

The roles of AMPK and LKB1 in hormone secreting cells

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A thesis presented for the degree of Doctor of Philosophy of the University of London and diploma of Imperial College London department of Cell Biology and Functional Genomics

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Abstract

Regulation of insulin secretion from the pancreatic β -cell and glucagon secretion from the α -cell is vital for the control of normal glucose homeostasis. Dysfunction may lead to some forms of Diabetes. Liver kinase B1 (LKB1) is a tumour suppressor that is mutated in the premalignant disorder Peutz-Jeghers Syndrome (PJS). PJS is characterised by the formation of polyps in the gastrointestinal tract. LKB1 exerts its biological effect via direct phosphorylation of AMP-activated protein kinase (AMPK) and related kinases.

The incretin hormones glucagon-like-peptide 1 (GLP-1), secreted from proglucagon-expressing enteroendocrine L-cells, is involved in the regulation of appetite and glucose homeostasis. Therefore, GLP-1 is considered of therapeutic value in the treatment of Type 2 Diabetes. Both LKB1 and AMPK have been shown to play some role in insulin secretion. However, the role of LKB1 and AMPK in proglucagon-expressing cells in regulating GLP-1 secretion and glucose homeostasis has yet to be studied *in vivo*.

In this study, we investigate how LKB1 contributes to insulin secretion in pancreatic β -cells by deleting LKB1 using a β -cell selective Ins1Cre promoter in mice. I also explore the roles of LKB1 and AMPK α 1 α 2 in proglucagon-expressing cells including enteroendocrine L-cells of the gut and pancreatic α -cells using a proglucagon specific iGluCre promoter.

Deletion of LKB1 in β -cells resulted in two opposing effects. (1) Glucose tolerance and β -cell mass were increased and (2) the cytosolic ATP/ADP and Ca²⁺ response to glucose was impaired. Despite these effects, insulin secretion *in vitro* remained unaffected. Moreover, deletion of LKB1 resulted in amplification of a glutamate-induced signalling pathway. Therefore, ablation of LKB1 amplifies other pathways which compensates for impairments in cytosolic and mitochondrial function.

Deletion of LKB1 in proglucagon-expressing cells has no effect on GLP-1 secretion or glucose homeostasis but result in the development of large gastro-duodenal polyps. On the other hand, deletion of AMPK from these cells improves glucose tolerance as well as L-cell mass and GLP-1 secretion.

Therefore, both LKB1 and AMPK play roles in normal insulin secretion. Targeting inhibition of these kinases in β - and L-cells may thus provide a new therapeutic strategy in some forms of Type 2 Diabetes.

Table of Contents

Abstract	4
Table of Contents	5
List of Figures	11
List of Tables	13
Abbreviations.....	14
Chapter 1	18
Introduction	18
1.1 The Pancreas	18
1.1.1 The endocrine pancreas.....	18
1.1.2 The development of the pancreas	18
1.1.3 Islets of Langerhans	20
1.2 The β -cell and glucose stimulated insulin secretion	21
1.2.1 The pancreatic β -cell.....	21
1.2.2 The insulin granule	22
1.2.3 Glucose sensing and phosphorylation in the β -cell.....	23
1.2.4 Biphasic insulin secretion.....	26
1.2.5 Metabolic activation of mitochondria	26
1.2.5.1 The Krebs/ Citrate cycle	26
1.2.5.2 The Electron transport chain	26
1.2.6 ATP-sensitive K^+ channels	27
1.2.7 Insulin exocytosis	28
1.2.8 Disallowed genes	29
1.2.9 Type 2 Diabetes.....	30
1.2.9.1 Mechanisms of β -cell loss	31
1.2.9.2 Treatment and prevention of Type 2 Diabetes.....	32
1.3 Glucagon and the α -cell	32
1.3.1 Discovery and production	32
1.3.2 Glucagon in development.....	33
1.3.3 Glucagon secretion from the α -cell	33
1.3.3.1 K_{ATP} channel-dependent glucose sensing.....	34
1.3.3.2 Ca^{2+} dependent glucose sensing.....	35

1.3.4	Glucagon secretion during hyperglycaemia.....	36
1.3.4.1	Reduced inhibition of glucagon secretion	36
1.3.4.2	Pulsatile glucagon release during hyperglycaemia	37
1.3.5	Influence of glucagon in Type 2 Diabetes	37
1.3.6	Glucagon as a therapeutic target.....	38
1.4	Glutamate signalling	38
1.4.1	Glutamate-stimulated insulin secretion	39
1.4.2	Glutamate-induced activation of glucagon secretion.....	40
1.5	AMP-activated protein kinase (AMPK)	40
1.5.1	The heterotrimeric structure of AMPK	41
1.5.2	Regulation of AMPK in the β -cell	42
1.5.3	Expression of AMPK in the β -cell	43
1.5.4	The role of AMPK in insulin secretion	44
1.5.4.1	AMPK inhibits GSIS.....	45
1.5.4.2	AMPK promotes GSIS.....	46
1.5.5	The role of AMPK in apoptosis.....	46
1.5.6	Deletion of AMPK α 1 and α 2	47
1.5.7	Downstream mechanism of AMPK action in β -cells	48
1.5.8	The role of AMPK in the control of food intake by the hypothalamus.....	48
1.5.9	AMPK and glucagon secretion	49
1.6	Liver Kinase B 1(LKB1)	49
1.6.1	Protein structure and localisation of LKB1.....	49
1.6.2	The role of LKB1 in cell polarity	50
1.6.3	The role LKB1 in cell growth and proliferation	51
1.6.4	LKB1 in diabetes.....	52
1.6.5	Roles of LKB1 in the β -cell.....	52
1.6.5.1	LKB1 and β -cell polarity.....	52
1.6.5.2	LKB1 and β -cell size.....	53
1.6.6	Peutz Jeghers Syndrome	53
1.7	Glucagon-Like Peptide 1	54
1.7.1	The GLP-1 receptor (GLP-1R) and its presence on the β -cell.....	54
1.7.2	GLP-1 mediated regulation of insulin secretion	55
1.7.3	Effect of GLP-1 in β -cell proliferation, differentiation and apoptosis.....	56
1.7.3.1	GLP-1 stimulates β -cell proliferation	57

1.7.3.2	GLP-1 affects β -cell differentiation	57
1.7.3.3	GLP-1 on β -cell apoptosis	58
1.7.4	Actions of GLP-1 in pancreatic α -cells.....	58
1.7.5	The role of GLP-1 in Type 2 Diabetes	59
1.7.6	GLP-1 treatment for Type 2 Diabetes	59
Aims of thesis.....		61
Chapter 2		62
Materials and Methods.....		62
2.1	In vivo mouse experiments.....	62
2.1.1	Generation of mice selectively lacking LKB1 in pancreatic β -cells	62
2.1.2	Generation of mice selectively lacking LKB1 in proglucagon-expressing cells	62
2.1.3	Generation of mice selectively lacking AMPK α 1 and α 2 in proglucagon-expressing cells	62
2.1.4	Mice maintenance and diet	63
2.1.5	Body weight	63
2.1.6	Oral and Intraperitoneal glucose tolerance test.....	63
2.1.7	Insulin tolerance test (ITT) and plasma glucagon measurement.....	63
2.1.8	Plasma glucagon and insulin measurement	63
2.2	In vitro cell experiments	64
2.2.1	Islet isolation and dissociation	64
2.2.2	Treatment of islets for glucagon and insulin measurements	64
2.2.3	Insulin and glucagon radioimmuno-assay.....	64
2.2.4	Ca ²⁺ , TMRE and ATP imaging using confocal microscopy	65
2.2.5	Detection of ATP	66
2.2.6	Seahorse Bioscience XF24 assay	66
2.2.7	Metabolomic measurements.....	67
2.2.8	Electron microscopy of islets	67
2.3	Protein Experiments.....	67
2.3.1	Immunohistochemistry of whole pancreas and ileum sections	67
2.3.2	Optical Projection Tomography and determination of relative β -cell and L-cell mass	68
2.4	Molecular Biology experiments	68
2.4.1	DNA extraction and polymerase chain reaction (PCR) for genotyping studies	68
2.4.2	RNA extraction and real-time (RT) PCR.....	69

2.4.3	Quantitative Real-Time PCR (qRT-PCR).....	69
2.4.4	Amplification and purification of high titre adenovirus.....	70
2.5	Statistical analysis	70
Chapter 3		71
LKB1 deletion in β -cells enhances β -cell mass despite impaired Ca^{2+} and ATP flux		71
Abstract.....		71
3.1	Introduction	72
3.2	Results	74
3.2.1	Ins1LKB1KO improves β -cell mass and has no effect on insulin secretion in vitro ..	74
3.2.2	Defects in ATP and Ca^{2+} in Ins1LKB1KO occur independently of insulin secretion....	77
3.2.3	LKB1 is required for normal mitochondrial function	83
3.2.4	Glutamate signalling is up-regulated in Ins1LKB1KO mice	90
3.3	Discussion.....	93
3.3.1	The Ins1Cre promoter deletes selectively in the β -cell at E11.5	93
3.3.2	LKB1 deletion improves glucose tolerance and β -cell mass however, GSIS is unaffected	94
3.3.3	Cytosolic signalling and mitochondrial defects are present in Ins1lkb1ko islets.....	95
3.3.4	Glutamate release and action may compensate in LKB1 deficient β -cells	96
3.4	Conclusions	98
Chapter 4		99
Deletion of LKB1 in α - and L-cells and its effects on glucagon and GLP-1 levels.....		99
Abstract.....		99
4.1	Introduction	100
4.2	Results	101
4.2.1	Deletion of LKB1 from preproglucagon-expressing cells causes dramatic tumour growth in the duodenum	101
4.2.2	GluLKB1KO mice have normal glucose tolerance and in vivo GLP-1 secretion	105
4.2.3	Glucagon secretion and α -cell mass is unaffected in GluLKB1KO mice	110
4.3	Discussion.....	114
4.3.1	GluCre expression is localised in α - and L-cells.....	114
4.3.2	Deletion of LKB1 causes dramatic tumour growth in the duodenum	114
4.3.3	GluLKB1KO display normal glucose tolerance and GLP-1 levels.....	115
4.3.4	GluLKB1KO mice display normal glucagon secretion.....	116
4.4	Conclusions	116

