</td>
</tr>
<tr>
<td>10</td>
<td><img src="image10.png" alt="Motif 10" /></td>
<td>ARNT (HLH)</td>
<td>10^{-4}</td>
<td>441 (1.65)</td>
<td>293.7 (1.37)</td>
</tr>
</tbody>
</table>

Depolarization induces profound changes in cellular activity, and NPAS4 as an immediate early gene is implicated in the direct transcriptional response to these changes. The most significantly enriched known motif in KCL ChIP:NPAS4 samples was 5′-TGA(G/C)TCA-3′ (Table 5.2). This bZIP motif is recognized by proteins of the heterodimeric activator protein 1 (AP-1) complex (JUN, FOS, ATF families), which is regulated by cellular activity. The second most enriched motif 5′-(A/T)CTAC-3′ belonged to the forkhead transcription factor family (e.g. FOXA2). Although these observations were partly unexpected, it raises the possibility that NPAS4:ARNT participates in regulating transcription by binding close to sites with AP-1 and FOX motifs.
Table 5.2. Known motifs in stimulated (KCL) ChIP:NPAS4 samples.

Identification and ranking (#) of known DNA binding motifs based on significant (p) enrichment of sequences in KCL ChIP:NPAS4 fractions (Target Seq) over KCL input fractions (Background Seq). n = 4.

<table>
<thead>
<tr>
<th>#</th>
<th>Motif</th>
<th>Name</th>
<th>p</th>
<th>Target Seq [%]</th>
<th>Background Seq [%]</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td><img src="motif1.png" alt="Motif" /></td>
<td>BATF (bZIP)</td>
<td>10^{-197}</td>
<td>396 (11.10)</td>
<td>664.9 (1.57)</td>
</tr>
<tr>
<td>2</td>
<td><img src="motif2.png" alt="Motif" /></td>
<td>JunB (bZIP)</td>
<td>10^{-185}</td>
<td>361 (10.12)</td>
<td>579.4 (1.37)</td>
</tr>
<tr>
<td>3</td>
<td><img src="motif3.png" alt="Motif" /></td>
<td>Fra1 (bZIP)</td>
<td>10^{-184}</td>
<td>354 (9.92)</td>
<td>556.8 (1.32)</td>
</tr>
<tr>
<td>4</td>
<td><img src="motif4.png" alt="Motif" /></td>
<td>Atf3 (bZIP)</td>
<td>10^{-184}</td>
<td>375 (10.51)</td>
<td>640.4 (1.52)</td>
</tr>
<tr>
<td>5</td>
<td><img src="motif5.png" alt="Motif" /></td>
<td>Fra2 (bZIP)</td>
<td>10^{-174}</td>
<td>322 (9.03)</td>
<td>477.5 (1.13)</td>
</tr>
<tr>
<td>6</td>
<td><img src="motif6.png" alt="Motif" /></td>
<td>AP-1 (bZIP)</td>
<td>10^{-173}</td>
<td>397 (11.13)</td>
<td>787.2 (1.86)</td>
</tr>
<tr>
<td>7</td>
<td><img src="motif7.png" alt="Motif" /></td>
<td>FOXA1 (forkhead)</td>
<td>10^{-167}</td>
<td>449 (12.59)</td>
<td>1066.6 (2.53)</td>
</tr>
<tr>
<td>8</td>
<td><img src="motif8.png" alt="Motif" /></td>
<td>Fosl2 (bZIP)</td>
<td>10^{-165}</td>
<td>267 (7.49)</td>
<td>324.8 (0.77)</td>
</tr>
<tr>
<td>9</td>
<td><img src="motif9.png" alt="Motif" /></td>
<td>FOXA1 (forkhead)</td>
<td>10^{-162}</td>
<td>394 (11.05)</td>
<td>833.4 (1.97)</td>
</tr>
<tr>
<td>10</td>
<td><img src="motif10.png" alt="Motif" /></td>
<td>FOXM1 (forkhead)</td>
<td>10^{-158}</td>
<td>396 (11.10)</td>
<td>864 (2.05)</td>
</tr>
</tbody>
</table>

A similar picture emerged in KCL ChIP:ARNT samples, in which the predominant known motifs belonged to the AP-1 family (Table 5.3). As before, a likely explanation was that DNA fragments enriched by ChIP:ARNT contained regulatory elements with binding sites for both AP-1 and ARNT. While it is intriguing that AP-1 motifs were much more significantly enriched than the anticipated bHLH or E-Box motifs (not shown), any potential biological relevance, such as cooperative binding of ARNT with AP-1, would have to be tested experimentally.
Table 5.3. Known motifs in stimulated (KCL) ChIP:ARNT samples.
Identification and ranking (#) of known DNA binding motifs based on significant (p) enrichment of sequences in KCL ChIP:ARNT fractions (Target Seq) over KCL input fractions (Background Seq). n = 4.

<table>
<thead>
<tr>
<th>#</th>
<th>Motif</th>
<th>Name</th>
<th>P</th>
<th>Target Seq [%]</th>
<th>Background Seq [%]</th>
</tr>
</thead>
<tbody>
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<td>1</td>
<td>[CATGACTCA]</td>
<td>Fra1 (bZIP)</td>
<td>10^{-102}</td>
<td>368 (3.30)</td>
<td>278.7 (0.84)</td>
</tr>
<tr>
<td>2</td>
<td>[CATGACTCA]</td>
<td>Fra2 (bZIP)</td>
<td>10^{-98}</td>
<td>321 (2.88)</td>
<td>223.6 (0.68)</td>
</tr>
<tr>
<td>3</td>
<td>[CATGACTCA]</td>
<td>BATF (bZIP)</td>
<td>10^{-96}</td>
<td>420 (3.76)</td>
<td>368.6 (1.12)</td>
</tr>
<tr>
<td>4</td>
<td>[CATGACTCA]</td>
<td>AP-1 (bZIP)</td>
<td>10^{-95}</td>
<td>445 (3.99)</td>
<td>411.3 (1.24)</td>
</tr>
<tr>
<td>5</td>
<td>[CATGACTCA]</td>
<td>Atf3 (bZIP)</td>
<td>10^{-94}</td>
<td>402 (3.60)</td>
<td>346.8 (1.05)</td>
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<tr>
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<td>[CATGACTCA]</td>
<td>Jun-API1 (bZIP)</td>
<td>10^{-92}</td>
<td>205 (1.84)</td>
<td>94.8 (0.29)</td>
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<td>7</td>
<td>[CATGACTCA]</td>
<td>Fosl2 (bZIP)</td>
<td>10^{-90}</td>
<td>251 (2.25)</td>
<td>148.5 (0.45)</td>
</tr>
<tr>
<td>8</td>
<td>[CATGACTCA]</td>
<td>JunB (bZIP)</td>
<td>10^{-90}</td>
<td>367 (3.29)</td>
<td>305.8 (0.93)</td>
</tr>
<tr>
<td>9</td>
<td>[CATGTTTACA]</td>
<td>Foxa2 (forkhead)</td>
<td>10^{-42}</td>
<td>438 (3.92)</td>
<td>627 (1.90)</td>
</tr>
<tr>
<td>10</td>
<td>[TGCTGACTCA]</td>
<td>Bach2 (bZIP)</td>
<td>10^{-40}</td>
<td>123 (1.10)</td>
<td>81.6 (0.25)</td>
</tr>
</tbody>
</table>

Exposure of MIN6 cells to hypoxia is expected to stabilize HIF1α and enable HIF1α:ARNT dimerization and DNA binding. Indeed, HYPO ChIP:ARNT samples showed an enrichment of HIF1α motifs compared to background, but another dominant known motif was the forkhead family motif, particularly FOXA1 (5′-AAAGTAAACA-3′) (Table 5.4). FOXA1 can uncouple mitochondrial metabolism through direct regulation of UCP2 [853], while HIF1α:ARNT upregulates expression of genes for anaerobic glycolysis, suggesting the two transcription factors could synergize in regulating the hypoxic stress response.
Table 5.4. Known motifs in HIF1α-stabilizing (HYPO) ChIP:ARNT samples.

Identification and ranking (#) of known DNA binding motifs based on significant (p) enrichment of sequences in HYPO ChIP:ARNT fractions (Target Seq) over HYPO input fractions (Background Seq). n = 2.

<table>
<thead>
<tr>
<th>#</th>
<th>Motif</th>
<th>Name</th>
<th>p</th>
<th>Target Seq [%]</th>
<th>Background Seq [%]</th>
</tr>
</thead>
<tbody>
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<td>1</td>
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<td>FOXA1 (forkhead)</td>
<td>$10^{-275}$</td>
<td>1036 (12.17)</td>
<td>1297.1 (3.31)</td>
</tr>
<tr>
<td>2</td>
<td><img src="image2" alt="Motif2" /></td>
<td>FOXM1 (forkhead)</td>
<td>$10^{-228}$</td>
<td>868 (10.20)</td>
<td>1087.1 (2.77)</td>
</tr>
<tr>
<td>3</td>
<td><img src="image3" alt="Motif3" /></td>
<td>Foxa2 (forkhead)</td>
<td>$10^{-205}$</td>
<td>720 (8.46)</td>
<td>838.7 (2.14)</td>
</tr>
<tr>
<td>4</td>
<td><img src="image4" alt="Motif4" /></td>
<td>Foxa3 (forkhead)</td>
<td>$10^{-176}$</td>
<td>392 (4.60)</td>
<td>283.2 (0.72)</td>
</tr>
<tr>
<td>5</td>
<td><img src="image5" alt="Motif5" /></td>
<td>Fox:Ebox (Forkhead,bHLH)</td>
<td>$10^{-160}$</td>
<td>725 (8.52)</td>
<td>1031.5 (2.63)</td>
</tr>
<tr>
<td>6</td>
<td><img src="image6" alt="Motif6" /></td>
<td>NeuroD1 (bHLH)</td>
<td>$10^{-116}$</td>
<td>509 (5.98)</td>
<td>704.6 (1.80)</td>
</tr>
<tr>
<td>7</td>
<td><img src="image7" alt="Motif7" /></td>
<td>Atoh1 (bHLH)</td>
<td>$10^{-100}$</td>
<td>617 (7.25)</td>
<td>1069.4 (2.73)</td>
</tr>
<tr>
<td>8</td>
<td><img src="image8" alt="Motif8" /></td>
<td>FOXK1 (forkhead)</td>
<td>$10^{-100}$</td>
<td>617 (7.25)</td>
<td>1070.9 (2.73)</td>
</tr>
<tr>
<td>9</td>
<td><img src="image9" alt="Motif9" /></td>
<td>HIF2α (bHLH)</td>
<td>$10^{-96}$</td>
<td>357 (4.19)</td>
<td>431.3 (1.10)</td>
</tr>
<tr>
<td>10</td>
<td><img src="image10" alt="Motif10" /></td>
<td>HIF1α (bHLH)</td>
<td>$10^{-96}$</td>
<td>302 (3.55)</td>
<td>315 (0.80)</td>
</tr>
</tbody>
</table>

Lastly, only few sequences were enriched in HYPO ChIP:NPAS4 samples (Table 5.5). This was expected, given that MIN6 cells are not as glucose-responsive as primary β-cells, requiring depolarization in addition to glucose for optimal NPAS4 induction \(^{[446]}\). Furthermore, NPAS4 is largely suppressed by hypoxia \(^{[3:4]}\), and thus it was noteworthy that some sequence motifs were enriched. The principal known motif in this condition was the forkhead family motif (Table 5.5), although the overall confidence in this prediction was low (compare p-values in Table 5.5 with Table 5.2). Considering that LG ChIP:NPAS4 samples showed no enrichment at all, these motifs might represent very low levels of NPAS4 induction and DNA binding rather than non-specific background. Furthermore, NPAS4 and ARNT interacted during hypoxia (Chapter 4), indicating
that NPAS4, present in low amounts, could retain its DNA-binding activity (barring changes in subcellular localization).

Table 5.5. Known motifs in HIF1α-stabilizing (HYPO) ChIP:NPAS4 samples.
Identification and ranking (#) of known DNA binding motifs based on significant (p) enrichment of sequences in HYPO ChIP:NPAS4 fractions (Target Seq) over HYPO input fractions (Background Seq). n = 3.

<table>
<thead>
<tr>
<th>#</th>
<th>Motif</th>
<th>Name</th>
<th>p</th>
<th>Target Seq [%]</th>
<th>Background Seq [%]</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>[Motif Image]</td>
<td>FOXA1 (forkhead)</td>
<td>10^-9</td>
<td>14 (22.58)</td>
<td>1038.9 (2.46)</td>
</tr>
<tr>
<td>2</td>
<td>[Motif Image]</td>
<td>Foxa3 (forkhead)</td>
<td>10^-7</td>
<td>7 (11.29)</td>
<td>191.9 (0.45)</td>
</tr>
<tr>
<td>3</td>
<td>[Motif Image]</td>
<td>FOXM1 (forkhead)</td>
<td>10^-7</td>
<td>11 (17.74)</td>
<td>832.7 (1.97)</td>
</tr>
<tr>
<td>4</td>
<td>[Motif Image]</td>
<td>Foxa2 (forkhead)</td>
<td>10^-7</td>
<td>10 (16.13)</td>
<td>696.9 (1.65)</td>
</tr>
<tr>
<td>5</td>
<td>[Motif Image]</td>
<td>FoxL2 (forkhead)</td>
<td>10^-4</td>
<td>7 (11.29)</td>
<td>627.5 (1.48)</td>
</tr>
<tr>
<td>7</td>
<td>[Motif Image]</td>
<td>FOXX2 (forkhead)</td>
<td>10^-4</td>
<td>6 (9.68)</td>
<td>472.9 (1.12)</td>
</tr>
<tr>
<td>8</td>
<td>[Motif Image]</td>
<td>Foxo3 (forkhead)</td>
<td>10^-3</td>
<td>6 (9.68)</td>
<td>641.2 (1.52)</td>
</tr>
<tr>
<td>9</td>
<td>[Motif Image]</td>
<td>Foxf1 (forkhead)</td>
<td>10^-3</td>
<td>6 (9.68)</td>
<td>675.3 (1.60)</td>
</tr>
<tr>
<td>10</td>
<td>[Motif Image]</td>
<td>Foxo1 (forkhead)</td>
<td>10^-3</td>
<td>9 (14.52)</td>
<td>1714.9 (4.06)</td>
</tr>
</tbody>
</table>

Together, these results confirmed that DNA sequences were specifically enriched based on which transcription factor was immunoprecipitated and which stimulus cells were exposed to. While useful for initial analysis of ChIP-Seq samples, some limitations apply. For example, while comparing results to known motifs might work for HIF1α:ARNT, which has been well characterized, there was no database entry for an NPAS4 consensus binding motif in the analysis. Thus, looking at de novo predicted binding motifs might be a more informative approach, especially for NPAS4. Notably, available ChIP-Seq data could not discriminate which
DNA strand NPAS4:ARNT binds to, hence the actual NPAS4-bound sequence might have appeared in reverse after sequencing. Indeed, the top \textit{de novo} motif “GTCACGAT” in KCL ChIP:NPAS4 samples matched the previously reported preferred NPAS4 binding site in reverse orientation (Table 5.6) with a few bases appended on either side, resulting in 5’-ATCGTGAC-3’ (previous consensus underlined)\cite{539}. The second most enriched \textit{de novo} motif was again an AP-1 motif (Table 5.6). At this point, it was interesting to speculate that the AP-1 complex attached to one strand of DNA with the core sequence 5’-TGAGTCA-3’, and NPAS4:ARNT bound the complementary strand with 5’-ATCGTGAC-3’ (overlap of reverse complement underlined). While it would explain co-enrichment of NPAS4 and AP-1 binding sites in ChIP:NPAS4 samples, this possibility has to be investigated experimentally. The third most enriched motif in KCL ChIP:NPAS4 samples was a forkhead-like motif, indicating co-operative binding with members of that family. In HYPO ChIP:NPAS4 samples, a core NPAS4 motif (5’-TCGTG-3’) was found in all three predicted motifs, suggesting that residual NPAS4 was interacting with ARNT (Chapter 4) and binding DNA even under hypoxia.

\textbf{Table 5.6. \textit{De novo} predicted binding motifs of NPAS4 and ARNT under different physiological conditions.}

Prediction and ranking (#) of \textit{de novo} DNA binding motifs based on significant (p) enrichment of sequences in LG, KCL or HYPO ChIP:NPAS4 and ChIP:ARNT fractions.

<table>
<thead>
<tr>
<th>#</th>
<th>Motif</th>
<th>Reverse Motif</th>
<th>p</th>
<th>Target Seq [%]</th>
<th>Background Seq [%]</th>
</tr>
</thead>
<tbody>
<tr>
<td>LG ChIP:ARNT</td>
<td></td>
<td></td>
<td>10^{-2107}</td>
<td>20.34</td>
<td>3.65</td>
</tr>
<tr>
<td>1</td>
<td>AAAAAAAAAAGGGAG</td>
<td>TTICCTTTTTTTTTTT</td>
<td></td>
<td>10^{-56}</td>
<td>0.18</td>
</tr>
<tr>
<td>2</td>
<td>GGAGAACAACCTGTC</td>
<td>GACAGGGTTTCTCC</td>
<td></td>
<td>10^{-38}</td>
<td>0.13</td>
</tr>
</tbody>
</table>
On the other hand, the most significant *de novo* motifs predicted for ChIP:ARNT samples were more heterogeneous. In both LG and KCL conditions, an SRF2-like motif became the most significantly enriched over background. It is unknown if this represents biological relevance or could be a sequencing artefact due to the repetitive sequence. The second and third most predicted sequences in LG ChIP:ARNT samples were most closely related to IRF4 and ZFX.

<table>
<thead>
<tr>
<th>#</th>
<th>Motif</th>
<th>Reverse Motif</th>
<th>p</th>
<th>Target Seq [%]</th>
<th>Background Seq [%]</th>
</tr>
</thead>
</table>
| KCL ChIP:NPAS4
| 1  | GTCACCAGC      | ATCGTGAC      | 10^{-314} | 14.44          | 1.55               |
| 2  | ATGACTCA       | TGACTCAT      | 10^{-196} | 11.24          | 1.63               |
| 3  | TGTTTTACAT     | TGTTTAACAT    | 10^{-186} | 17.49          | 4.42               |
| KCL ChIP:ARNT
| 1  | CGGCCTTTTTT    | AAAAAAAAAAAA  | 10^{-106} | 14.71          | 1.34               |
| 2  | ATGAGTCA       | GTGACTCAT     | 10^{-119} | 2.63           | 0.46               |
| 3  | TACTATGTAAAA   | TGTTTACATTAGC | 10^{-69}  | 0.80           | 0.06               |
| HYPO ChIP:NPAS4
| 1  | GTCGTGACT      | ATGCACGAC     | 10^{-23}  | 19.35          | 0.10               |
| 2  | TTACTAATCGTA   | TCACGATTAGTA  | 10^{-12}  | 9.68           | 0.04               |
| 3  | CGATTGCGTGGTTG | CAAGCAGCCGATC | 10^{-12}  | 6.45           | 0.00               |
| HYPO ChIP:ARNT
| 1  | TGTTTACCTA     | TAACTAACAT    | 10^{-279} | 12.96          | 3.68               |
| 2  | GCCATTATAG     | CATTATAGC     | 10^{-126} | 6.63           | 2.03               |
| 3  | ATCCATCTCGT    | ACGTGCCGCCGC  | 10^{-125} | 5.57           | 1.49               |
motifs, respectively, but only found in a very limited number of target sequences; thus, they might not be relevant. The second and third most enriched motifs in KCL ChIP:ARNT samples resembled AP-1 and FOXA2 motifs, respectively, and might be enriched due to ARNT interaction with NPAS4. Lastly, a motif resembling a forkhead transcription factor binding site was predicted the most enriched de novo motif in HYPO ChIP:ARNT samples, possibly representing cooperative gene regulation of bHLH-PAS and forkhead transcription factors. The second motif resembled the NEUROD1 binding sequence 5'-CAGATGG-3', an E-Box motif. This could indicate either HIF1α:ARNT binding near NeuroD1 binding sites, such as the insulin promoter, or that a NEUROD1-like DNA sequence was the bona fide HIF1α:ARNT motif in MIN6 cells. The third most represented de novo motif was a variation of a HIF1α binding site.

Altogether, binding motif analysis gave us some first clues as to which sequences each transcription factor was preferably binding, and under which physiological condition. Comparing enriched target sequences to known transcription factor binding sites worked well for HIF1α:ARNT complexes, as its DNA binding motif was well established, whereas an NPAS4 DNA binding sequence was best predicted de novo in absence of an available consensus. It appeared that the most common binding site for NPAS4 in MIN6 β-cells had the sequence 5'-TCGTGA-3'. This information may be useful in pinpointing or confirming possible NPAS4 target genes in β-cells in future studies.

5.3 NPAS4 and ARNT share binding sites under different physiological conditions

One question that I asked at the beginning of the ChIP-Seq experiment was whether there would be any significant competition between NPAS4 and HIF1α at DNA binding sites. Since ChIP for HIF1α was not carried out, ChIP:ARNT under hypoxia served as a proxy, as this would be the main bHLH-PAS heterodimer under these conditions [454; 464; 516]. HIF2α:ARNT heterodimers
were also possible [480], although HIF2α expression in murine β-cells is low [483; 484]. Initially, heatmaps of ChIP:ARNT peaks were assessed for significant overlapping patterns in all ChIP-Seq samples. LG ChIP:ARNT peaks predictably showed the strongest overlap with LG ChIP:ARNT samples (Figure 5.2 A1, Figure 5.3 A1&2), followed by KCL ChIP:ARNT and HYPO ChIP:ARNT (Figure 5.2 A2&3, Figure 5.3 A1, A3&4). Peaks enriched in LG ChIP:ARNT were not seen as frequently in ChIP:NPAS4 samples (Figure 5.2 A4-6, Figure 5.3 A1, A5-7), suggesting that they did not result from interaction with NPAS4. Following stimulation with KCL, ChIP:ARNT peaks still shared similar binding patterns in LG and KCL conditions (Figure 5.2 B1&2, Figure 5.3 B1-3), but depolarization-induced ARNT binding differed noticeably from hypoxic conditions (Figure 5.2 B3, Figure 5.3 B1&4). KCL ChIP:ARNT peaks overlapped more closely with KCL ChIP:NPAS4 peaks than those in LG or HYPO ChIP:NPAS4 samples (Figure 5.2 B4-6, Figure 5.3 B1, B5-7); which was predicted given that this is when NPAS4 is most induced and interacts with ARNT (Chapter 4). HYPO ChIP:ARNT peaks were more unique and most clearly overlapped with HYPO ChIP:ARNT samples, followed by LG and KCL (Figure 5.2 C1-3, Figure 5.3 C1-4). However, there were also noticeable similarities between peaks found in HYPO ChIP:ARNT and KCL ChIP: NPAS4 (Figure 5.2 C5, Figure 5.3 C1&6); indicating that NPAS4:ARNT and HIF1α:ARNT could bind similar sites, albeit under different physiologic stimuli.
Figure 5.2. ChIP peak binding around LG, KCL, and HYPO ChIP:ARNT peak summits.
ChIP peak signals of ChIP:ARNT and ChIP:NPAS4 samples were visualized using EaSeq. (A1-A6) Heatmaps showing distribution of ChIP:ARNT and ChIP:NPAS4 peaks ±1,000 bp around LG ChIP:ARNT peak summits. (B1-B6) Heatmaps showing distribution of ChIP:ARNT and ChIP:NPAS4 peaks ±1,000 bp around KCL ChIP:ARNT peak summits. (C1-C6) Heatmaps showing distribution of ChIP:ARNT and ChIP:NPAS4 peaks ±1,000 bp around HYPO ChIP:ARNT peak summits. Peaks were sorted by score.

In summary, this analysis suggested that NPAS4 and ARNT share overlapping binding patterns during stimulatory conditions, as they regulated gene expression together. More intriguingly, sites enriched in ChIP:ARNT samples under hypoxia, and thus likely due to interaction with HIF1α, shared a significant overlap with KCL ChIP:NPAS4 samples, further supporting the idea that NPAS4 competed with HIF1α to regulate transcription.

Figure 5. ChIP peak signals of ChIP:ARNT and ChIP:NPAS4 samples were visualized using EaSeq. (A1-A7) Overlay (A1) and individual tracks (A2-A7) of average ChIP peak density profiles ±1,000 bp around LG ChIP:ARNT peak summits. (B1-B7) Overlay (B1) and individual tracks (B2-B7) of average ChIP peak density profiles ±1,000 bp around KCL ChIP:ARNT peak summits. (C1-C7) Overlay (C1) and individual tracks (C2-C7) of average ChIP peak density profiles ±1,000 bp around HYPO ChIP:ARNT peak summits.

To corroborate these findings, the reverse analysis was conducted with ChIP:NPAS4 samples.

For LG treated samples, there was not sufficient enrichment to conduct a reliable analysis, due
to absence of NPAS4 expression (not shown). Strikingly, sequences enriched in KCL ChIP:NPAS4 samples not only overlapped with KCL ChIP:NPAS4, but also overlapped with similar efficiency with all ChIP:ARNT samples (Figure 5.4 A1-6, Figure 5.5 A1-7). This showed that binding sites occupied by NPAS4 under KCL were bound by ARNT without NPAS4 in LG and HYPO, providing a mechanistic foundation to explain the dichotomous role of ARNT in regulating metabolism.