Chapter 5	117
Deletion of AMPK α 1 and α 2 in proglucagon-expressing cells up-regulates GLP-1 levels and improves glucose homeostasis	117
Abstract.....	117
5.1 Introduction	118
5.2 Results.....	121
5.2.1 Deletion of AMPK α 1 α 2 from proglucagon-expressing cells does not lead to polyposis	121
5.2.2 Deletion of the AMPK α -subunits improves glucose tolerance after oral gavage but not after intraperitoneal injection.....	126
5.2.3 AMPK α 1 α 2 deletion in proglucagon expressing cells has no effect on insulin tolerance or circulating insulin levels	130
5.2.4 AMPK α 1 and α 2 deletion in proglucagon-expressing cells results in increased L-cell mass and circulating GLP-1 levels.	133
5.2.5 AMPK α 1 and α 2 deletion in proglucagon-expressing cells has no effect on α -cell mass or glucagon secretion	136
5.3 Discussion.....	139
5.3.1 Deletion of AMPK α 1 and α 2 does not stimulate polyposis	139
5.3.2 Deletion of AMPK α 1 and α 2 in L-cells of the gut improves GLP-1 secretion resulting in these mice to become more glucose tolerant	139
5.3.3 AMPK α 1 and α 2 deletion from pancreatic α -cells does not alter α -cell mass or glucagon secretion but increases L-cell mass	140
5.4 Conclusions	141
Chapter 6	142
Final Discussion.....	142
6.1 Investigating the relevance of AMPK and LKB1 in glucose homeostasis using tissue-specific knock-out mice.....	142
6.2 Roles of LKB1 in the pancreatic β -cell.....	143
6.3 Impact of LKB1 and AMPK deletion in enteroendocrine L-cells	145
6.3.1 Deletion of LKB1 in L-cells.....	145
6.3.2 Deletion of AMPK α 1 and α 2 in L-cells.....	147
6.4 Impact of AMPK and LKB1 in pancreatic α -cells	148
6.4.2 Deletion of LKB1 in α -cells	148
6.4.2 Deletion of AMPK in α -cells	148
6.5 Final conclusion.....	150
Bibliography.....	151

Appendix 1	176
Antibody List	176
Appendix 2	177
PCR primers (for genotyping) and T_m	177
Appendix 3	178
Real time qPCR primers	178
Appendix 4	179
In vivo insulin secretion and glucose tolerance improves in Ins1LKB1KO mice	179
Appendix 5	180
Nptx2 expression is up-regulated in islets of Ins1LKB1KO mice	180
Appendix 6	181
Recombination in enteroendocrine L-cells was demonstrated by staining for RFP in GluLKB1KO mice.....	181
List of Publications	182

List of Figures

Chapter 1

Figure 1.1 - Overview of organogenesis of the pancreas

Figure 1.2 - The localisation and anatomy of the pancreas and the islets of Langerhans

Figure 1.3 - Formation of preproinsulin and the insulin granule

Figure 1.4 - GSIS of the pancreatic β -cell

Figure 1.5 – Insulin exocytosis from the β -cell

Figure 1.6 - Glucose stimulated glucagon secretion from the pancreatic α -cell

Figure 1.7 Structure of the heterotrimeric AMPK complex

Figure 1.8 - The AMPK signalling pathway- controlled by LKB1 and AMPK intracellular signalling and metabolism

Figure 1.9 - Overall structure and interactions of the LKB1-STRADa-MO25a complex

Figure 1.10 – Polyps extracted from a patient with PJS

Figure 1.11- GLP-1 binding to and activating GLP-1R plays a role in GSIS

Chapter 3

Figure 3.1 β -cell mass is enhanced in Ins1LKB1KO mouse pancreata

Figure 3.2 Gene expression of *Ins1* and *Ins2* are up-regulated in Ins1LKB1KO islets

Figure 3.3 Insulin secretion is unaffected in Ins1LKB1KO islets

Figure 3.4 - Glucose-induced $[ATP/ADP]_{\text{cyt}}$ changed are drastically impaired in Ins1LKB1KO *versus* WT mouse islets

Figure 3.5 - Luciferase-based measurements of ATP concentrations in glucose-treated islets

Figure 3.6 - $[Ca^{2+}]_{\text{cyt}}$ changes are drastically impaired in Ins1LKB1KO islets

Figure 3.7 - *In vitro* rescue of LKB1 expression results in restoration of $[ATP/ADP]_{\text{cyt}}$ and $[Ca^{2+}]_{\text{cyt}}$ signalling in Ins1LKB1KO mice

Figure 3.8 - Mitochondrial defects are present in Ins1LKB1KO mice

Figure 3.9 - Metabolomic profiling data shows that several citrate cycle intermediates and FA's are down-regulated in Ins1LKB1KO islets whereas fructose-6-phosphate is up-regulated

Figure 3.10 - OCR measured by Seahorse XF24 analyser in the presence of glucose, oligomycin, FCCP, rotenone and antimycin

Figure 3.11 - Respiratory capacity is up-regulated in Ins1LKB1KO islets

Figure 3.12 - LKB1 is not essential for normal mitochondrial integrity in the β -cell

Figure 3.13 - Up-regulation of *Nptx2* expression and Ca^{2+} signalling in Ins1LKB1KO mice using the glutamate receptor agonist kainite

Figure 3.14 - Glutamate signalling enhances insulin secretion in Ins1LKB1KO mice

Figure 3.15 - Timeline of Ins1Cre, RIP2Cre and Pdx1 CreER mouse models *floxed* for LKB1

Figure 3.16 - Model showing an alternative signalling system in the β -cell after LKB1 deletion

Chapter 4

Figure 4.1 - Generation of GluLKB1KO mice use of a Rosa26tdRFP reporter to assess recombination

Figure 4.2 - Decreased body weight and life-span of GluLKB1KO mice

Figure 4.3 - Polyp formation in GluLKB1KO mice

Figure 4.4 - Glucose tolerance is unaffected in GluLKB1KO mice

Figure 4.5 - Oral glucose tolerance tests performed in iGluLKB1KO mice show no difference in glucose tolerance *versus* WT littermates

Figure 4.6 - L-cell mass is unaffected in iGluLKB1KO mice

Figure 4.7 - Plasma GLP-1 levels in fed and fasted mice had little effect in GluLKB1KO mice

Figure 4.8 - α : β cell ratio increases in GluLKB1KO mice, whilst there is no effect in cell mass

Figure 4.9 - Insulin tolerance and glucagon secretion *in vivo* are unaffected in GluLKB1KO mice

Figure 4.10 - Glucagon levels in plasma of GluLKB1KO mice both fed and fasted overnight are unaffected

Figure 4.11 - Glucagon secretion is unaffected in GluLKB1KO mice

Chapter 5

Figure 5.1 - GluAMPKKO mice did not display any weight loss or polyp development as found in GluLKB1KO mice

Figure 5.2 - AMPK expression is down-regulated in ~30% α -cells

Figure 5.3 - AMPK expression in enteroendocrine L-cells is down-regulated in GluAMPKKO *versus* WT mice

Figure 5.4 - iGlu Cre recombination does not cause deletion of AMPK in pancreatic β -cells

Figure 5.5 - Fed glycaemia is unchanged in GluAMPKKO mice

Figure 5.6 - Glucose tolerance is improved in GluAMPKKO mice after oral administration of glucose

Figure 5.7 - Glucose tolerance is unaffected in GluAMPKKO mice after intraperitoneal administration of glucose

Figure 5.8 - AMPK α 1 α 2 deletion has no effect on glycaemia after insulin injection

Figure 5.9 - Plasma insulin levels are unaffected in GluAMPKKO mice when fed and fasted overnight

Figure 5.10 - The iGlu Cre transgene has no effect on β -cells function

Figure 5.11 - L-cell mass is enhanced in GluAMPKKO mice

Figure 5.12 - GLP-1 secretion *in vivo* is enhanced in AMPK α 1 α 2KO mice

Figure 5.13 - α : β cell ratio increases in GluAMPKKO mice, whilst there is no effect in overall cell mass

Figure 5.14 - Glucagon levels in the plasma is unaffected in GluAMPKKO mice *in vivo*

Figure 5.15 - Glucagon secretion *in vitro* is unaffected in GluAMPKKO mice

Chapter 6

Figure 6.1 - The proposed pathway for the modulation of β -cell size, utilising the LKB1-AMPK-mTOR-rpS6 pathway

Figure 6.2 – An brief overview of LKB1 and AMPK regulation in the pancreatic β -, α - and enteroendocrine L-cell

List of Tables

Chapter 2

Table 2.1: Compounds added to each port of XF sensor cartridge

Abbreviations

AMPA	α -amino-5-methyl-4-isoxazolepropionic acid
AMPK-RK	AMPK-related kinases
GABA	γ -aminobutyric acid
AICAR	5-Aminoimidazole-4-carboxamide ribonucleotide
ACC	Acetyl CoA carboxylase
Acot	Acetyl-CoA thioesterase
AC	Adenylate cyclase
ANT	Adenine nucleotide translocator
ADP	Adenosine diphosphate ADP
ATP	Adenosine triphosphate
AMPK	AMP-activated protein kinase
ARK5	AMPK-related kinase 5
8ABCC8	ATP-binding cassette transporter sub-family C member
K _{ATP}	ATP-sensitive K ⁺ channel
BH	Bcl-2 homology
BMI	Body mass index
BRSK/SAD-1	Brain specific kinase 1
CAMKK	Calcium/calmodulin dependent protein kinase kinase
CRE	cAMP response element
ChREBP	Carbohydrate Response Element Binding Protein
CPH	Carboxypeptidase H
CPT1	Carnitine palmitoyltransferase 1
CAT	Catalase
CNS	Central nervous system
CA	Constitutively active
cAMP	cyclic adenosine monophosphate
CDK	Cyclin dependent kinases
COX-2	Cyclooxygenase-2
DAPI	4,6-diamidino-2-phenylindole
DMSO	Dimethylsulfoxide
DPP	Dipeptidyl peptidase
DN	Dominant negative