Figure 5.4. ChIP peak binding around KCL and HYPO ChIP:NPAS4 peak summits.
ChIP peak signals of ChIP:ARNT and ChIP:NPAS4 samples were visualized using EaSeq. (A1-A6) Heatmaps showing distribution of ChIP:ARNT and ChIP:NPAS4 peaks ±1,000 bp around KCL ChIP:NPAS4 peak summits. (B1-B6) Heatmaps showing distribution of ChIP:ARNT and ChIP:NPAS4 peaks ±1,000 bp around HYPO ChIP:NPAS4 peak summits. Peaks were sorted by score.
Few peaks were identified in HYPO ChIP:NPAS4, but those that were present matched sequences bound in all other samples equally well; indicating that even during chronic hypoxia, residual NPAS4 could, presumably with ARNT, occupy sequences that were likely targets of HIF1α:ARNT (Figure 5.4 B1-6, Figure 5.5 B1-7).

**Figure 5.5. Average binding profiles of ChIP peaks around KCL and HYPO ChIP:NPAS4 summits.**

ChIP peak signals of ChIP:ARNT and ChIP:NPAS4 samples were visualized using EaSeq. (A1-A7) Overlay (A1) and individual tracks (A2-A7) of average ChIP peak density profiles ±1,000 bp around KCL ChIP:NPAS4 peak summits. (B1-B7) Overlay (B1) and individual tracks (B2-B7) of average ChIP peak density profiles ±1,000 bp around HYPO ChIP:NPAS4 peak summits.

In summary, this analysis showed that NPAS4 likely competed with HIF1α at many target gene promoters. This provided an alternative mode of competition between these bHLH-PAS family members, in addition to competition for ARNT interaction, and might reflect a similar observation in neuronal cells [539].
Because NPAS4 mainly binds enhancers in neurons\textsuperscript{[315]}, I next asked whether this would be the case in β-cells as well. Indeed, the majority of peaks identified in KCL and HYPO ChIP:NPAS4 samples were annotated as intronic and intergenic regions, which included enhancer regions (Figure 5.6 A&B). Notably, NPAS4 peaks were relatively more abundant in promoter/transcriptional start site (TSS) regions than ARNT, which mainly bound intergenic and intronic regions (Figure 5.6 C&D). However, a noticeable shift occurred following hypoxic exposure, with a greater percentage of promoter sites occupied by ARNT trebling (Figure 5.6 E). There were marked differences in the absolute number of peaks between samples, but it remained unknown whether this influenced any of the observed trends.

Figure 5.6. NPAS4 binds relatively more promoter sites than ARNT in MIN6 cells. (A-E) HOMER was used to annotate genomic regions of ChIP:NPAS4 (A&B) and ChIP:ARNT (C-E) peaks. Percentage of total peaks is indicated for promoters/transcriptional start sites (TSS), introns, and intergenic regions (absolute number of identified peaks is indicated).

\[\text{Peaks: 4009}\]
\[\text{Peaks: 146}\]
\[\text{Peaks: 54195}\]
\[\text{Peaks: 17770}\]
\[\text{Peaks: 10575}\]
Lastly, the overall number of overlapping peaks between conditions was compared. Stimulated cells shared the most significant overlap; almost 60% of KCL NPAS4 peaks were also observed in KCL ChIP:ARNT samples, corresponding to 13.5% of ChIP:ARNT peaks (Figure 5.7). Despite the overall lower number of peaks, a similar percentage of ChIP:NPAS4-enriched sequences were also bound by ARNT under hypoxia (Figure 5.7 B). It will be interesting to test whether NPAS4 and ARNT can form heterodimers and bind DNA under hypoxic conditions, which canonically favour HIF1α.

![Figure 5.7](image)

Figure 5.7. NPAS4 and ARNT DNA binding overlaps following depolarization and hypoxia. (A&B) Bedtools was used to determine overlap of ARNT and NPAS4 peaks following KCL (A) or HYPO (B) treatment. Absolute numbers of ARNT and NPAS4 peaks found in the same genomic location (± 100 bp), as well as sites unique to either transcription factor, are indicated.

In conclusion, peak overlap analysis suggested that NPAS4:ARNT bound to sites occupied by ARNT during hypoxia, and thus, is likely able to compete with the HIF1α:ARNT heterodimer. While the majority (~60%) of peaks in ChIP NPAS4 samples overlapped with ARNT peaks, it is puzzling what the remaining 40% of peaks represent. NPAS4 interaction with ARNT2 has been shown in Chapter 4, but based on expression data from islets [296], it is unlikely that it can account for such a large proportion.
5.4 NPAS4 and ARNT are enriched at important β-cell genes

Three direct targets of NPAS4 DNA binding in β-cells were previously identified, namely near the Ins1, Ins2, and Rgs2 genes [446]. In neurons, NPAS4 was found at 28,000 unique sites, many of which were enhancers [315], and many putative NPAS4 target genes were classified as immediate early or activity-regulated genes themselves [562; 564; 565]. In support of these observations, the expression of numerous putative NPAS4 targets [315; 354; 546; 562; 564; 565; 854; 855] was changed in RNA-Seq data from N4 PKO islets, with the largest expression changes noted for activity-regulated genes Egr1, Egr4, Nptx2, and Rgs2 (Figure 5.8; in collaboration with Dr. Paul V. Sabatini). Some of these genes could be NPAS4 effectors in β-cells, but whether they were direct targets (such as Rgs2 [446]) remained unknown.

![Figure 5.8. RNA-Seq in N4 PKO mice shows expression changes in putative NPAS4 targets.](image)

Tamoxifen (or corn oil) was administered to 6-week old male Npas4^floxed/lox^-Pdx1-CreER^+ (N4 PKO) mice. KO (white bars) and control (black bars) islets were isolated two weeks later, RNA harvested and prepared for RNA-Seq analysis. Gene expression was normalized to control islets. Npas4 expression was partially lost in N4 PKO islets, correlating with expression changes of putative NPAS4 targets [315; 354; 546; 562; 564; 565; 854; 855] classified as immediate early genes (IEGs) and non-IEGs (Others). n = 2.

Based on my earlier studies and gleaning from the binding motif analysis, it was apparent that NPAS4 DNA binding was predominantly active in KCl-treated β-cells, and shared overlapping
DNA binding motifs with ARNT. To further validate the obtained data, differential NPAS4 and ARNT enrichment was assessed at key β-cell genes and previously identified targets in β-cells. First, it was validated whether ARNT was enriched near sites of canonical HIF1α target genes. Indeed, ChIP:ARNT samples were enriched in sequences mapping to open chromatin in promoters or intergenic regions of Aldoa, Gapdh, Ldha, Pdk1, Slc16a3 and Vegfa under hypoxic (HYPO), but not stimulated (KCL) conditions (Figure 5.9 A-F). Interestingly, KCL ChIP:NPAS4 samples were also enriched in some of these peaks, most notably at the Aldoa locus (Figure 5.9 A), whereas ARNT did not seem to bind this region as pronounced under stimulatory conditions. This raised the possibility that NPAS4 not only optimized β-cell metabolism by competing for ARNT, but also actively bound HIF1α target genes. It remained to be validated whether NPAS4 bound Aldoa, and crucially, whether it could act as transcriptional repressor, thus counteracting HIF1α activity. The idea of NPAS4 binding in the absence of ARNT was intriguing, but would require thorough follow-up investigation.
Figure 5.9. ARNT is enriched near HIF1α target genes under hypoxia.

(A-F) ChIP peak signals of ChIP:ARNT and ChIP:NPAS4 samples were visualized using the Integrative Genomics Viewer (IGV). Regions of active transcription in β-like cells (open chromatin; from β-TC6 ATAC-Seq [856]) and enhancers (based on Shen et al. (2012) [857]) are indicated. Specific peaks (red arrows) are observed near the TSS of Aldoa (A), Gapdh (B), Ldha (C), Pdk1 (D), Slc16a3 (E), and Vegfa (F).

Conditions: LG = basal; KCL = stimulatory; HYPO = HIF1α-stabilizing.

Furthermore, I observed enriched binding of NPAS4 and ARNT at open chromatin near promoters and enhancers of important β-cell genes (Figure 5.10). Binding near the K\textsubscript{ATP} channel subunit Abcc8 and the β-cell hormone Iapp (Figure 5.10 A&B) under stimulatory (KCL) conditions suggests NPAS4 may provide transcriptional feedback during β-cell activity to regulate stimulus-secretion coupling and satiety. NPAS4 was previously shown to indirectly regulate insulin expression by modulating protein expression of transcription factors important for insulin transcription [446]. Indeed, some peaks were detected near promoter and enhancer regions of Neurod1 and Nkx6.1 in KCL ChIP:NPAS4 (Figure 5.10 C&D); although overexpression
of Npas4 previously did not change Neurod1 and Pdx1 gene expression [446], warranting further investigation. Some of the aforementioned regions also showed ARNT enrichment following hypoxia (ChIP:ARNT HYPO; Figure 5.10 A-D); thus, HIF1α might regulate their expression. Lastly, several significant peaks in KCL ChIP:NPAS4 samples overlapped with open chromatin regions at TSS of genes involved in insulin exocytosis (Snap25, Syt7, Unc13a) (Figure 5.10 F); raising the possibility that NPAS4 provides transcriptional feedback enhancing insulin secretion during times of increased demand. Altogether, NPAS4:ARNT bound and likely regulated the expression of important β-cell genes, although this assertion remains to be validated in future studies.

Figure 5.10. NPAS4 is enriched near important β-cell genes.

(A-F) ChIP peak signals of ChIP:ARNT and ChIP:NPAS4 samples were visualized using the Integrative Genomics Viewer (IGV). Regions of active transcription in β-like cells (open chromatin; from β-TC6 ATAC-Seq [856]) and enhancers (based on Shen et al. (2012) [857]) are indicated. Specific peaks (red arrows) are observed at TSS and/or enhancers near Abcc8 (A), Iapp (B), Neurod1 (C), Nkx6.1 (D), Snap25 (E), Syt7 (F), and Unc13a (G). Conditions: LG = basal; KCL = stimulatory; HYPO = HIF1α-stabilizing.
As an immediate early gene, NPAS4 is implicated in the initial transcriptional response to stimulation. As such, it was possible that NPAS4 regulated β-cell activity and function through enhancing other activity-induced genes. For instance, NPAS4 was previously speculated to regulate its own expression in a positive feedback loop \[^{354; 539}\]. Indeed, peaks at open chromatin regions near the *Npas4* promoter were enriched in KCl ChIP:NPAS4 and to a lesser degree ChIP:ARNT (Figure 5.11 A), which supported the notion that NPAS4 regulates its own expression in β-cells. Notably, a minor peak at the same site was also seen in HYPO ChIP:ARNT, suggesting HIF1α:ARNT could bind under hypoxia to suppress *Npas4* expression (Figure 5.11 A). NPAS4 also regulates the activity-induced gene *Rgs2* \[^{446}\], and binding near this gene was mainly seen in KCl ChIP:NPAS4 samples (Figure 5.11 B). Notably, an area in the last exon of *Rgs2* showed strong enrichment in HYPO ChIP:ARNT; thus, it was tempting to speculate that *Rgs2* expression was also regulated by HIF1α. Peaks near TSS and enhancers of other putative neuronal NPAS4 target genes were also observed in β-like cells, including immediate early genes (*Junb, Fosl2, Nr4a1*), transcription factors (*Maff, Mef2a*) and ion channels (*Kcna4*) (Figure 5.11 C-H). ChIP peaks not previously seen in neurons included ER stress gene *Ddit3* (Figure 5.11 I), N-methyl-D-aspartate receptor subunit *Grin1* (Figure 5.11 J), and gap junction protein *Gjd2* \[^{858}\] (Figure 5.11 K), which have critical roles in β-cells. N.B. that NPAS4 is implicated in suppressing *Ddit3* expression \[^{446}\], and preliminary data from overexpression studies in mouse islets suggest NPAS4 might increase *Gjd2* expression (Figure 5.11 L). Together, I propose that NPAS4 could play a role in modulating ion currents (*Kcna4, Grin1*) and β-cell connectivity (*Gjd2*), but these predictions certainly have to be validated experimentally.
Figure 5.11. NPAS4 and ARNT are enriched near genes involved in activity regulation.