E	Embryonic day
EPAC2	Exchange Protein Activated by cAMP-2
ETCF	Electron transport chain
ER	Endoplasmic reticulum
EGFR	Epidermal growth factor receptor
FA	Fatty acid
FBS	Fetal bovine serum
FGF	Fibroblast growth factor
FKBP	FK binding protein complex
FACS	Fluorescence-activated cell sorting
GIP	Gastric inhibitory polypeptide
GLP	Glucagon like peptide
GLP-1R	Glucagon like peptide 1 receptor
GK	Glucokinase
G6-P	Glucose-6-phosphate
GSIS	Glucose-stimulated insulin secretion
GLUT	Glucose transporter
GDH	Glutamate dehydrogenase
GAD	Glutamic acid decarboxylase
GPX1	Glutathione peroxide 1
HbA1c	Glycated haemoglobin 1c
GSK3	Glycogen synthase kinase 3
GFP	Green fluorescent protein
IHC	Immunohistochemistry
IRS	Insulin receptor substrate
ITT	Insulin tolerance test
ICU	Intracerebrovascular
IPGTT	Intrapritonal glucose tolerance test
CREB	Involving cAMP response element-binding protein
IAPP	Islet amyloid polypeptide
KO	Knock-out
KRB	Krebs-Ringer bicarbonate buffer
LDHA	Lactate dehydrogenase
LKB	Liver kinase B

mTOR	Mammalian target of rapamycin
MODY2	Maturity-onset diabetes of the young
FCCP	Mesoxalonitrile 4-trifluoromethoxyphenylhydrazone
mGluR	Metabotropic glutamate synthetic receptors
MBCD	Methyl- β -cyclodextrin
MARK	Microtubule-affinity regulating kinase
FMN	Mitochondrial flavin
MAPK	Mitogen activated protein kinase
MCT	Monocarboxylate/ lactate transporter
MO25	Mouse protein 25
MLPA	Multiplex ligation dependent probe amplification
MRLC	Myosin regulatory light chain
DNPI	Na ⁺ -dependent inorganic phosphate
NMDA	N-methyl-D-aspartate
NS	Not significant
Nfib	Nuclear factor I/B
OPT	Optical projection tomography
OGTT	Oral glucose tolerance test
OCR	Oxygen consumption rate
PTF1A	Pancreas transcription factor 1 alpha subunit
PRX	Pancreatic and duodenal homeobox
PDX1	Pancreatic duodenum homobox-1
PASK	PAS kinase
PJS	Peutz jeghers syndrome
PTEN	Phosphatase and tensin homolog
PI3K	Phosphoinositide 3-kinase
PCR	Polymerase chain reaction
KCNJ11	Potassium Channel, Inwardly Rectifying Subfamily J, Member 11
PPG	Preproglucagon
PHC	Prohormone convertase
PC	Protein convertase
PKA	Protein kinase A
AKT	Protein kinase B
PKC	Protein kinase C

PP1	Protein phosphatase 1
PC	Pyruvate carboxylase
PDH	Pyruvate dehydrogenase
QIK	Qin-induced kinase
qRT-PCR	Quantitative Real-Time PCR
RIA	Radioimmunoassay
RIP	Rat insulin promoter
ROS	Reactive oxygen species
RRP	Readily releasable pool
RT	Real time
RFP	Red fluorescent protein
RFX	Regulatory factor X
RYR	Ryanodine receptor
SIK	Salt-induced kinase
SRCA	Sarco(endo)plasmic Ca^{2+} ATPase
SNARK	SNF/AMPK related kinase
SEM	Standard error of the mean
STRAD	Ste20-related adaptor
STZ	Streptozotocin
SNAP	Synaptosomal-associated protein
TORC2	Target of rapamycin
TMRE	Tetramethylrhodamine, ethyl ester
TTX	Tetrodotoxin
TGFB	Transforming growth factor β TGFB
VGLUT2	Vesicular glutamate transporter 2
WT	Wild Type

Chapter 1

Introduction

1.1 The Pancreas

1.1.1 The endocrine pancreas

The pancreas is a glandular organ vital for nutrient metabolism and digestion. It has both exo- and endocrine functions [1] and consists of acinar and ductal cells. The acinar cells secrete enzymes such as amylase, trypsin and carboxypeptidase-A to catalyse the breakdown of carbohydrates, lipids and proteins [2]. The endocrine pancreas functions independently of exocrine cells and is responsible for the regulation of glucose homeostasis by releasing hormones such as insulin, glucagon and somatostatin into the blood stream. The endocrine pancreas is made up of cells (α -, β -, δ -, PP- and ξ -cells, discussed in more detail later) residing in small clusters known as the islets of Langerhans [3].

1.1.2 The development of the pancreas

During development, the pancreas first becomes morphologically evident on the 26th day after gestation in humans and on the 9th day in mice as the epithelial buds emerge, overlaying the dorsal aspect of the endodermal gut tube in the duodenal anlage of the foregut [4]. As this is happening, the pancreatic buds, which are surrounded by mesenchymal cells, continue to elongate and change their shape forming branched tubular structures; this process is known as the primary transition [5].

The secondary transition lasts from the 12th day of embryogenesis in mouse and the 37th day of embryogenesis in humans until birth. The endocrine cell number greatly expands, especially the β -cells, and rapid branching morphogenesis will occur. As well as this, gene expression in acinar enzyme increases and large amounts of rough endoplasmic reticulum (ER) and zymogen granules will form [1]. At this stage, endocrine, acinar and ductal cells become fully differentiated [3]. The development of the pancreas during embryogenesis is illustrated in Figure 1.1.

Disturbances in pancreatic development, though rare, may result in alterations in exocrine or endocrine pancreas. Developmental abnormalities in the pancreas are most likely to occur during morphogenesis of the foregut or due to mutations of genes such as regulatory factor X (RFX)6, Pancreatic duodenum homobox-1 (PDX1) and pancreas transcription factor 1 alpha subunit (PTF1A) [6].

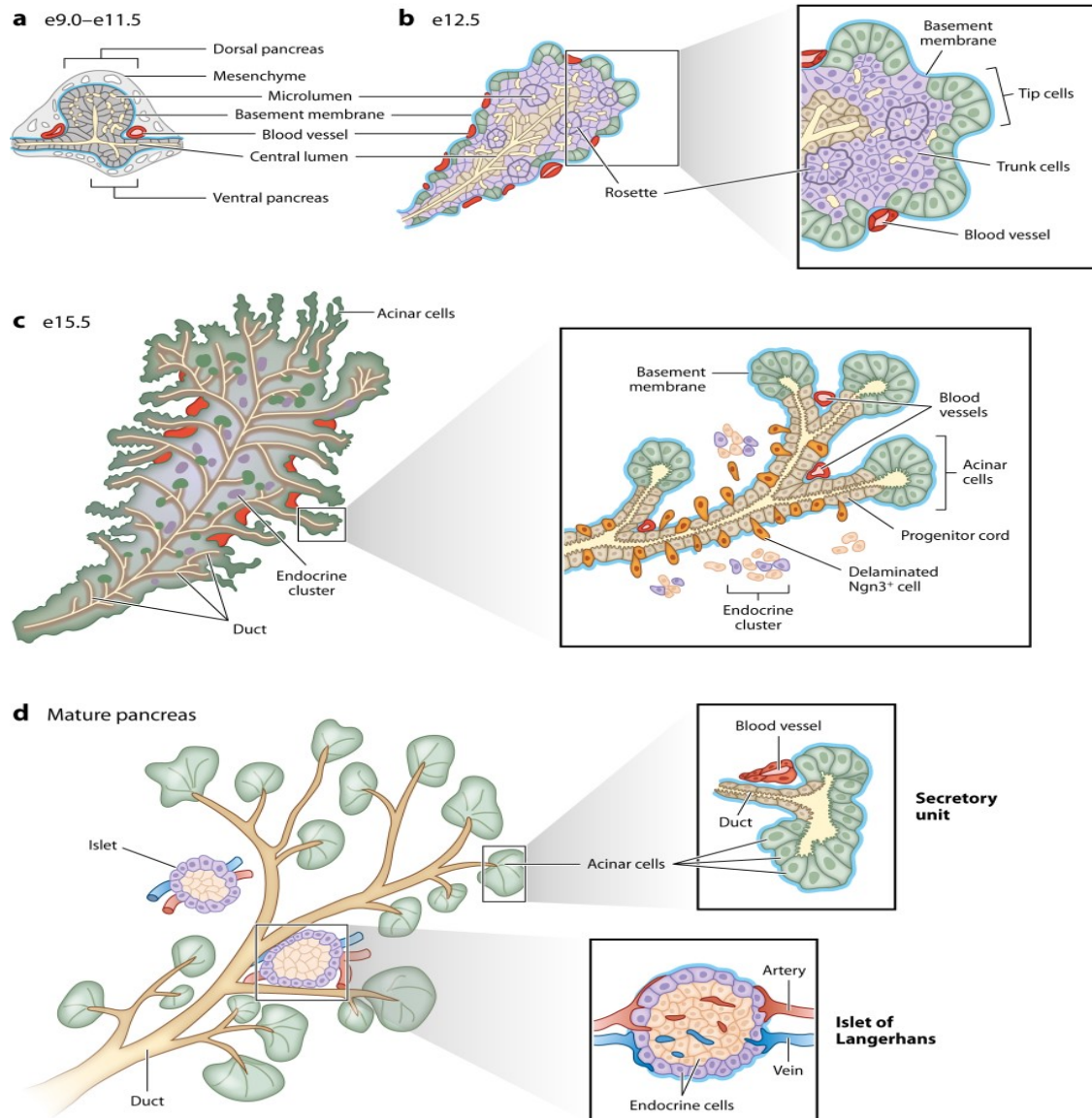


Figure 1.1 - Overview of organogenesis of the pancreas

(A) By embryonic days (e) 9–22.5 the dorsal and ventral pancreatic epithelium evaginate into the adjoining mesenchyme. The pancreatic epithelium is made up of a multi-layered core containing unpolarised cells that are submerged by the basement membrane. Blood vessels surround the epithelial buds but have not yet penetrated. (B) By e12.5, the outer tip (green) layer of the epithelium forms branch protrusions and the primitive plexus forms in the trunk where newly polarised cells are organised into rosettes surrounding the lumen (purple). At this point, blood vessels intercalate into the epithelium and come into contact with the cells at the trunk. (C) At e15.5, the luminal plexus becomes a single-layered epithelium which consists of branched primitive ducts and acinar cells. Ngn3-expressing endocrine precursors (orange) migrate from the progenitor cords and form the endocrine clusters. Blood vessels become intercalated between the pancreatic ductal tree and the nascent branches. (D) Mature pancreas: acinar cells cap the endings of small terminal ducts and form functional exocrine secretory units. Islets of Langerhans are formed and endocrine cells become clustered and penetrated by the a dense network of blood vessels [3].

1.1.3 Islets of Langerhans

The endocrine pancreas is formed by five different cell types aggregated to resemble 'islands', known as the "Islets of Langerhans", named after the German anatomist Paul Langerhans in 1869 [7]. These cell types are; the β -cells (65-80% of total islet cells) which produce the hormone insulin, the α -cells (15-20%), which produce the hormone glucagon, the δ -cells (3-10%) which produce the hormone somatostatin (3-10%) , the PP cells (3-5%) producing pancreatic polypeptides and the ξ -cells (<1%) producing the hormone ghrelin [8]. The structure of the pancreas and the Islets of Langerhans is illustrated in figure 1.2. Islets have a dense network of sinusoidal capillaries: larger islets receive blood from one to three afferent arterioles and the capillaries of smaller islets are integrated with exocrine capillaries [9]. Islets tend to have an oval or clover-leaf shape indicating a complex pattern of subunits. It has been suggested that in rodents blood flows from within the core of islets outwards going from β - to α - to δ - cells ("B-A-D" pathway) [9]. Whether this pathway exists in humans remains unclear, although it has been suggested that β -cells are perfused before non- β cells indicating an opposite path of microcirculation [10]. Other studies suggest that blood enters at the arterial pole of the islet and travels through the islet capillaries to the venous pole in humans [11]. As the different cell types in the islet are located in specific locations, the pattern of blood flow within the islet can impact on the ability of cells to communicate with each other within the islet [11].

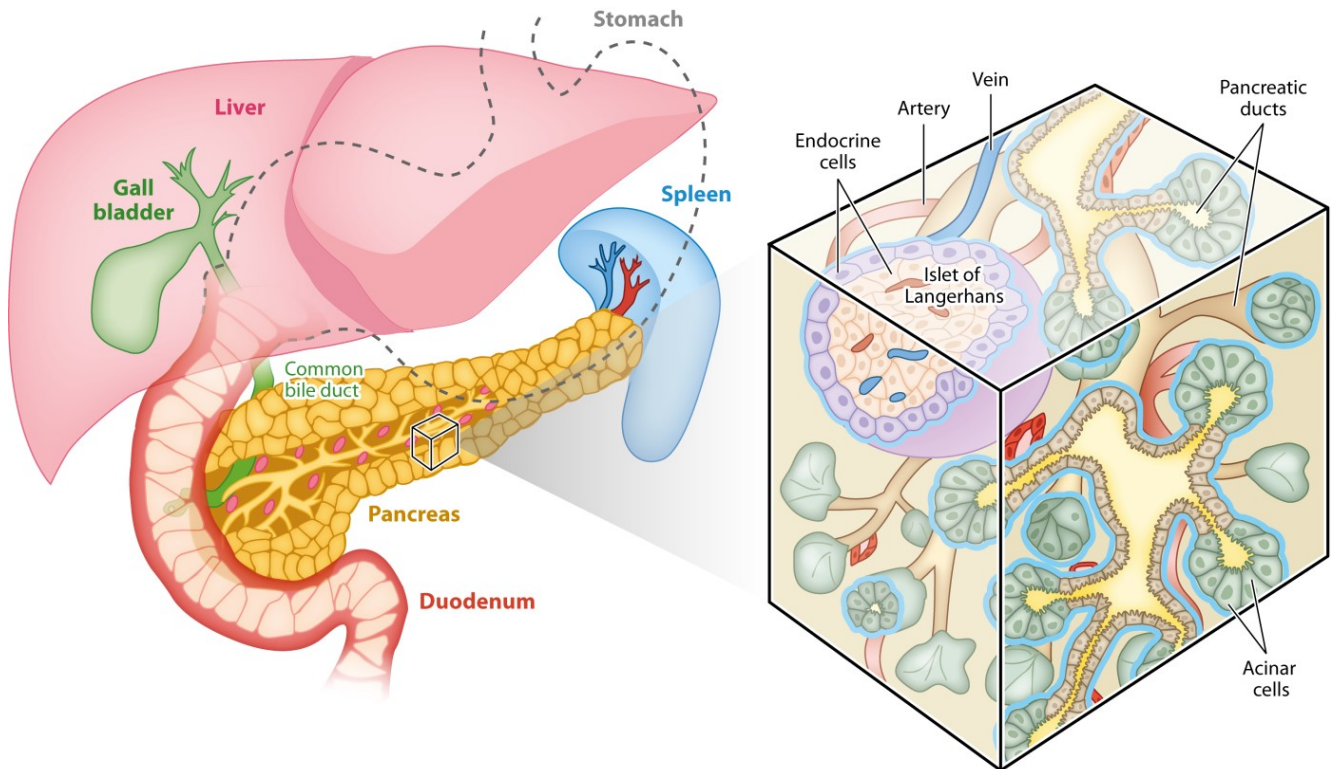


Figure 1.2 - The localisation and anatomy of the pancreas and the islets of Langerhans

In humans, the pancreas is situated behind the stomach (outlined with dashed line). Exocrine acinar cells secrete enzymes for the breakdown of carbohydrates, proteins and lipids and the endocrine pancreas contains clusters of cells known as the Islets of Langerhans (magnified section). These cells include, glucagon secreting α -cells and insulin secreting β -cells. Islets are surrounded by a thick network of capillaries where hormones are secreted into the blood stream [3].

1.2 *The β -cell and glucose stimulated insulin secretion*

1.2.1 *The pancreatic β -cell*

The primary function of the pancreatic β -cell is in the secretion of insulin in response to nutritional, hormonal and nervous stimuli in order to maintain homeostatic plasma glucose levels. This ensures that glucose is efficiently taken in and stored (as glycogen) by peripheral tissues (e.g. muscle, adipose tissue). The full mechanism of glucose-stimulated insulin secretion is explained in full later in this chapter. In brief, increasing glucose concentrations in the blood initiates the transport of glucose into the β -cell, stimulating the generation of ATP and Ca^{2+} influx into the cell via the closure of

voltage-gated K^+ channels and opening of Ca^{2+} channels. This Ca^{2+} influx stimulates insulin exocytosis from the β -cell [12].

Each individual β -cell contains 10,000-13,000 secretory granules [13] with an average diameter of 350nm. These granules contain an electron-dense Zn_2 -insulin₆ crystal, 10^5 mol of insulin [14] and roughly another 50 polypeptides. Some of these polypeptides will have biological functions, for example the islet amyloid polypeptide (IAPP), responsible for decelerating gastric emptying, and chromogranin A which promotes the generation of secretory granules. Several low-molecular weight compounds are stored in these granules such as adenosine triphosphate (ATP), γ -aminobutyric acid (GABA), serotonin and glutamate as well as metal ions including Ca^{2+} and Zn^+ [15].

1.2.2 *The insulin granule*

Insulin plays a fundamental role in glucose homeostasis throughout the entire body. In humans, insulin is synthesised as a 110 amino acid precursor, known as preproinsulin, in the rough ER of the β -cell [12]. After the removal of a 24 residue signal sequence and packaging in the golgi body, insulin becomes stored as proinsulin in immature secretory granules. Pro-insulin is converted into its mature form of insulin and c-peptide via Ca^{2+} -dependent endoproteases, proprotein convertases (PC)1-2 and the exoprotease, carboxypeptidase H (CPH), which is a carboxypeptidase B-type metalloprotease that removes the COOH-terminus from peptide precursors [16, 17]. This process of insulin granule maturity is illustrated in figure 1.3. Mature insulin can be stored for days before being secreted from the β -cell or degraded by crinophagia (destruction by lysosomes) [18]. The last of the granules to be generated are the ones most likely to be secreted when β -cells are stimulated, however the reason for this remains unclear [19, 20].