(A-K) ChIP peak signals of ChIP:ARNT and ChIP:NPAS4 samples were visualized using the Integrative Genomics Viewer (IGV). Regions of active transcription in β-like cells (open chromatin; from β-TC6 ATAC-Seq (856)) and enhancers (based on Shen et al. (2012) (857)) are indicated. Specific peaks (red arrows) are observed at TSS and/or enhancers near putative NPAS4 targets (315, 354, 562, 564, 565, 854, 855) Npas4 (A), Rgs2 (B), Junb (C), Fosl2 (D), Nr4a1 (E), Maff (F), Mef2a (G), and Kcna4 (H). Specific peaks are also observed near ER stress gene Ddit3 (I), N-methyl-D-aspartate receptor subunit Grin1 (J), and gap junction protein Gjd2 (K). Conditions: LG = basal; KCL = stimulatory; HYPO = HIF1α-stabilizing. (L) Wild-type (B6) islets were infected with Ad-Npas4 or control (Ad-EGFP) at an MOI of 20:1. Gene expression was measured via qPCR after 24 h, normalizing Gjd2 to Gusb expression (n = 2).
Altogether, ChIP experiments showed that NPAS4 and ARNT bind similar DNA motifs under stimulatory conditions, further establishing their shared regulation of gene expression. On the other hand, ARNT had a divergent role in binding HIF1α targets under hypoxic and low glucose conditions, elegantly tying cellular activity and energy requirements to a transcriptional switch. Both ARNT and NPAS4 were enriched at sites near important β-cell genes, implicating their involvement in regulating expression; although this remains to be tested, particularly whether they promote or inhibit transcription. The sequencing data obtained from these ChIP studies thus represent a valuable resource to further investigate the roles of NPAS4:ARNT in β-cell function, and a more in-depth and systematic analysis of the data is warranted.

5.5 Discussion

This initial examination of NPAS4 and ARNT ChIP-Seq data represents an encouraging starting point for future experiments and analysis. For example, LG ChIP:NPAS4 fractions perform well as negative control, without any significantly enriched peaks, since NPAS4 is not expressed under basal conditions. Interestingly, however, enough NPAS4 was expressed under HYPO conditions to distinguish a few enriched sites with an NPAS4 binding motif; suggesting that if present, NPAS4 is still potent to heterodimerize with ARNT and thus limit HIF1α transcriptional activity. Since both NPAS4:ARNT and HIF1α:ARNT complexes share similar consensus binding motifs, it also opens up the possibility that NPAS4:ARNT is occupying canonical HIF1α target genes, as was seen at the Aldoa promoter, and vice versa. Future analyses will have to systematically analyze the overlap of ChIP:NPAS4 and ChIP:ARNT enriched peaks under KCL and HYPO conditions to obtain a global picture of the dimension of co-enrichment, and thus, competition for target gene binding. In addition, I would ask whether any significant differential transcriptional regulation (i.e. repressor versus activator) between NPAS4 and HIF1α exists.
Regarding the frequent identification of AP-1 consensus motifs following KCL treatment, Pruunsild et al. (2011)\(^\text{[859]}\) noticed an AP1-like element (TCACTCA) which partly overlapped (underlined bases) an asymmetric E-Box-like element in reverse orientation (CACGAC) in promoter I of \(BDNF\), an NPAS4 target gene. This E-Box element was bound by NPAS4:ARNT2 and was required for promoter activity\(^\text{[859]}\). In this chapter, the top \textit{de novo} predicted motif in KCL ChIP:NPAS4 samples also contained a reverse E-Box (bold) -like motif (GT\textit{CACGAT}), which partly overlapped (underlined bases) the dominantly identified motif in CHIP:ARNT samples, an AP-1 motif (TG\textit{AGTCA}). Whether there is a biological relevance to the overlap of these elements is unknown. However, AP-1 consensus motifs can also be recognized by the CREB/ATF family of transcription factors. An asymmetric E-box-like element (CTCGTG) in the promoter IX of BDNF partly overlapped with a CRE-like element (TGACAGCA)\(^\text{[859]}\). The authors showed that this element was jointly regulated by NPAS4:ARNT2 and CREB1 binding; suggesting that transcriptional regulation by NPAS4 involves co-operative DNA binding with other transcription factor families. Cooperative DNA binding has been demonstrated for NFAT and other transcription factors\(^\text{[339]}\). Thus, it is possible that E-Box and AP-1/CREB motifs in promoter regions occur in close proximity to allow for co-operative binding of transcription factors and thus, robust transcription. Based on my own analysis and experimental evidence by Pruunsild et al. (2011)\(^\text{[859]}\), I propose a similar mechanism for NPAS4:ARNT transcriptional regulation in MIN6 cells. Corroborating this view, ARNT interacts with the transcriptional co-activator EP300\(^\text{[572]}\), which also binds many other transcription factors such as HIF1\(\alpha\)\(^\text{[860; 861]}\), and CREB/ATF family members\(^\text{[860]}\), and thereby promotes co-operative regulation of transcription\(^\text{[862]}\). Thus, enrichment of the AP-1 motif in my ChIP-Seq samples might simply indicate that AP-1 and NPAS4:ARNT, binding to proximal sites, may both be bridged to the mediator and core transcriptional machinery by EP300. This is also supported by a study showing that NPAS4 TAD activity was enhanced by EP300 expression\(^\text{[539]}\).
The mapping and identification of enriched peaks in ChIP:NPAS4 and ChIP:ARNT samples near promoter or enhancer sites of important β-cell genes is largely correlative at this stage and requires follow-up work to assess biological significance. However, many of the peaks I identified match previously reported findings and predictions, such as those near HIF1α target genes in HYPO ChIP:ARNT samples, as well as NPAS4 binding its own promoter[^354; 539], the Rgs2 gene[^446], and other putative targets[^315; 354; 546; 562; 564; 565; 854; 855]. Together, these findings increase the confidence in newly identified binding sites.

Notably, peaks near the Iapp gene are seen in HYPO ChIP:ARNT and KCL ChIP:NPAS4 samples. Glucose-induced human IAPP promoter activity requires calcium influx through L-VDCCs[^863], supporting the notion that calcium-dependent NPAS4 might regulate IAPP expression. Hypoxia also increases Iapp expression in mice[^864], and whether HIF1α and NPAS4 might be competing for the same DNA binding sites remains to be tested.

Lastly, NPAS4 is predicted to bind predominantly at enhancer regions in neurons[^315], and if this holds true in islets, it will not be sufficient to study promoter regions of genes to draw conclusions about their potential regulation by NPAS4. In that case another technology such a chromosome conformation capture will be beneficial to correlate enhancer-promoter interactions. Altogether, while many of the enriched peaks can be explained, their authenticity remains to be tested experimentally. In future studies, I intend to analyze available ChIP-Seq data systematically to tease out all differentially and mutually enriched sites between the different conditions, to get a clearer picture of the extent of overlap or exclusive DNA binding of the different transcription factors.
Chapter 6: Role of RGS2 in β-cell function

6.1 Background

Insulin secretion depends on elevated intracellular calcium levels, and thus a balance in calcium homeostasis is crucial for β-cell function. This is reflected in animal studies of mice overexpressing the calcium sensor CaM, as they develop hyperglycemia due to impaired glucose metabolism and loss of GSIS \cite{394, 395}. Notably, mice expressing inactive CaM likewise show insulin secretion defects, both in response to glucose and KCl \cite{390}. In this model, the muscarinic receptor agonist carbachol still elicits calcium release from intracellular stores \cite{390}. While I focused on the transcriptional regulation of β-cell function by the calcium-dependent transcription factor NPAS4 in previous chapters, the latter finding highlights that intracellular calcium levels are not only regulated by extracellular influx through VDCCs, but also by ligand-activated GPCRs. Loss of Gαq/Gαs-coupled GPCRs that amplify insulin secretion (M3R \cite{865}, P2Y1R \cite{642}, GLP1R \cite{622}) or high expression of Gαi-coupled inhibitory GPCRs (Prostaglandin E2 Receptor \cite{866}, α2A-adrenergic receptor \cite{867}) both reduces insulin release, and thus predisposes to impaired glucose homeostasis and diabetes. In contrast, loss of inhibitory G-proteins or Gαi-coupled GPCRs enhances β-cell function and mass \cite{629, 745, 868}. Together, their roles in regulating calcium levels, downstream signalling pathways, or direct actions on the exocytotic machinery, makes β-cell GPCRs important therapeutic targets to modulate insulin secretion \cite{54, 55}.

GPCR function is fine-tuned by the RGS family of proteins. This extra layer of regulation is essential, as loss of RGS proteins causes functional impairments in β-cells \cite{576, 577}. Because Npas4 overexpression causes a decrease in GLP1R-dependent insulin secretion \cite{446}, it was hypothesized that this effect is mediated through an NPAS4 target gene regulating GPCR signalling. One established direct target of NPAS4 in β-cells and neurons is Rgs2 \cite{446}. Indeed, it is one of the most highly differentially expressed genes in RNA-Seq data from Npas4 knockout
mice (Figure 5.8). Gene expression studies in MIN6 cells and islets demonstrated that NPAS4 positively affects Rgs2 transcription \(^{[446]}\). This correlated with NPAS4 DNA binding to an upstream enhancer and a region in the first intron of Rgs2, with 22-fold and 3-fold enrichment, respectively \(^{[446]}\). It was thus speculated that RGS2 was partly responsible for modulating effects on β-cell function downstream of NPAS4 \(^{[446]}\). Like Npas4, Rgs2 is an immediate early gene which is induced by a wide range of stimuli (Table 1.1). While Rgs2 expression is not directly dependent on calcium \(^{[669]}\), calcium does have synergistic effects on its induction in β-cells \(^{[670]}\). This is most commonly achieved through calcium-dependent kinases (PKC, PKA, MAPK). Moreover, germline loss of Rgs2 leads to disruption of calcium homeostasis in various tissues \(^{[739; 741]}\) and impaired insulin secretion from islets \(^{[646]}\). Canonically, RGS2 is a negative regulator of Gαq-coupled signalling \(^{[723]}\). Because Gαq signalling generally stimulates insulin secretion \(^{[54]}\), I hypothesized that increased RGS2 would lead to a dampening of Gαq signalling, reduced intracellular calcium, and thus, lower insulin secretion. In contrast, ablation of Rgs2 should enhance insulin secretion. In this chapter, I define the role of RGS2 in regulating β-cell function.

6.2 RGS2 expression in healthy and diabetic islets

To determine whether RGS2 expression had any relevance in human β-cells, I studied its expression in islets from non-diabetic human donors. Rgs2 is an immediate early gene whose rapid expression in murine islets and other cells is predominantly induced by AC, i.e. cAMP generation and PKA activity \(^{[670; 692]}\). In addition, RGS2 expression was induced by glucose in mouse islets \(^{[446]}\). Thus, human islets were cultured with glucose or the AC activator forskolin, and RGS2 mRNA and protein levels assessed. RGS2 mRNA was significantly induced following two-hour exposure to forskolin (Figure 6.1 A). RGS2 expression levels following high glucose treatment were higher than basal levels, but not statistically significant. These findings were
generally reflected at the protein level (Figure 6.1 C-E), where forskolin induced RGS2 after both 1 h and 2 h. Glucose stimulation did not significantly increase RGS2 protein, whereas NPAS4 expression was markedly induced (Figure 6.1 C-E). This indicated that while human islets were functional and glucose-responsive, RGS2 expression was primarily stimulated by adenylate cyclase activity, likely downstream of cAMP-PKA signalling as suggested by other studies [670]. When examining available scRNA-Seq data from adult human islet samples [296], RGS2 was expressed in acinar, ductal and endocrine cells. In endocrine cells, its expression was highest in β-cells and δ-cells (Figure 6.1 F). Together, these findings confirmed that RGS2 is expressed similarly in islets from mice and humans, except that glucose was not a potent driver for human as opposed to mouse RGS2 expression.
Figure 6.1. RGS2 expression in human islets is induced by AC activation.

(A&B) Human islets were stimulated for 1-2 h in KRBH with glucose (− = 2.8 mM, + = 16 mM) or forskolin (− = 0 μM, + = 10 μM). RGS2 expression was normalized to GUSB and is shown as fold basal (2 h 2.8 mM glucose) expression (n = 6). (C-E) Treatments as in (A), followed by immunoblot for RGS2 and NPAS4. Multiple immunoreactive bands are detected for RGS2 (black arrows). Relative expression was quantified densitometrically and normalized to GAPDH (D&E; n = 4). (F) Modified tSNE plots from Figs 1B and 1E of Segerstolpe et al. (2016) [296]. Significance was determined using a one-way ANOVA with Dunnett’s multiple comparisons test (A, D&E), or Kruskal-Wallis test with Dunn’s multiple comparisons test (B). * p ≤ 0.05; ** p ≤ 0.01; *** p ≤ 0.001.
RGS2 occurs in different subcellular locations \([673; 714; 715]\), which influences its interactome and thus affects how it regulates cellular functions. As RGS2 expression is induced by activity, and to understand its subcellular location during β-cell stimulatory conditions, pancreatic sections were prepared from mice given an oral glucose bolus. Glucose infusion raises circulating levels of glucose and incretins, and thus was predicted to raise levels of RGS2 (Figure 6.1) \([446; 670]\). Staining for RGS2 and INS in these mice revealed that RGS2 was enriched in the endocrine islet, and was expressed in all β-cells (Figure 6.2 A-H). Subcellularly, several groups have demonstrated nuclear localization for RGS2 \([673; 715]\), arguing against a canonical role in regulating membrane-bound G-proteins. Co-localization with RGS2 was indeed seen in some acinar nuclei (Figure 6.2 B), raising the prospect of a role in regulating transcription there. However, in the islet, RGS2 was largely absent from nuclei and was instead mainly seen in the cytoplasm and at prospective membrane structures (Figure 6.2 F), suggesting a canonical role in regulating G-protein signalling in the islet. RGS2 was also expressed at the islet periphery, co-localizing with GCG and SST (Figure 6.2 I-P), confirming the human scRNA-Seq data showing enrichment of RGS2 message in δ-cells (Figure 6.1 F).
Figure 6.2. RGS2 is enriched in pancreatic endocrine cells.