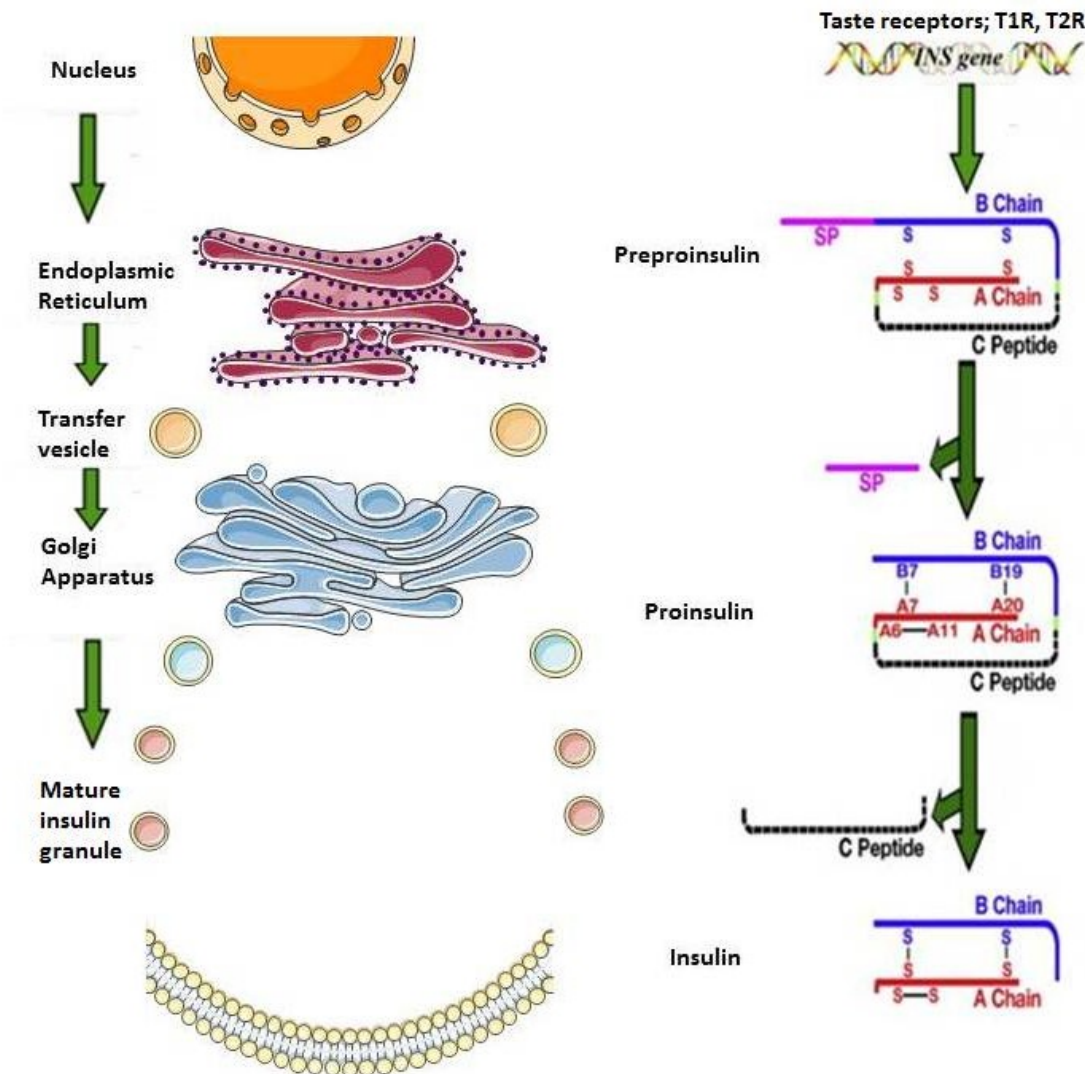


Figure 1.3 - Formation of preproinsulin and the insulin granule

Preproinsulin is converted to proinsulin after the removal of the N-terminal in the ER where it is folded and oxidised. Proinsulin is then transported via transport vesicle to the golgi apparatus where it is packaged into insulin storage granules. Protein convertase 1/2 and carboxypeptidase H cleaves proinsulin into mature insulin and C-peptide, ready for release upon stimulation. This figure was partially generated using "Servier Medical Art" (<http://www.servier.com/servier-medical-art>) [21].

1.2.3 Glucose sensing and phosphorylation in the β -cell

The study of glucose-stimulated insulin secretion (GSIS) from pancreatic β -cells was introduced in the 1960s after the production of insulin radioimmunoassays (RIA) [22]. Two hypothesis exist as to how GSIS occur; (1) putative cell surface receptor analogues will bind both hormones and neurotransmitters and (2) the model known as the "fuel hypothesis" which proposes that insulin secretion is mediated via the metabolism of sugar [23]. It is now largely believed that the latter hypothesis is the primary mediator of insulin secretion [24]. This theory is supported by the fact that

insulin secretion after a rise in blood glucose concentration is accompanied by elevated glucose utilisation and oxidation [25], and that the inhibition of glucose consumption results in the inhibition of insulin secretion [22]. Furthermore, non-metabolic glucose analogues are ineffective in stimulating insulin secretion, whilst metabolic amino acids, e.g. glutamate, leucine and certain branched chain amino acids such as hetero-isocaproate, are potential secretagogues [26].

Pancreatic β -cells express, predominantly, the glucose transporter isoform 2 (GLUT2) [27]. It remains contested whether other GLUT isoforms, such as GLUT1 or 3, play an equal or more important role than GLUT2 in human β -cells [28]. GSIS is initiated by the rapid movement of glucose across the β -cell membrane; phosphorylation, in comparison, is thought to be a slow and rate-limiting step [29]. Glucose is phosphorylated to form glucose-6-phosphate (G6-P) by glucokinase (GK), thereby promoting glycolysis [30]. GK is a type IV hexokinase [31] and presents strongly cooperative kinetics. Given its flux-generating role in β -cells glycolysis, GK has been dubbed “The β -cell glucose sensor”. Inactivation of the GK gene in β -cells in mice leads to impaired glucose sensitivity and decreased insulin release *in vivo* [32, 33]. In man, inactivating mutations in the GK gene leads to maturity-onset diabetes of the young (MODY2)[34]. The full process of GSIS in the pancreatic β -cell is represented in the schematic shown in figure 1.4 and described in more detail in later sections.

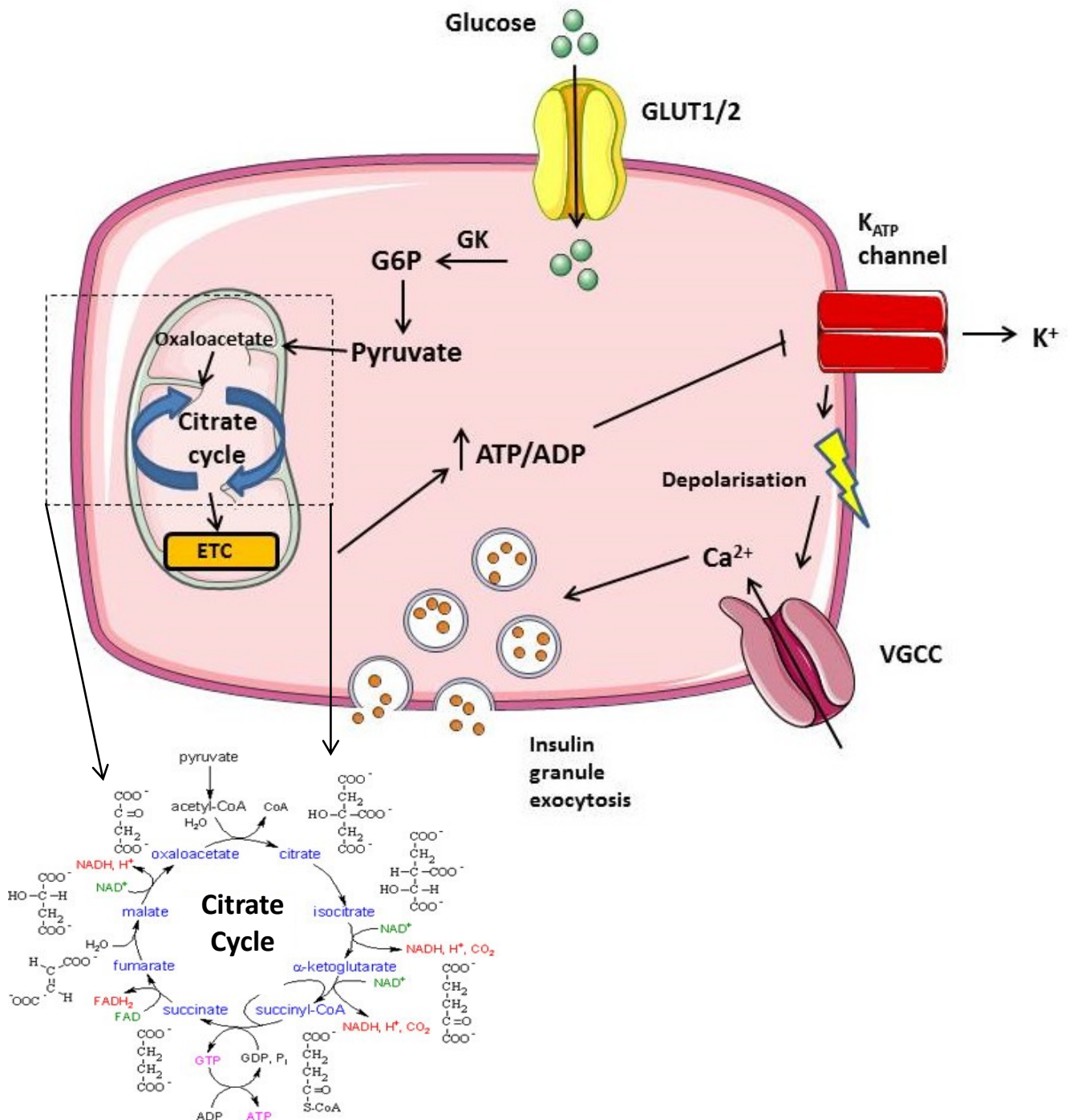


Figure 1.4 - GSIS of the pancreatic β -cell

Increasing concentrations of extracellular glucose initiate the transport of glucose into the cell through glucose transporter 1/2 and is phosphorylation to glucose-6-phosphate via glucokinase (1.2.3). After glycolysis, pyruvate enters the mitochondria where ATP is generated through the citrate cycle (1.2.5). This up-regulation of ATP in the cytosol results in closure of K_{ATP} channels and depolarisation of the cell (1.2.6). Voltage gated L-type Ca^{2+} channels will open, resulting in an influx of Ca^{2+} into the cytosol and triggering insulin exocytosis (1.2.7). The figure was generated using images from "Servier Medical Art". (<http://www.servier.com/servier-medical-art>).

1.2.4 *Biphasic insulin secretion*

Insulin secretion in response to high glucose concentrations takes place in a biphasic manner. The two stages consist of (1) transient secretion, (which is dependent on a rise in $[Ca^{2+}]_{cyt}$) causing a rapid and short insulin secretion and (2) a longer lasting second phase [35]. Additional signals are necessary for this second phase to occur, in particular an “amplifying pathway” (known as K_{ATP} -channel independent stimulation of insulin secretion) [36]. The second phase of insulin secretion involves the supply of new insulin granules for release by ATP- and time-dependent granule preparation and is a rate-limiting step of insulin exocytosis [37].

The exact mechanisms (and coupling factors) involved in the stimulation of the second phase of insulin secretion remains uncertain in both human and rodent β -cells [38]. As mentioned above, this phase exhibits a dependency on Ca^{2+} [39, 40] as well as a strong tetrodotoxin-sensitive sodium conductance, in humans from voltage-gated Na^+ ($Na_v1.6/Na_v1.7$)- channels which contribute to the firing of action potentials in β -cells [41].

1.2.5 *Metabolic activation of mitochondria*

ATP is the primary metabolic factor during GSIS, causing closure of K_{ATP} channels leading to Ca^{2+} elevation and insulin exocytosis [42]. Mitochondria generates metabolic signals and allows the generation of ATP which is necessary for the elevation of $[Ca^{2+}]_{cyt}$ and insulin secretion during processes known as the citrate/ krebs cycle and the electron transport chain (ETC). The role of mitochondria in β -cell stimulus-secretion coupling is discussed in more detail below.

1.2.5.1 The Krebs/ Citrate cycle

After glycolysis takes place in the cytoplasm (1.2.3) of pancreatic β -cells, pyruvate enters the mitochondria resulting in depolarisation of the mitochondrial membrane [43] (Figure 1.4). After its entry into the mitochondria, pyruvate loses either a carbon to generate acetyl-CoA or gain a carbon to form oxaloacetate (catalysed via pyruvate dehydrogenase (PDH) [44] and pyruvate carboxylase (PC), respectively [45]). 4-carbon oxaloacetate then enters the krebs/ citrate cycle where citrate is generated via condensation of the 2-carbon acetyl group of acetyl CoA catalysed by citrate synthase [45].

1.2.5.2 The Electron transport chain

The citrate cycle extracts reducing equivalents from the metabolic intermediates of this cycle, using these to generate NADH and $FADH_2$ for oxidation by the ETC. The ETC consists of five complexes such as; Complex I (the electron acceptor/ NADH dehydrogenase), Complex II (succinate dehydrogenase) which transfers electrons to coenzyme-Q from $FADH_2$, and Complex V which catalyses ATP formation. ATP and glycerol 3-phosphate dihydrogenase is then translocated to the cytosol via the adenine

nucleotide translocator (ANT) in exchange for adenosine diphosphate (ADP). NADH electrons are transferred to the ETC to supply the energy needed for the proton electrochemical gradient that drives ATP synthesis. Enhanced NADH generation during GSIS thus follows increased glucose metabolism and results in mitochondrial hyperpolarisation [46]

Complex 1 causes continued reoxidation of mitochondrial NADH to NAD^+ to maintain a reduced redox state in order to maintain pyruvate input to the citrate cycle during glucose stimulation. However, complex I activity is restricted by the inherent thermodynamic constraints of proton gradient formation [47] and so additional NADH are reoxidised via other dehydrogenases such as through cataplerotic functions (confirmed by use of NMR spectroscopy) [48].

The reducing equivalents cause hyperpolarisation of the mitochondrial membrane as well as generation of ATP. Electrons are transferred by NAD^+ and FAD, in the mitochondrial membrane of note, several citrate cycle dehydrogenases are activated via Ca^{2+} [49]. This stimulation may contribute to the sustained stimulation of ATP synthesis and insulin release [50].

1.2.6 *ATP-sensitive K^+ channels*

The ATP-sensitive K^+ channel (K_{ATP}) is a hetero-octameric protein comprising a channel-forming subunit ($\text{K}_{\text{ir}6.2}$) and a larger transmembrane protein which is part of the “ABC cassette family”. These subunits are encoded in β -cells by the K^+ channel, Inwardly Rectifying Subfamily J, Member 11 (*KCNJ11*) and the sulphonylurea receptor 1 (*SUR*) genes [51-53]. When glucose levels in plasma are low, K_{ATP} channels are open, thereby allowing K^+ to exit the cell and maintaining resting membrane potential at a hyperpolarised level ($\sim -70\text{mV}$) [54].

Generation of ATP from the mitochondria results in closing K_{ATP} channels and suppresses K^+ ion efflux. This leads to depolarisation of the plasma membrane [55] and the firing of action potentials resulting in a decrease in voltage and the opening of voltage-sensitive, L-type Ca^{2+} channels. This, in turn, will increase cytosol free Ca^{2+} ($[\text{Ca}^{2+}]_{\text{cyt}}$) concentrations [56]. The latter surge in $[\text{Ca}^{2+}]_{\text{cyt}}$ concentrations is the main trigger for insulin exocytosis (1.2.7) [57].

As well as pancreatic β -cells, K_{ATP} channels have been found in a variety of cell types including pancreatic α -cells, intestinal L-cells, cardiac muscles, brain, kidneys, skeletal and smooth muscles [55]. Therefore, any mutations in K_{ATP} channels may affect multiple systems leading to diseases such as Diabetes, Epilepsy and Neurodevelopment [58].

In β -cells with mutations in *SUR1* (and therefore lacking K_{ATP} channels), glucose readily promotes insulin release and there was no apparent defects in K^+ or Ca^{2+} voltage-gated channels [59]. Which

indicates that the sustained release of insulin depends particularly on the activation of K_{ATP} channels, but is dependent on metabolic factors. Nonetheless an elevation of $[Ca^{2+}]_{cyt}$ is required to observe significant rates of insulin release [36].

1.2.7 *Insulin exocytosis*

Exocytosis of insulin from the β -cell is dependent on increases in $[Ca^{2+}]_{cyt}$. L-type Ca^{2+} channels form tight complexes with the secretory granule to ensure high exposure of $[Ca^{2+}]_{cyt}$ [60]. However, low-affinity Ca^{2+} sensor allows exocytosis when $[Ca^{2+}]_{cyt}$ levels are relatively low [61, 62].

The process of insulin release from their secretory vesicles relies on intracellular ATP and the translocation of vesicles to the cells surface before they fuse with the plasma membrane. The pancreatic β -cell is of neuroendocrine origin, therefore exocytosis of insulin bears a strong resemblance to the release of neurotransmitters in synapses and many SNAP (Soluble NSF Attachment Protein) receptor (SNARE) proteins which are vital for synaptic vesicle release are also expressed in the β -cell [63]. This includes the t-SNAREs syntaxin 1 and synaptosomal-associated protein (SNAP)-25 [64] which concentrate in cholesterol-rich membrane regions, causing removal of cholesterol from the plasma membrane using methyl- β -cyclodextrin (MBCD) inhibits insulin secretion and exocytosis [65]. Phosphorylation of SNAP-25 via protein kinase C (PKC) has been found to induce exocytosis in insulin-secreting cell lines [66]. High concentrations of cyclic adenosine monophosphate (cAMP) ($<10 \mu M$) has been found to enhance insulin exocytosis through a PKA-independent mechanism [67, 68], therefore SNAP-25 plays a key role in the adjustment of β -cell exocytosis over a range of cAMP concentrations [57].

As mentioned above, GSIS occurs in two phases, during the first phase of insulin secretion, granules are pre-docked on the plasma membrane in what are referred to as the 'readily releasable pool' (RRP) and are fused rapidly (Figure 1.5). Granules deeper within the cell, known as the 'storage granule pool's move to the periphery of the cell in order to replenish RRP at the cells surface [69, 70]. The RRP make up a small pool, approximately 50-200 β -cells and $<10,000$ mature insulin granules [71]. Replenishment of RRP takes place during the first phase with the mobilisation, docking and priming of previous non-RRP to support the second phase of insulin secretion [69]. Note, however, that this relatively simple model based in part on electrophysiological approaches including capacitance measurements [72] has been questioned by measurements using total internal reflection of fluorescence (TIRF) measurements which suggest that "deeper" granules also contribute to first phase insulin secretion [73].

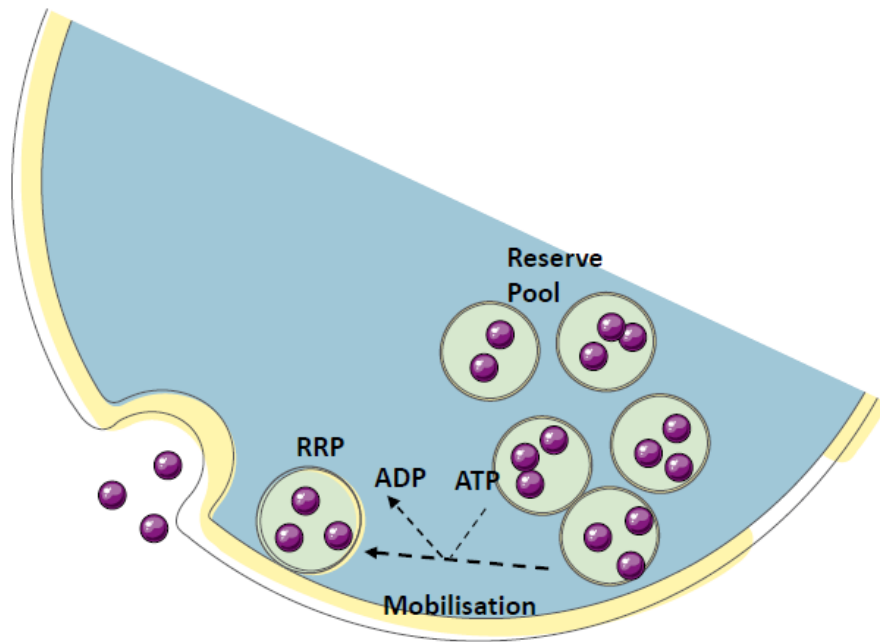


Figure 1.5 – Insulin exocytosis from the β -cell

During the first phase of insulin secretion, insulin granules are pre-docked on the plasma membrane, these granules are known as the readily releasable pool (RRP) and fuse rapidly with the plasma membrane before insulin secretion via exocytosis [72].