Following a one-hour hyperglycemic clamp (60 mg glucose bolus followed by continuous glucose infusion), pancreatic sections were prepared from control mice (Npas4flox/flox; Dr. Paul V. Sabatini, unpublished). (A-H) Co-staining for RGS2 and insulin (INS). White arrowheads highlight RGS2 in acinar nuclei (B) or putative membrane-localized RGS2 in INS-positive cells (F). (I-L) RGS2 and glucagon (GCG) co-localize (white arrowheads). (M-P) RGS2 and somatostatin (SST) co-localize (white arrowheads). Nuclei are stained with TO-PRO-3 iodide (DNA). Objective (A-D) = 20x/0.75, scale bar = 30 μm; objective (E-P) = 63x/1.40, scale bar = 15 μm. Representative images of n = 3.

Next, I investigated if there was any correlation between T2D and islet RGS2 expression in collaboration with Dr. Paul V. Sabatini. Notably, HFD feeding impaired glucose-stimulated
Rgs2 induction in mouse islets (Figure 6.3 A). In addition, human islets from individuals with T2D expressed significantly lower amounts of RGS2 compared to islets from healthy controls (Figure 6.3 B), suggesting reduced expression of RGS2 is associated with diabetes pathology.

Figure 6.3. RGS2 in diabetes.
(A) Rgs2 expression in islets from mice on regular chow (CHW) or high fat diet (HFD) (n = 3). (B) RGS2 expression in non-diabetic control and T2D diabetic islets (n = 4). Significance was determined using an unpaired two-tailed Student’s t test. * p ≤ 0.05.

In summary, RGS2 was enriched in the cytoplasmic and membrane fraction of endocrine islet cells, and its expression increased in response to β-cell stimuli. Furthermore, perturbations in RGS2 expression were associated with diabetes in mice and humans. Based on these results and previous literature, it seemed likely that RGS2 plays a role in modulating β-cell function through regulation of G-protein signalling.

6.3 RGS2 is a negative regulator of insulin secretion

To test this hypothesis, I constructed an adenovirus driving Rgs2 expression (Ad-Rgs2). To test whether it affected β-cell function through modulation of G-protein signalling, Rgs2 was initially overexpressed in MIN6 cells. G-protein signalling amplifies or restricts GSIS, but in the
absence of ligand, it was predicted that GSIS itself would not be changed by Rgs2 overexpression. Surprisingly, Ad-Rgs2 MIN6 cells exposed to increasing concentrations of glucose failed to release insulin in significant amount compared to control-infected (Ad-βGal) cells (Figure 6.4 A). This was not through direct inhibition of the L-VDCC, as depolarization with KCl still induced insulin secretion (Figure 6.4 A). Insulin content (Figure 6.4 B) and cell number (Figure 6.4 C-E) remained the same between control and Ad-Rgs2 cells, ruling out drastic effects on insulin production, proliferation or apoptosis. The same effect was reproduced in primary cells, as transduction of intact wild-type (CD-1) islets with Ad-Rgs2 significantly lowered glucose-stimulated but not KCl-induced insulin secretion, nor insulin content (Figure 6.4 F&G).
Figure 6.4. RGS2 suppresses glucose-stimulated insulin secretion.

(A-E) MIN6 cells were transduced with Ad-βGal (control) or Ad-Rgs2 at a multiplicity of infection (MOI) of 5:1 and let to recover for 1 day. After 1 h preincubation in KRBH with low glucose (2.8 mM), GSIS was performed with various glucose concentrations or 2.8 mM glucose with 40 mM KCl (KCl) for 2 h. Insulin content was measured after acid-ethanol extraction. Protein content was measured via BCA, DNA extracted from trypsinized cells, or trypsinized cells counted (n = 5). (F&G) Approximately 40 islets from wildtype (CD-1) mice were transduced and treated as in (A-E), except using an MOI of 10:1 (n = 6). Significance was determined using an unpaired two-tailed Student’s t test (B-G) with Welch’s correction (A). * p ≤ 0.05.

RGS2 has previously described roles in modulating intracellular calcium levels [726; 732; 739; 741; 869-871], which, calcium influx being essential to β-cell function, might explain why RGS2 inhibits insulin secretion. Even though KCl still elicited insulin secretion in Ad-Rgs2 cells, it did not preclude the possibility of a defect upstream of the L-VDCC, leading to insufficient depolarization and calcium influx. Consequently, intracellular calcium influx was measured in
response to glucose in a perifusion assay using the ratiometric probe Fura-2-AM. During the first 10 min of the assay, basal intracellular calcium levels in the presence of 2.8 mM glucose remained stable and were equivalent in control-infected (Ad-βGal) and Ad-Rgs2 cells (not shown). Following high glucose exposure of Ad-βGal cells, the F₃₄₀/F₃₈₀ ratio rose, indicating an increase in Ca²⁺cyt levels (Figure 6.5 A&B) and thus β-cell activity. In comparison to controls, Ad-Rgs2 cells exhibited a significantly dampened rise in Ca²⁺cyt during 20 min of high glucose stimulation (Figure 6.5 A&B). The subsequent recovery period in low glucose, as well as KCl-stimulated calcium influx, was similar to controls (Figure 6.5 A&B). These findings explained the defect seen in the insulin secretion assay.

A possible explanation for reduced Ca²⁺cyt levels in Ad-Rgs2 transduced cells is failure to mobilize intracellular calcium stores via Gαq-dependent signals, such as IP₃R- and RyR-mediated ER calcium release. Notably, metabolites such as ATP can activate RyRs [247]. Calcium
transfer from ER to mitochondria via microdomains is an important mechanism for optimization of mitochondrial ATP production \[^{[274; \text{279}; \text{280}]}\], as the activity of some mitochondrial enzymes is calcium-dependent \[^{[265]}\]. Greater amounts of ATP production then promote continued closure of the K\(_{\text{ATP}}\) channel to promote further extracellular calcium influx \[^{[92]}\], and thus amplification of insulin release. As reduced intracellular calcium mobilization impairs ATP production, Ad-Rgs2 transduced islets were investigated for impairments in metabolic activity. As described in Chapter 4, this was done by measuring oxygen consumption in response to glucose, as a measure for oxidative phosphorylation. As MIN6 cell metabolism is more skewed towards glycolytic glucose utilization, primary islet cells were used. OCR rates increased in a glucose-dependent manner in both Ad-\(\beta\)Gal and Ad-Rgs2 dispersed islet cells, but Ad-Rgs2 cells did not further increase oxygen consumption from glucose concentrations of 11 mM (Figure 6.6 A&B). Thus, Ad-Rgs2 reduced oxygen consumption compared to controls at higher glucose concentration, potentially reflecting lower mitochondrial calcium levels. While hypoxia had ambivalent effects on RGS2 \[^{[696; \text{872}]}\], expression of HIF1\(\alpha\) target genes was not affected by overexpression of Rgs2 in mouse or human islets (Figure 6.6 C-F), ruling out metabolic impairment due to HIF1\(\alpha\) signalling. In conjunction with impaired insulin secretion at higher glucose concentration in islets (Figure 6.4 F), this indicated that an underlying defect in calcium homeostasis and, secondary to that, impaired mitochondrial ATP generation, was the cause for reduced \(\beta\)-cell function in Ad-Rgs2 cells.
To delineate how RGS2 modulates calcium homeostasis and insulin secretion, agonists of prominent GPCRs known to amplify insulin secretion were investigated. The $G_{\alpha_s}$-coupled incretin receptor GLP1R stimulates insulin release through the second messenger cAMP and
activation of PKA \cite{607}. When the GLP1R agonist exendin-4 was used in conjunction with glucose, control and Ad-Rgs2 transduced islets showed no difference in insulin secretion (Figure 6.7 A). Rather, exendin-4 amplified GSIS to a greater extent in islets overexpressing Ad-Rgs2 compared to Ad-βGal (Figure 6.7 B). Since the ability of exendin-4 to amplify insulin secretion over glucose was not impaired in Ad-Rgs2 cells (Figure 6.7 A), RGS2-mediated suppression of insulin release was not through the GLP1R and likely independent of Gαs-coupled signalling. This finding contradicted a previous report \cite{692}, but was in line with other studies highlighting a role for RGS2 in primarily inhibiting Gαq-coupled signalling \cite{668; 723; 726}. As such, it remains unknown whether lower calcium levels in Ad-Rgs2 cells were solely due to repression of glucose-stimulated calcium influx, or additional repression of Gαq signalling.

![Graph A](image)

![Graph B](image)

**Figure 6.7. RGS2 does not suppress Gαs-coupled incretin signalling.**

\(\text{(A&B), Approximately 40 islets from wildtype (CD-1) mice were transduced with Ad-βGal (control) or Ad-Rgs2 at an MOI of 10:1 and let to recover for 1 day After 1 h preincubation in KRBH with low glucose (2.8 mM), GSIS was assayed with low glucose (2.8 mM), medium glucose (8 mM), or medium glucose plus 50 nM exendin-4 (Ex4). Insulin content shown in Figure 6.4 G (n = 6). Significance was determined using a one-way ANOVA with Tukey’s multiple comparisons test (A), or using an unpaired two-tailed Student’s t test (B).} \) * p ≤ 0.05; ** p ≤ 0.01; *** p ≤ 0.001.
In summary, these data demonstrate that RGS2 repressed insulin secretion due to lowering of Ca$^{2+}$_cyt and ATP production. Questions remain about the intracellular target through which RGS2 mediated its effects. Whereas this did not appear to be through action on the $\alpha_s$-dependent GLP1R receptor, a role for a $\alpha_q$-dependent mechanism required further study. Supraphysiological expression of Rgs2 had been successfully employed for the initial characterization of the role of RGS2 in $\beta$-cells, but a loss-of-function model might be more suitable for prospective mechanistic studies. As a mouse with conditional knockout allele was not available at the time of study, a strategy was devised to knock out Rgs2 in the MIN6 $\beta$-cell line.

6.4 Generation of MIN6 cell lines with loss of Rgs2 expression

The advent of CRISPR-Cas9 for genome editing has made generating knockout cell lines more time efficient and manageable. All that is needed is the ability to introduce a plasmid driving expression of the Cas9 enzyme and a single-guide RNA (sgRNA) into cells. The sgRNA provides specificity, as it contains a short (around 20 bp) sequence complementary to a sequence in the genome, to which it will bind. The sgRNA then binds and guides the Cas9 enzyme to the specified sequence, and, if a protospacer adjacent motif (PAM; base code NGG) is recognized, the DNA strand will be cut by the Cas9 nuclease. Endogenous DNA repair enzymes proceed to repair the defect, but occasionally small insertions or deletions (indels) occur during non-homologous end joining (NHEJ). These mutations may be disruptive, especially if targeted to a TSS of a gene. This approach was initially taken, targeting the transcriptional start codon of Rgs2 (Figure 6.9); without success (not shown). Alternatively, a repair template containing a sequence homologous to a few hundred base pairs on either side of the DSB (“donor sequence”) can be co-transfected with CRISPR-Cas9. The donor sequence may be designed to contain disruptive mutations, such as stop codons or frameshift mutations. If the strategy is successful,
the donor sequence is incorporated during the repair of the DSB-affected endogenous sequence in what is referred to as homology-directed repair (HDR).

Specifically, I designed two separate sgRNAs (sg720/21, sg722/23) targeting a similar site in the center of Exon 1 of mouse Rgs2 (Figure 6.9). The donor sequence contained homology arms of approximately 250 bp on either side of the DSB. In addition, a short sequence near the 3’ end of Exon 1 corresponding to an alternative translational start site was altered to instead contain a stop codon in each of three open reading frames (Figure 6.8 A). If incorporated successfully, this mutation was predicted to terminate translation at the end of Rgs2 Exon 1. Early passage MIN6 cells were then transfected with CRISPR-Cas9 and donor sequence plasmids. As the CRISPR-Cas9 plasmid also expresses GFP, cells were FACS-sorted to enrich for successful transfection. After extended culture to expand clonally derived colonies, several hundred clones were available to screen for disrupted Rgs2 expression.