1.2.8 Disallowed genes

Several β -cell specific genes are essential for β -cell function and GSIS, when mutated lead to abnormalities in insulin secretion and some forms of diabetes. Examples of these genes include; GK [74], pancreatic duodenum *homeobox-1* (PDX1) [75] and ATP-binding cassette transporter sub-family C member 8 (ABCC8) [76]. These genes are known as “allowed/ β -cell specific genes”. In recent years, it has become apparent that alongside the expression of these “allowed/ β -cell specific genes” the repression or silencing of certain genes known as “disallowed genes” [77] which are normally expressed in all other cell types are important for normal β -cell action .

Examples of “disallowed genes” include; monocarboxylate/ lactate transporter (MCT-1) [78] and lactate dehydrogenase (LDHA) [79]. Expression of these genes is reduced in β -cells in comparison to α -cells and the liver. Both these enzymes play key “housekeeping” roles in the majority of cell types, therefore it seems likely that their suppression in β -cells is a requirement for normal GSIS and/or β -cell survival [80]. In other tissues MCT1 and LDHA are vital for anaerobic production of lactate and is therefore critical for cell survival when at low oxygen concentrations [81]. Due to the absence of these enzymes from the β -cell, compensatory mechanisms must be present to ensure that glucose is sufficiently oxidised and circulation of metabolites, such as lactate, cannot stimulate insulin release

[80]. Expression of glycerol phosphate dehydrogenase which creates an alternative metabolic fate (via mitochondrial oxidation at complex II of the respiratory chain reaction) for NADH production during glycolysis [82, 83] was found in β -cells. As well as in β -cells, low expression of *LDHA* was also found in other oxidative cell types such as insect flight muscle cells, emphasising the likely role of oxidative metabolism and ATP production in coupling glucose metabolism to insulin secretion [84]. Studies have confirmed that β -cells produce very little pyruvate [45, 83] and that the CO_2 needed for lipid and amino acid synthesis is obtained via pyruvate carboxylase, acting to “top-up” the citrate cycle [80].

Overexpression of *Mct-1* and *Ldha* selectively in β -cells has been found to greatly impair GSIS in both MIN6 cells and isolated islets [85-87], supporting the importance of these genes. In Type 2 Diabetes, up-regulation of disallowed genes were identified in β -cells [88] for example, Acyl-coA thioesterase (Acot) 7 and Nuclear factor I/B (Nfib) [80]. Furthermore, up-regulation of several disallowed genes has been observed in β -cell enriched tissue for Type 2 Diabetic patients [89]. Modulation of some of these genes may provide a new therapeutic target for some forms of Type 2 Diabetes as well as contribute to the development of new sources of transplantable β -cells for Type 1 Diabetes.

1.2.9 Type 2 Diabetes

Both Type 1 and 2 Diabetes are diseases associated with malfunctions of the endocrine pancreas resulting in uncontrolled hyperglycaemia. Whilst the development of Type 1 Diabetes is due to autoimmune destruction of pancreatic β -cells, Type 2 Diabetes occurs during early insulin resistance and the failure of β -cells to compensate for the resulting hyperinsulinemia. The most successful therapy for uncontrolled hyperglycaemia is the use of insulin injection in Type 1 diabetes and in the later stages of Type 2 diabetes. Insulin was first introduced as a treatment in 1922 by Frederick Banting and Charles Best. However, a permanent “cure” for diabetes is yet to be discovered. In the early stages of Type 2 Diabetes disease progress can be delayed with exercise and improved diet.

It is believed that several factors including genetic components, the fetal environment and the nutritional environment in early life are all critical factors affecting the functional β -cell mass and susceptibility to diabetes in adult life. Obesity-induced insulin resistance occurs when insulin demand increases, resulting in an increase in insulin secretion and β -cell mass. Chronic exposure of hyperglycaemia to β -cells leads to the loss of β -cell function and dedifferentiation due to glucolipotoxicity and β -cell death [90]. Mechanisms for measuring β -cell mass *in vivo* remain to be established [91, 92]. Therefore, measurements rely chiefly on histological analysis of pancreas obtained post mortem. As insulin resistance and hyperinsulinemia are enhanced in patients with Type 2 Diabetes it is assumed that β -cell mass will also increase. However, histological analysis has shown that β -cell mass decreases by approximately 65% in obese patients with Type 2 Diabetes in comparison

to non-diabetics with matching body mass index (BMI) [93, 94]. Apoptosis of β -cell is enhanced in Type 2 Diabetes [95], whereas β -cell replication and neogenesis is unchanged, indicating that the main cause of declining β -cell mass is β -cell loss.

Various mechanisms that initiate β -cell loss have been suggested for this reduction in β -cell mass such as hyperglycaemia (glucotoxicity), fatty acids (lipotoxicity), oxidative stress, mitochondrial dysfunction, ER stress and dedifferentiation of β -cells.

1.2.9.1 Mechanisms of β -cell loss

Chronic hyperglycaemia (glucotoxicity) leads to enhanced glucose metabolism via oxidative phosphorylation, causing mitochondrial dysfunction and the production of reactive oxygen species (ROS) which may damage cell structure [96].

Due to the abundance of ROS in the islet in response to high concentrations of glucose, and the low expression of anti-oxidant enzyme defence mechanisms, β -cells are highly susceptible to oxidative stress. Thus antioxidant enzymes such as, superoxide dismutase 1 and 2 (SOD1-2), glutathione peroxidase 1 (GPX1) and catalase (CAT) are more weakly expressed in islets in comparison to other tissues [97]. In addition, islets have a poor DNA repair capacity against oxidative stress [98]. In patients with Type 2 Diabetes, markers of oxidative stress were found to be significantly up-regulated in comparison to healthy controls, levels of expression of these markers positively correlate with the degree of impairment in insulin secretion [99, 100].

Oxidative stress is a critical factor in the induction of β -cell dysfunction. Isolated islets exposed to high glucose concentrations resulted in an increase in intracellular levels and reduction of insulin content and secretion [101]. In contrast, β -cell function in the leptin deficient db/db mouse model and islets isolated from diabetic mice when treated with antioxidant agents was found to improve function [99, 100].

The mechanism by which ROS reduce β -cell mass and function remains unclear. Oxidative stress results in a decrease in the transcription of the insulin gene by decreasing MaFA and PDX1 binding activity to the insulin gene [102]. ROS will also activate stress inducing pathways such as, NF- κ B, JNK and mTOR pathways which affect insulin gene expression [103]. Oxidative stress can also lead to the induction of ER stress [104] and trigger the formation of the inflammasome complex.

These studies indicate that ROS play an important role in the impairment of β -cell function during hyperglycaemia. However, it is important to note that ROS may also be required for normal β -cell

function. Low concentrations of H_2O_2 in INS1 cells and mouse islets increase GSIS at low concentrations of glucose [105].

1.2.9.2 Treatment and prevention of Type 2 Diabetes

The best proposed treatment for Type 2 Diabetes emphasises on improving the lifestyle of the patient by reducing body weight which in turn cuts down on the β -cell workload. Prior to the introduction of metformin treatment in 1995 (which prevents hepatic gluconeogenesis), the only oral anti-hyperglycaemia drugs available which target the β -cell were sulfonylureas. Sulfonylureas work by binding to the SUR1 on the β -cell resulting in closure of K^+_{ATP} channels and subsequent insulin secretion [106]. There are indications that sulfonylurea treatment may be associated with heart failure since patients on high doses of sulfonylurea were admitted to hospital for heart failure more than patients receiving metformin treatment [107].

Glucose like peptide (GLP)-1 analogues have been found to up-regulate GSIS and β -cell mass in rodents by activating the mitogen activated protein kinase (MAPK) and PKC pathways inducing β -cell proliferation [108]. In human islets GLP-1 inhibits β -cell apoptosis [109], however has no effect on β -cell mass. GLP-1 is discussed in more detail in section 1.7 later in this chapter.

A greater understanding of the mechanisms and function of GSIS and β -cell mass are needed to develop more efficient targets for treating Type 2 Diabetes.

1.3 *Glucagon and the α -cell*

Glucagon is a 29-amino acid peptide hormone is secreted by the pancreatic α -cell [110]. Glucagon has a strong stimulatory effect on hepatic glucose production, resulting in a surge in blood glucose levels [111] and acts as a counter-regulatory hormone to insulin. The balance between insulin and glucagon is important for normal glucose homeostasis [112]. Dysfunction in this balance may lead to some forms of diabetes.

1.3.1 *Discovery and production*

The term hyperglycaemia was defined in 1922 after secretion extracts were taken from the pancreas and an unknown substrate separated, this substrate was injected into dogs resulting in a surge of blood glucose. These finding lead to the discovery of a second pancreatic hormone which was later termed glucagon [111]. Glucagon, GLP-1, GLP-2 and glicentin are all encoded by the preproglucagon gene. The structure of this preproglucagon gene was established in 1983 [113], with 160 amino acids and it is expressed predominantly in the central nervous system (CNS), L-cells and pancreatic α -cells.

In α -cells, maturation of the preproglucagon hormone occurs by a post-translational cleavage by prohormone convertase (PHC) 2 [114]. The main products of this cleavage are glucagon and glucagon related polypeptides [115].

1.3.2 *Glucagon in development*

α -cell granules containing glucagon, are formed in the pancreas during evagination from the foregut [116] and are similar to that of the adult pancreas [117], suggesting that glucagon plays a role in growth and differentiation of early embryonic development [116]. Evidence for this has been obtained in *in vitro* studies of cultured pancreas suggesting that glucagon is needed in the early formation of insulin cells (E11-13) but not in the later E15 pancreas.