Initially, DNA was obtained from sg720/21- and sg722/23-transfected colonies and genotyping PCR performed using a primer (LL703) specific to the mutated site (Figure 6.8 A, Figure 6.9). A single 350 bp band, corresponding to the calculated PCR product size of 346 bp, was detected in a few colonies (e.g. 2A, 7G, 7H, 10D, 12A, 12C; Figure 6.8 B), whereas others had additional bands of different sizes and unknown identity (e.g. 2B, 2D, 7D, 10A; Figure 6.8 B). Based on these results, 28 out of 188 sg720/21 clones and 38 out of 192 sg722/23 clones showed a single 350 bp band, and were considered to have incorporated the donor sequence successfully, without alterations resulting in additional bands. Further genotyping was conducted on a few of these clones.

Correct homologous integration of the donor sequence destroyed an alternative translational start site and replaces it with stop codons and a KpnI restriction site (CCATGG; Figure 6.8 A). Primers binding outside either side of this target site were used for PCR in putative mutant and
control cell lines, and a KpnI digest was performed (Figure 6.8 A). In a few colonies (2A, 7G, 12A, 12C), incubation with KpnI completely digested the PCR product, whereas others showed no sign of digestion (WT, 9B, 7H, 8C, 9H) or partial digestion (10D) (Figure 6.8 C). Altogether, 8 out of 25 sg720/21 clones and 12 out of 28 sg722/23 clones showed the expected restriction pattern (397 bp + 176 bp). For those clones showing complete digestion of the PCR product, I speculated that they were potentially homozygous mutants, while a partial digest would indicate heterozygous mutation, and no digestion wild-type sequence.

Figure 6.8. Screening for Rgs2 mutant cell lines. 
(A) Targeting and screening strategy (LL515 also used later for sequencing). (B) PCR with primers LL703 and LL705; correct insertion yields a band of 346 bp (black arrowheads). Red boxes highlight clones with correct band size. (C) PCR with primers LL515 and LL514, followed by KpnI digestion; expected restriction pattern 397 bp + 176 bp (black arrowheads). Red boxes highlight occurrence of restriction.
Based on the restriction digest, a few control (“no cut”) and mutant (“cut”) clones were sent for Sanger sequencing across the target locus. Out of the 8 sequenced sg720/21 lines, a single colony had successfully incorporated the donor sequence (9D), while 4 out of 12 (2A, 7G, 12C, 9E) sg722/23 lines matched the predicted mutant sequence (Figure 6.9 A). Notably, in addition to replacing the alternative TSS with stop codons, all homozygous colonies showed further homozygous indel mutations at the sgRNA target locus, suggesting sgRNA-Cas9 interactions continued creating DSBs after HDR had been achieved, leading to further repair through NHEJ (Figure 6.9 A). All detected indels resulted in a frameshift, and were thus predicted beneficial in attempting to knock out RGS2 protein expression. The remaining colonies either had a sequence reading corresponding to the MIN6 wild-type sequence (e.g. 9B, WT MIN6), or a mix of several different sequences (seen as convoluted basecalls), indicating these were colonies with heterozygous mutations (not shown). The Rgs2 intron boundaries are marked by canonical 5′-GT-3′ (intron start; Figure 6.9 A) and 5′-CAG-3′ (intron end) motifs and, as sequencing revealed no mutation in these elements, splicing was predicted to produce mature Rgs2 mRNA for all clones. However, whereas wild-type Rgs2 mRNA is translated into a 211 aa protein (Figure 6.9 B), cell lines with homozygous mutations in exon 1 were predicted to only produce truncated peptides (Figure 6.9 C&D). Finally, freezer stocks were prepared for all colonies, and those that had returned a wild-type sequence were used as controls, whereas homozygous mutants were used for further research into the role of Rgs2.
Figure 6.9. Sanger sequencing reveals homozygous Rgs2 mutant cell lines.

(A) Sanger sequencing reveals homozygous mutations in several clones. Potential start codons are underlined. (B-D) Open reading frames within Rgs2 exon 1-5 in wildtype (WT, clone 9B; B) and Rgs2 mutant (clones 7G, 9E; C&D) cell lines. Mutations within Rgs2 Exon 1 are predicted to disrupt expression of full-length RGS2 protein. Predicted translated regions are highlighted in light red; translation initiation sites (“M”) are highlighted in green and termination sites shown in red (“*”). The only regions predicted to be translated and homologous to wild-type RGS2 are found at the N-terminus (MQSAMFLAVQHDVCVPMDK) and C-terminus (MENNNSYPRFLESEFYQDLCKKQITTEPHAT).

Before proceeding to test the biological consequences of loss of RGS2, RGS2 immunoblots were performed to confirm the predicted absence of full-length protein in homozygous Rgs2 mutant cell lines (Figure 6.9 C&D; henceforth called “KO”). Following stimulation with glucose and forskolin, RGS2 was robustly induced compared to basal levels in control cells, whereas KO cell
lines did not show the specific band corresponding to full-length RGS2 (Figure 6.10 A-D). Thus, RGS2 expression was successfully ablated. Lastly, control and Rgs2 KO cells did not show any obvious morphological abnormalities as seen under light microscopy (Figure 6.11 A-H). Growth and viability appeared normal (not shown).

**Figure 6.10. RGS2 expression is lost in Rgs2 mutant cell lines.**

(A-D) Cell lines were preincubated in KRBH without glucose for 1 h, followed by treatment with low glucose (2.8 mM; LG) or high glucose with forskolin (25 mM + 10 μM; HG+Fors) for 1 h (or 2 h, D). Immunoblots were performed for RGS2 and GAPDH (black arrowheads = RGS2; # = non-specific band). Representative blots of n = 3.

In summary, several sub-clones of control and Rgs2 KO cells were derived from the MIN6 parental cell lines following genome editing by CRISPR-Cas9. As sg722/23 transfected cells yielded homozygous Rgs2 KO lines more efficiently than sg720/21, further research was conducted using only the four sg722/23-derived KO lines (2A, 7G, 12C, 9E).
Figure 6.11. Morphological appearance of control and *Rgs2* KO cells.

WT = parental MIN6 cells of same passage. * = *Rgs2* KO.

6.5 Loss of *Rgs2* increases insulin secretion

Because *Rgs2* overexpression suppressed GSIS, loss of *Rgs2* should increase it. Studies conducted in germline *Rgs2* knockout mice supported this hypothesis, as islets from 8-10 week old mice had higher serum insulin levels after a glucose bolus. However, β-cell function was not characterized closely at that age, leaving open the question of the role of RGS2 in islets versus insulin target tissues. Consequentially, GSIS was examined in *Rgs2* KO cells. When GSIS was measured, several KO cell lines showed slightly enhanced GSIS over control cells (Figure 6.12 A), although heterogeneity was seen in both genotypes. Insulin content was compared across all cell lines to exclude the possibility of *Rgs2* KO cells secreting more insulin due to increased content. Most control and KO cell lines showed comparable insulin content, but one control line (10C) had notably increased insulin content (Figure 6.12 B). This could drive an increase in insulin release; further results from this cell line needed to be viewed in light of this finding. When insulin secretion results from all cell lines were combined (by genotype), the
predicted increase of GSIS in Rgs2 KO cells held up (Figure 6.12 C&D). This supported the idea that RGS2 is a negative regulator of insulin secretion.

Figure 6.12. Glucose-stimulated insulin secretion is enhanced in Rgs2 KO cells. (A) Control (CTR) or Rgs2 mutant (KO) cell lines were preincubated in KRBH with low glucose (2.8 mM) for 1 h, and GSIS was performed with low glucose (2.8 mM), high glucose (25 mM), or low glucose plus KCl (2.8 mM + 40 mM) for 2 h. Insulin secretion (A, C&D) and acid-ethanol extracted insulin content (B) were normalized to DNA content. Individual replicates are displayed for each cell line (A, n = 3-5), and for CTR versus KO genotype (B, n = 13). Average insulin secretion by genotype is shown (C, n = 3). Significance was determined using a two-way ANOVA (A) or one-way ANOVA (B) with Tukey’s multiple comparisons test, respectively, or an unpaired two-tailed Student’s t test (C&D). * p ≤ 0.05; ** p ≤ 0.01; *** p ≤ 0.001.
As calcium influx was reduced in *Rgs2* overexpression studies, I investigated whether an increase would be seen in *Rgs2* KO cells. Fura-2-AM calcium imaging was therefore conducted as previously described. Overall, no striking differences in glucose- or KCl-induced calcium influx were observed (Figure 6.13 A-F). Some heterogeneity occurred in this assay as well. Control line 10C again differed most from the other cell lines, as a clear glucose-stimulated calcium peak was evident together with complete calcium clearance in low glucose (recovery period). This stimulation would be considered above average when compared to passage-matched parental MIN6 cell line (WT; Figure 6.13 B). Its higher calcium influx paired with increased insulin content (Figure 6.12 A) suggested that this may be driving part of the clone 10C glucose-stimulated response, which may otherwise be even lower than other control cell lines. Compared to control cell lines other than 10C, *Rgs2* KO cell lines exhibited very minor trends towards increased calcium levels, but these were not significant overall (Figure 6.13 B-F).

![Figure 6.13. Intracellular calcium levels are similar in control and *Rgs2* KO cells.](image_url)
(A-F) Control and Rgs2 KO cells were seeded onto glass cover slips and let to attach for 1 day. Following 30 min preincubation with Fura-2-AM (5 μM in KRBH with 2.8 mM glucose) and 30 min low glucose perfusion, recording of F$_{340}$/F$_{380}$ ratio was started. Cells were perfused with low glucose (2.8 mM), high glucose (25 mM), and low glucose plus KCl (2.8 mM + 40 mM) (A-D, n = 3-6; E&F, n = 12-13). Significance was determined using multiple t tests corrected for multiple comparisons using the Holm-Sidak method.

All data considered so far were largely supportive of a role for RGS2 as negative regulator of insulin secretion. An intriguing detail is that RGS2 is thought to act by inhibiting Gα₉ signalling, yet no agonist of known Gα₉-coupled receptors was supplemented together with glucose. This was the case not only in primary islets, but MIN6 cells as well, therefore excluding effects of hormone secretion from other endocrine cell types such as δ-cells. This suggested that glucose itself or its metabolites were responsible for inducing GPCR signalling and providing positive feedback on insulin secretion. Adenosine nucleotides, which make up a fraction of insulin granule content and are co-released upon granule exocytosis, were considered a possible ligand class to explain these observations [77; 376]. ATP, for instance, can stimulate certain Gα₉-coupled receptors such as P2Y1R in a positive feedback loop to amplify insulin release [642]; and can also lead to induction of Rgs2 [685]. Thus, I determined whether a purinergic agonist, in combination with glucose, could elicit higher insulin release in Rgs2 KO cells. KO cells already exhibited a trending increase in insulin release at medium glucose levels (16G; Figure 6.14), but only addition of the purinergic agonist, α,β-MeATP, led to significant differences between controls and KO cells. The effect was certainly mild and difficult to separate from release of endogenous ATP, but gave some indication that RGS2-mediated inhibition of endogenous purinergic signalling was reduced in KO cells. Insulin secretion in response to carbachol, a general muscarinic agonist that signals via Gα₉, was not statistically significantly different between KO and control cells; although a trending increase was noted at low glucose levels (Figure 6.14).
Figure 6.14. *Rgs2* KO cells co-stimulated with glucose and a purinergic agonist show increased insulin release.

Control (CTR) or *Rgs2* mutant (KO) cell lines were preincubated in KRBH with low glucose (2.8 mM) for 1 h, and GSIS was performed with low glucose (2.8 mM), medium glucose (16 mM), or medium glucose plus α,β-MeATP (100 μM) or carbachol (100 μM) for 2 h. Insulin secretion and inulin content were normalized to DNA as shown in Figure 6.12. Individual replicates for CTR versus KO genotype are shown (n = 6–9). Significance was determined using multiple t tests corrected for multiple comparisons using the Holm-Sidak method. * p ≤ 0.05.

Overall, while effects of CRISPR-Cas9 treatment, clonal selection and passaging led to some heterogeneity between clones, the overall goal of validating higher GSIS in *Rgs2* KO cells was achieved. These cells might be useful tools for future studies to uncover the elusive target of *Rgs2* in β-cells.

### 6.6 Discussion

Differences in cytoarchitecture and cell composition between mouse and human islets can complicate the attempt to extrapolate results found in mouse models to human disease. In the first section of this chapter, I demonstrated that RGS2 expression in human islets largely mirrors that in mice[446; 670]. Induction of RGS2 by forskolin in human islets was also largely in agreement with other studies finding induction of *Rgs2* by GLP1 to be PKA-dependent in mice[670]. The induction of *Rgs2* was correlated with direct regulation by the transcription factor
NPAS4, through binding of an intronic and an enhancer region of Rgs2 \[446\]. Interestingly, feeding-induced pancreatic Rgs2 expression was inhibited by the CaN inhibitor FK-506 \[743\], which matches findings that NPAS4, which drives Rgs2 expression \[446\], is inhibited by FK-506 (Chapter 3). Calcium-dependent expression of NPAS4 could explain the partial calcium-dependence of Rgs2 observed by \[670\], whereas Rgs2 induction generally depends on PKA and PKC signalling \[716\]. It was further shown that RGS2 expression was perturbed in an obesity-driven mouse model of diabetes and human T2D islets. This resembles and corroborates another study, which showed higher basal but lower forskolin-induced Rgs2 expression in islets from HFD-fed and high-carb fed, and following in vitro culture of mouse and human islets with high glucose for 3 days \[393\]. Thus, it seems that the mouse is an acceptable model for studying the role of RGS2 in islets as well as pathological changes pertaining to RGS2 during diabetes disease progression.

To test whether RGS2 modulates insulin secretion in β-cells, I overexpressed it in MIN6 cells to exclude paracrine influences of other endocrine cell types, such as α-cells or δ-cells. I demonstrated that overexpression of Rgs2 inhibits insulin secretion at a range of glucose concentrations, independent of effects on the L-VDCC or insulin production. Furthermore, my finding was confirmed in primary mouse islets, a more physiologically relevant and glucose-sensitive model. Of note, inhibition of GSIS was not as pronounced in whole islets as in MIN6 cells, likely reflecting limited viral penetration to the center of the islet.

My findings are supported by previously published knockout studies. First, higher insulin secretion was reported in islets from Rgs2 null mice \[646\]. As RGS2 is expressed in other endocrine cell types, including SST-secreting δ-cells (section 6.2), inhibition of insulin secretion could theoretically originate from higher SST release in KO islets. Efforts were thus made to generate Rgs2 KO MIN6 cells (section 6.5). As expected, they secreted more insulin than control
cells in response to glucose, without changes to KCl-induced insulin release and insulin content, corroborating the previous study. However, insulin release was not increased as much in KO MIN6 cells (1.8-fold control) as in KO islets (~5-fold control) [646]. There are several possible explanations for this finding. First, loss of Rgs2 in other endocrine cells may have contributed to higher insulin secretion in KO islets. Second, passaging of MIN6 cells may have led to a gradual loss of glucose sensitivity which limited the effects of loss of Rgs2. The role of Rgs2 in other islet cells has not yet been characterized, but high passage MIN6 cells are known to exhibit reduced insulin secretion and glucose utilization [873]. I speculate that reduced excitability in high passage MIN6 reduced RGS2 levels similarly to pathological changes seen in T2D (Figure 6.3); thus, limiting differences between control and knockout cells. A third possibility could be differences in the expression or activity of GPCRs between primary islets and MIN6, as is seen for human and mouse islets [874]; but without knowledge which GPCR is inhibited by RGS2, this remains challenging to test. Altogether, my results firmly establish RGS2 as a negative regulator of insulin secretion.

In previous studies, Rgs2 KO islets were protected against hypoxia-induced apoptosis, with older mice exhibiting lower β-cell mass and serum insulin, presumably due to β-cell exhaustion [646]. However, whether RGS2 affects β-cell function was not mechanistically shown. While one study suggested that RGS2 bound Gαs in the immortalized β-cell line βTC-tet [692], and islets from Rgs2 null mice secreted more insulin than controls in response to glucose and exendin-4 [646], no decrease in insulin secretion was seen in Rgs2 overexpressing islets following exendin-4 treatment (Figure 6.7 A). Different mouse strains express variable amounts of certain GPCRs, but CD-1 islets utilized for overexpression studies and B6 KO islets express similar levels of Gαs-coupled GLP1 receptors [874]. When compared relative to absolute stimulation by glucose, it seems rather that exendin-4 is less potent in Rgs2 KO cells [646], suggesting the increase in insulin secretion is mainly glucose-driven. As a conclusion, RGS2 likely does not
modulate G\(\alpha_s\) directly. However, it raises an interesting possibility. If exendin-4 stimulation is relatively less potent in Rgs2 KO islets, but comparatively more potent in Rgs2 overexpressing islets (Figure 6.7 A), it is likely that a G\(\alpha_i\)-coupled receptor is inhibited by RGS2. A possible candidate in islets would be the SSTR, as SST is released from \(\delta\)-cells in response to glucose to counteract G\(\alpha_s\). In the absence of \(\delta\)-cells, i.e. in MIN6 cell culture, a G\(\alpha_i\) coupled receptor with tonic activity in the absence of ligand binding, such as \(\alpha_{2A}\)AR \([875]\), is more likely. RGS2 exhibits GAP activity towards G\(\alpha_i\) \([668]\), which can contribute to enhanced calcium influx \([727]\), but it is relatively more potent towards G\(\alpha_q\) \([714]\). RGS2 expression levels and GPCR affinity may be contributing factors in this \([715; 876]\). Further studies are needed to ascertain a role for RGS2 in suppressing G\(\alpha_i\) in \(\beta\)-cells.

The role of RGS2 in modulating calcium homeostasis has been well documented \([726; 732; 739; 741; 869-871]\), and has been confirmed in overexpression studies in this chapter. This was correlated with decreased oxygen consumption independent of changes in HIF1\(\alpha\) signalling, thus likely a consequence of lower mitochondrial activity following reduced mitochondrial calcium influx through ER-mitochondrial microdomains. Notably, while increased calcium influx was predicted as a mechanism of why Rgs2 KO cells secrete slightly more insulin, no significant increase was seen. This observation could be due to transcriptional changes to intracellular calcium handling proteins following loss of Rgs2, because in other cell types, increased expression of Serca2a and Serca2b \([739; 741; 869]\), a switch in expression from a high to a low sensitivity IP3R isoform \([741]\), and changes in PMCA activity \([739; 741]\) and expression \([739]\) were observed. In combination, these changes lower intracellular calcium through increased calcium efflux and ER uptake, and less IP3-dependent ER calcium release. It was proposed that these changes are a protective adaptation to the initially excessive calcium influx following loss of Rgs2 \([741]\). It remains to be tested whether Rgs2 KO cells indeed express higher levels of the
calcium transporters, as it would help explain why they exhibit calcium influx resembling those of control cells.

When considering alternative candidate GPCRs that could be regulated by RGS2 during exposure to glucose, the field was narrowed down to purinergic receptors, stimulated by adenosine nucleotides following insulin granule exocytosis, and muscarinic receptors, some of which exhibit tonic activity in the absence of ligand \[^{875}\]. The two compounds to probe these receptor families were an ATP analogue and carbachol. Although no difference was seen with the latter, carbachol stimulates not only $\text{G}_{\alpha_q}$-coupled receptors (M1R, M3R, M5R), but also $\text{G}_{\alpha_i}$-coupled receptors (M2R, M4R), complicating interpretations of results. Human islets also express different levels of muscarinic receptors than mice \[^{874}\], although traces for the expression of most receptors can be found \[^{877}\]; yet it is a factor to consider when trying to extrapolate results from mouse islets into the human condition. Until more selective agonist are available, knockdown approaches or antagonists for individual receptors may shed further light on their individual involvement. Finally, an ATP analogue induced a slight increase in insulin secretion over glucose alone in $\text{Rgs2}$ KO MIN6 cells, indicating purinergic receptors may be a target of RGS2 in $\beta$-cells. Adenosine nucleotides are co-secreted with insulin following glucose exposure, but whether they exhibit stimulatory or inhibitory influence on insulin secretion is debated \[^{635}\]. Endogenous $\text{Rgs2}$ is induced by purinergic signalling \[^{685}\] and inhibits ATP-stimulated calcium influx and GAP activity in other tissues \[^{717; 726}\], so this mechanism appears as a likely possibility.

While the studies described in this chapter both confirm and expand on previous reports, there are still many unanswered questions remaining. While RGS2 is a negative regulator of insulin secretion by lowering calcium influx, it is unclear whether a specific or a combination of GPCRs
are targeted by RGS2. RGS2 may also exert additional non-canonical effects. Further research needs to be conducted to unravel the exact role of Rgs2 in β-cells.
Chapter 7: Conclusion

7.1 Research Summary

In the natural course of type 2 diabetes, hyperglycemia and insulin resistance drive increased insulin secretion and β-cell mass expansion, but eventually β-cells become dysfunctional and undergo dedifferentiation and apoptotic cell death [201]. Calcium-dependent signalling pathways and transcription factors have been shown to be involved in all these processes [316; 391; 393; 398; 411; 422; 775; 878; 879], and GWAS studies have found T2D risk variants associated with calcium signalling or β-cell activity [426-430; 880; 881]. It is important to realise that neither excessive elevation nor reduction in calcium signalling is beneficial: long-term treatment of T2D patients with KATP channel-blocking sulfonylureas worsens glycemic control and β-cell function [162; 441; 442], and while promoting β-cell rest is beneficial in the short term [410; 439; 440], permanently reduced calcium signalling impairs β-cell viability and insulin secretion [1; 397; 418]. To preserve calcium homeostasis and β-cell function, the goal must therefore be to restore the balance which is disturbed during diabetes, rather than excessively stimulate or deplete essential calcium signals. This of course begs the question of what defines this optimal balance. How exactly does a typical β-cell work? How do we even define a “typical” β-cell? These questions exemplify the limit of our current state of knowledge. While we have a good understanding of basic β-cell function and calcium signalling, an increasing body of literature in the field of β-cell calcium homeostasis [1] gradually uncovers new roles for old players, as well as new factors. Potential new therapeutics to preserve β-cell survival and function may be informed by these studies.

Ultimately, the aim of my thesis, and the experiments and results described herein, was to further our understanding of how β-cells work. In particular, I expanded on the role of calcium-related processes for β-cell function, by studying the transcription factor NPAS4 and the regulator of GPCR signalling RGS2. Both were previously shown to be activity-regulated
immediate early genes with cytoprotective effects [354; 446; 648; 667; 733; 735] whose expression is induced or enhanced by calcium [446; 670]; whereas RGS2 is also a known modulator of intracellular calcium levels [726; 732; 739; 741; 869-871]. Considering these properties, I asked how NPAS4 and RGS2 might be relevant to normal β-cell physiology and diabetes disease progression.

To start answering this question, I first examined the calcium-dependent signalling pathways and post-translational modifications controlling NPAS4 expression and stability in β-cells. In Chapter 3, I demonstrated that Npas4 mRNA and protein expression depend on CaN, PKB (in collaboration with Dr. Paul V. Sabatini) and CaMKII. The roles for CaN and CaMKII pathways in Npas4 induction are novel discoveries, and it remains to be seen if neuronal Npas4 is regulated in a similar way. In contrast, a role for PKB and an upstream kinase, PI3K, was noted in the induction of Npas4 message by neurotrophic growth factor (NGF) in neuronal cells [550]; although another study showed that Npas4 protein expression was not detected following NGF exposure [354]. These results would not be inconsistent with a model where growth factor stimulation is required, but not sufficient, for NPAS4 induction. Indeed, insulin alone does not stimulate Npas4 induction (section 3.2.1). Instead, depolarization was the main stimulus to elicit NPAS4 expression in both β-cells [446] as well as neurons [354], and it was following this stimulus that PKB inhibition elicited its negative effect on NPAS4 expression (Chapter 3). This indicates that NPAS4 may be a coincidence detector of depolarization and PI3K-PKB signalling (Figure 7.1). A limitation of my studies was that some of the inhibitors that were used have reported off-target effects, which could have led to false-positive results, as is suspected to be the case for reduced NPAS4 induction with the AMPK inhibitor Compound C. Efforts were made in selecting the best inhibitors at the time of study, but as more specific inhibitors become available, it may be worth repeating some of these expression studies. Also, a few signalling
pathways that may play a role in Npas4 induction, such as PKA or stress-induced p38 MAPK, remain untested in β-cells.

Figure 7.1. Working model of NPAS4 and RGS2 actions in β-cells.

Following glucose-stimulated calcium influx, CaMK and CaN are activated, leading to induction of Npas4 gene expression via unknown transcription factors. PKB enhances NPAS4 protein levels. In the presence of NPAS4, transcriptional activity of HIF1α is blocked, likely via competition for the common heterodimerization partner ARNT. By suppressing HIF1α activity, and/or other unknown target genes, NPAS4 optimizes OXPHOS. Another NPAS4 target gene, RGS2, is a net negative regulator of insulin secretion, as it reduces Ca^{2+}_{cyt} and OCR. Whether RGS2 mediates its effects through GAP activity towards GPCRs is unknown; it may suppress ATP-evoked Gαq-coupled purinergic signalling. Through their combined actions, NPAS4 and RGS2 fine-tune insulin secretion, promoting optimal β-cell function.
Furthermore, I found that NPAS4 is ubiquitinated by an unknown ubiquitin ligase and degraded via the proteasome. This helps explain the rapid decay of NPAS4 upon termination of stimulation, and may be a potential target for NPAS4 stabilization by small molecules; although the specific enzymes involved have to be uncovered first. NPAS4 stabilization may be desirable to maintain β-cell viability, as NPAS4 was previously shown to inhibit ER stress and apoptosis. In Chapter 3, this finding is expanded on by showing that CaN signalling, which promotes β-cell survival, may do so by inducing NPAS4 (Figure 7.1); as overexpression of Npas4 reverted the apoptotic effects of a CaN inhibitor. This is relevant in the context of organ transplantation, where FK-506 is used as an immunosuppressant, but reduces β-cell function and compromises survival. These experiments need to be confirmed in human islets, and it remains to be tested whether NPAS4 expression is also able to maintain β-cell function during FK-506 treatment.

A mechanistic role for NPAS4 in β-cell function, and its role in β-cells in vivo, was further explored in Chapter 4. In vivo, it was previously shown that loss of Npas4 in the islet and brain of Npas4lox/lox;Pdx1-CreER+ (N4 PKO) mice causes several metabolic defects, including increased feeding, insulin resistance and glucose intolerance. In that study, initial data from the β-cell specific Ins1Cre mouse model indicated that the individual contributions from β-cells differs from loss in the brain. Here, I corroborated this work, showing that β-cell specific loss of Npas4 in a diet-induced model of obesity does not affect body weight, fed and fasting glycemia, or insulin resistance, but led to mild glucose intolerance. This was associated with increased HIF1α signalling in islets from Npas4 KO mice, and resulted in reduced oxygen consumption, a measure of metabolic activity. In contrast, Npas4 overexpression reduced HIF1α target genes. Mechanistically, I demonstrated that this was likely due to HIF1α and NPAS4 competing for their common heterodimerization partner ARNT (Figure 7.1). Such a mechanism may be part of a wider crosstalk between bHLH-PAS proteins. For example,
previous studies showed antagonistic behaviour between HIF1α and AHR due to limited ARNT availability\textsuperscript{[834]}, competitive DNA binding between SIM2 and NPAS4 \textsuperscript{[539]}, and inhibition of AHR activity by AHRR \textsuperscript{[882]}. Because HIF1α stabilization also inhibits NPAS4 induction, this is likely part of a feedback cycle which ensures that metabolism is optimal under normal physiological conditions. Altogether, I showed that NPAS4 supresses HIF1α signalling and thereby optimizes metabolism.

Building on these findings of antagonistic behaviour between NPAS4 and HIF1α, I asked what are their genomic targets in Chapter 5. Genome-wide NPAS4 and ARNT binding during different physiological conditions were revealed, which greatly expanded on the few previously known NPAS4 target genes in β-cells \textsuperscript{[446]}. In addition, I identified a likely NPAS4 DNA binding motif for β-cells. In its core sequence (NCGTG), it is identical to previously published NPAS4:ARNT2 motifs \textsuperscript{[539; 573]}, but a couple bases on either end could mean NPAS4 DNA binding specificity is slightly higher in β-cells. These data will be a useful resource in future studies of the inverse roles of NPAS4 and HIF1α during β-cell activity and oxidative stress, respectively, and can be used to confirm bona fide NPAS4 target sites. To complement available ARNT and NPAS4 ChIP data, ChIP for HIF1α should also be performed. Even then, observing NPAS4 and ARNT peaks at the same site remains correlative; to determine whether they bind DNA as a heterodimer requires further experiments, such as ChIP-on-ChIP. Another clear limitation of my studies is that MIN6 cells are an insulinoma cell line, and thus metabolically more skewed towards HIF1α signalling. Thus, it will be important to repeat these experiments in primary islets cells.

Lastly, I provided evidence that RGS2 is a negative regulator of insulin secretion in Chapter 6. Mechanistically, this was due to its role in reducing intracellular calcium levels and oxygen consumption rate (Figure 7.1). The effect on calcium homeostasis is consistent with previously
published roles for RGS2 in modulating calcium homeostasis. It would be interesting to also examine expression of calcium channels such as SERCA, IP3R, or PMCA, to see if expression changes of these proteins also occur in β-cells. While the subcellular localization of RGS2 in β-cells needs to be examined in greater detail, initial evidence presented in Chapter 6 points to localization at the plasma membrane rather than in the nucleus; suggesting any changes in ion channel expression would be secondary to changes in intracellular calcium levels. Reduced oxygen consumption following Rgs2 overexpression was also interpreted to be secondary to reduced release of calcium from intracellular stores; because calcium is normally transported into mitochondria through ER-mitochondrial microdomains, where it is able to enhance ATP production. However, this assumption has not been tested experimentally. Finally, I showed that RGS2 does not mediate its effects through the GLP1R, but perhaps through inhibition of Gαq-coupled GPCRs such as purinergic receptors. Further research is required to reveal the identity of the GPCRs directly regulated by RGS2. In the absence of a β-cell specific mouse model, the Rgs2 KO MIN6 cell lines I generated serve as useful tools to assist in this endeavour.

There are a number of other limitations that should not go unmentioned in my thesis. First, overexpression studies utilizing Ad-Npas4 and Ad-Rgs2 adenoviruses yielded supra-physiological levels of expression, which may result in artifacts. Where possible, knockout mouse models were used to complement overexpression studies to avoid false interpretations; however, it may be required to follow up some experimental findings with loss-of-function studies in the future. Second, there are various differences between mouse and human islets and β-cells in terms of cytoarchitecture, function and gene expression [7; 874]. Therefore, drawing conclusions from mouse models to inform on human biology may not always yield correct results. In my and Dr. Paul V. Sabatini’s hands, mouse and human NPAS4 and RGS2 expression was regulated largely identical, suggesting that the results obtained with mouse models have validity for the human condition. Third, studies using mice utilized HFD as a model of obesity-
driven diabetes, which may not necessarily reflect the natural course of disease. However, because HFD feeding and obesity induce HIF1α in other tissues \[^{839; 840}\], this model turns out to be quite suitable for studies on the role of NPAS4 in suppressing HIF1α signalling. Lastly, I only used male mice in my studies. As there are considerable sex differences in metabolism and diabetes susceptibility, such as a higher predisposition of males to develop diabetes \[^{883}\], it appears worthwhile to repeat some of my studies in mice of the opposite sex.

Whereas glycemic control declined in T2D patients between 1988-2000 despite treatment \[^{884}\], treatment options for diabetes have improved since. It is now feasible to maintain stable HbA1C levels in the majority of patients using a combination of lifestyle changes, blood glucose lowering oral antidiabetic drugs, and insulin \[^{225; 885}\]. However, some patients still show deteriorating glycemic control, and few an improvement, despite treatment \[^{225; 885}\]. Thus, novel therapeutic agents to protect the patient’s own β-cell population and preserve long-term insulin secretion through the use of β-cell cytoprotective factors are still needed. The transcription factor NPAS4 and its target gene Rgs2 may be such viable therapeutic targets because 1) NPAS4 has proven cytoprotective effects in β-cells (Chapter 3) \[^{446}\], 2) NPAS4 reduces oxidative stress by limiting excessive HIF1α signalling (Chapter 4) \[^{4}\], 3) NPAS4 binds near promoter sites of important β-cell genes and modulates their expression (Chapter 5) \[^{446}\], 4) RGS2 could alleviate excessive insulin release (Chapter 6) \[^{646}\], and 5) both NPAS4 and RGS2 expression are reduced in T2D islets (Chapter 6) \[^{4}\]. It may be desirable to stabilize or restore expression of NPAS4 or its target gene Rgs2 using pharmacological approaches, as even supraphysiological expression was well tolerated in insulinoma and islet cells without obvious cytotoxic effects at least after a few days of in vitro culture (Chapter 3, 6) \[^{446}\]. Proof-of-principle regarding RGS2 stabilization has been provided \[^{691}\], supporting the prospect of therapeutic applications to stabilize RGS2 in β-cells of those with diabetes. The results in this thesis provide the basis for future experiments designed to test this hypothesis.
7.2 Future Directions

In those T2D patients with excessive HIF1α signalling, temporarily stabilizing NPAS4 expression may be beneficial to alleviate hyperglycemia. While I identified the calcium-dependent kinases and phosphatase involved in NPAS4 expression, the downstream transcription factors and NPAS4 promoter elements remain unknown. It will be informative to uncover these, as upstream kinases have very broad roles, and if conceiving of NPAS4 stabilization as a β-cell therapy approach, off-target effects of targeting these kinases may be intolerable. In the end, it may be preferable to design an artificial transcription factor to drive NPAS4 expression specifically, but first identifying the relevant endogenous elements regulating NPAS4 transcription will be helpful in this endeavour. There may possibly also be further, as yet unidentified calcium signalling pathways impacting on NPAS4 expression. Alternatively, identifying and inhibiting a potentially specific ubiquitin ligase responsible for NPAS4 ubiquitination may allow for targeted stabilization of NPAS4 protein.

Regarding the antagonistic roles of NPAS4 and HIF1α, it would be informative to determine to which extent competition is due to competitive ARNT binding versus consensus motif binding [833]. I also propose identifying promising NPAS4 candidate target genes based on promoter or enhancer binding, and conduct functional studies to confirm them as such and study their role in β-cells. Since ChIP-Seq studies in this thesis were conducted in MIN6 cells, it will also be important to confirm my findings in islets, and extend them to HIF1α ChIP-Seq. While I found NPAS4 and ARNT binding sites near important β-cell genes, whether the heterodimer directly regulates the nearest gene is not strictly known. To obtain this spatial information, NPAS4 and ARNT ChIP-Seq data needs to be compared against chromosome conformation capture results, which can uncover physical linkage between genomic regions. The
above results could be complemented with scRNA-Seq of knockout and control islets to identify cell-type specific differences (NPAS4 is expressed in other endocrine cells).

As was shown, loss of \textit{Npas4} in β-cells using the \textit{Ins1}\textsuperscript{Cre} strain resulted in mild glucose intolerance, whereas knockouts generated with the Pdx1-CreER strain showed more pronounced defects. As recombination was noticed in the hypothalamus, which regulates food intake, it suggests the involvement of central loss of \textit{Npas4}. Thus, metabolic defects should be assessed in brain-specific HFD-fed \textit{Npas4} KO mice. \textit{In vitro}, it remains to be tested whether lower oxygen consumption in \textit{Npas4} KO islets can be corrected for by co-treatment with a HIF1α inhibitor, such as PX478 \cite{886}. If this were the case, application of PX478 would also be predicted to ameliorate hyperglycemia and glucose intolerance in HFD-fed N4 PKO mice \textit{in vivo}.

For RGS2 studies, there are plenty of unanswered questions. First and foremost, the interaction partners of RGS2 need to be determined. As an alternative to measuring insulin secretion following treatment with GPCR agonists, it may be feasible to narrow down several candidates in CoIP studies with cells overexpressing \textit{Gαq}-coupled GPCRs and RGS2. However, a lack of specific GPCR antibodies may limit the utility of this approach \cite{887}. Alternatively, RGS2 immunoprecipitation combined with mass spectrometry has the potential to identify all RGS2 interacting proteins (or post-translational modifications), but is time- and resource intensive. Furthermore, it needs to be determined experimentally whether the RGS2-induced decrease in oxygen consumption is truly due to lower mitochondrial calcium uptake, or perhaps an indirect consequence of altered calcium homeostasis. As HIF1α target genes were not changed, the changes in oxygen consumption are not likely due to an imbalance between NPAS4 and HIF1α, but mitochondrial function could certainly be affected by expression changes in other calcium-dependent genes. In the long term, a β-cell specific KO mouse model would be beneficial to study an \textit{in vivo} role of RGS2 in β-cells.
7.3 Final Thoughts

It appears to be a common paradox when investigating a particular problem or hypothesis, that the few answers we can obtain are in stark contrast with the vast number of questions which are left unanswered, and the new questions coming up along the way. Yet, we continue to make new discoveries, incrementally filling the gaps in our knowledge. It is a truly humbling experience to study the complexity of the seemingly simple biological process of insulin secretion from the islet, which had first been postulated almost 120 years ago. It is thus with utmost humility that I must now submit my thesis in this stimulating field. Nonetheless, I managed to define the calcium-dependent signals modulating NPAS4 expression, uncovered a novel role for NPAS4 in modulating metabolism, and provided an initial characterization of the role of RGS2 as negative regulator of insulin secretion. Furthermore, I provided some initial β-cell NPAS4 ChIP-Seq data. It is my hope that these results and resources will be of help for future generations of scientists studying and dissecting stimulus-secretion coupling in β-cells and beyond.
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