1.3.3 *Glucagon secretion from the α -cell*

Glucose transporter 1 (*GLUT1*) mRNA and protein has been detected in rodent α -cells, rather than *GLUT2* which is chiefly expressed in β -cells [118]. Overall, rates of glucose transport are 10-fold lower in α - than β -cells [118]. Activity of GK in α -cells is similar to that of β -cells with its main role being as a metabolic glucose sensor [119]. Hexokinase IV is also expressed in α -cells and is involved in the sensing of low glucose concentrations [119]. Glycolytic flux in the β -cell is aerobic but is anaerobic in the α -cell [45]. Therefore, glucose induces smaller changes in cytoplasmic ATP [120-122], NADPH [123] and FAD [124] in α - than β -cells. However, glucose metabolism is necessary for glucose recognition as glucose-inhibited glucagon release is mimicked via a GK activator, and secretion is not affected by a non-metabolisable glucose analogue [125].

In the absence of glucose, α -cells become electrically active and display Ca^{2+} oscillations which initiate glucagon exocytosis. Action potentials start at more negative potentials in α - than in β -cells due to the presence of T-type Ca^{2+} channels, which activate at -60mV and tetrodotoxin (TTX)-sensitive Na^+ channels that activate at potentials more positive than -30mV [126, 127]. As with β -cells, the action potential in α -cells leads to the opening of L- and N-type Ca^{2+} channels [128]. These channels contribute to the majority of Ca^{2+} entry into α -cells. However, the relationship between cytosolic $[\text{Ca}^{2+}]_{\text{cyt}}$ and glucagon secretion in α -cells is less clear than for insulin secretion in β -cells. These channels in the α -cell are illustrated in Figure 1.6.

In rodents, L-type Ca^{2+} channels act as the dominant channel (80%). However when these channels are inhibited in α -cells, there is little effect on glucagon secretion [128]. By contrast, inhibitors of non-L-type channels despite having little effect on $[\text{Ca}^{2+}]_{\text{cyt}}$ inhibit glucagon secretion as efficiently as if glucose was elevated to 6mM [128-130]. This may be due to the close association between non-L-type Ca^{2+} channels and the secretory granules [131]. In human α -cells, non-L-type channels open very

briefly and mediate only a small fraction of Ca^{2+} entry [132]. In the presence of adrenaline, Ca^{2+} entry into the α -cell occurs through L-type channels and strongly triggers glucagon secretion [130, 133]. Importantly, glucose inhibition of glucagon secretion is associated with small changes of $[\text{Ca}^{2+}]_{\text{cyt}}$ signalling [123] suggesting that other signals or microdomains of $[\text{Ca}^{2+}]$ are important in triggering exocytosis in these cells.

1.3.3.1 K_{ATP} channel-dependent glucose sensing

It has been suggested that an increase in glucose concentration in the α -cell increases $[\text{ATP}/\text{ADP}]_{\text{cyt}}$ resulting in K_{ATP} channel closure. However, unlike the situation in β -cells, depolarisation causes inhibition of secretion from α -cells. K_{ATP} channel conduction is low when α -cells are exposed to high glucose concentrations. High threshold voltage-dependent channels will only trigger exocytosis if low-threshold voltage-dependent channels are opened. These low-threshold voltage-dependent channels undergo inactivation upon sustained depolarisation [123, 126]. Therefore, closure of K_{ATP} channels in response to glucose-induced ATP formation leads only to a small degree of depolarisation, which is unable to open voltage-dependent Ca^{2+} channels. Instead this sustained depolarisation inactivates low threshold T-type Ca^{2+} and Na^+ channels.

In the above model, change in glucagon secretion depends on the frequency and amplitude of action potentials and implies that these cause glucagon secretion under a narrow window of membrane potentials. Action potentials are prevented by sustained depolarisation which inactivates the T-type Ca^{2+} channels. K_{ATP} channel closure, and slight depolarisation, at high glucose mimic the inhibitory effect of glucose [129, 134], and weaker hyperpolarisation with low concentrations of the K_{ATP} channel opener diazoxide causes glucose inhibition of glucagon secretion. Additionally, more prominent hyperpolarisation inhibits glucagon secretion [129, 135]. Notably, the inhibitory effect of glucose is lost following functional knock-out (KO) of the K_{ATP} channel [134, 135].

Although quite widely accepted, there is evidence that question this model of K_{ATP} channel dependent glucagon secretion. For example, though glucose decreases $[\text{Ca}^{2+}]_{\text{cyt}}$ in α -cells, several studies show that K_{ATP} channel closure by sulfonylureas leads to β -cell like $[\text{Ca}^{2+}]_{\text{cyt}}$ elevations instead of the expected decrease [123, 125, 136, 137]. Also, glucose maintains its inhibitory effect on glucagon secretion when K_{ATP} channels are closed in the presence of high sulfonylurea concentrations [138] and this is associated with a lowering of $[\text{Ca}^{2+}]_{\text{cyt}}$ [123, 138]. Glucose was found to maintain its inhibitory effect on glucagon secretion in islets blocked for the K_{ATP} channel using tolbutamide [125, 139]. Tolbutamide prevents glucagon secretion via two mechanisms 1) direct stimulation of α -cells and 2) indirect inhibition of somatostatin secretion from δ -cells [140]. Thus, tolbutamide effect is reinforced

by the disruption of somatostatin signalling which eradicates its inhibitory effect on glucagon secretion [125].

The fact that glucose depolarises the α -cell has also been met with disagreement. Electrophysiological measurements have suggested that both depolarising [134] and hyperpolarising [126, 141, 142] effects occur. This may be related to the increased input resistance of small α -cells [126], therefore the potential is sensitive to small currents which may be due to the invasive approaches carried out on these cells. When using dye-based techniques that are not invasive, α -cells were found to hyperpolarise after glucose stimulation [143]

1.3.3.2 Ca^{2+} dependent glucose sensing

As discussed above, lack of glucose results in the opening of voltage-dependent Ca^{2+} channels and the entry of Ca^{2+} that will trigger glucagon secretion from α -cells [126, 137]. In guinea-pig α -cells, adrenaline causes mobilisation of glucose-incorporated Ca^{2+} due to ATP-energised Ca^{2+} sequestration into the ER which lowers $[\text{Ca}^{2+}]_{\text{cyt}}$ [144]. However, the ER has a limited capacity for Ca^{2+} sequestration suggesting that use of uptake and release is only temporary.

Due to this temporary nature of the above mechanism, a store-operated mechanism made of Ca^{2+} influx is involved. Thus, Ca^{2+} store depletion has been suggested to trigger depolarisation and lead to Ca^{2+} influx. This depolarising current is minor causing only moderate $[\text{Ca}^{2+}]_{\text{cyt}}$ elevation [137, 145, 146] and nonetheless activation of this current after sarco(endo)plasmic Ca^{2+} ATPase (SERCA-pump) inhibition exerts a pronounced effect on $[\text{Ca}^{2+}]_{\text{cyt}}$ in α -cells [137], resulting in glucagon release but minor changes in β -cells [138, 147]. The higher sensitivity to SERCA subunit of α -cells is probably due to their higher input resistance [137], causing the membrane potential to be more sensitive to small currents which can trigger voltage-dependent Ca^{2+} influx. In hypoglycaemic conditions, the α -cell ER contains little Ca^{2+} and the store-operated pathway depolarises the cell stimulating glucagon release. In normo-glycaemic conditions, the SERCA pump becomes energised to fill the ER and prevent this stimulatory cascade [148].

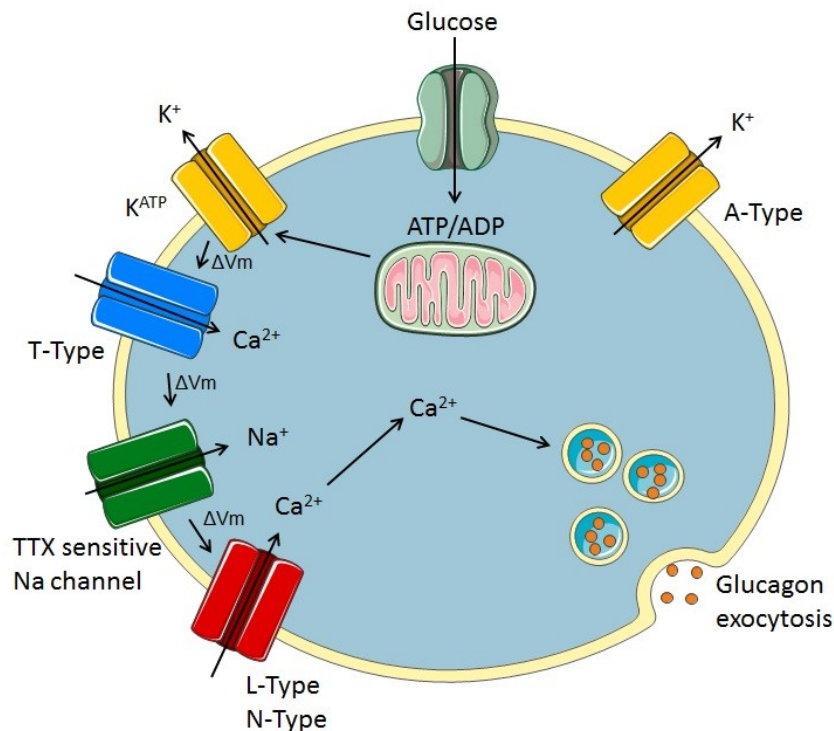


Figure 1.6 - Glucose stimulated glucagon secretion from the pancreatic α -cell

When extracellular concentrations of glucose increase, the α -cell becomes electrically inactivated after closure of K_{ATP} channels results in the opening of T-type Ca²⁺ and tetrodotoxin (TTX)-sensitive Na⁺ channels, suppressing action potentials, this activates L- and N- type Ca²⁺ channels (1.3.3). Closure of, the latter, channels restrict Ca²⁺ influx into the cell which in turn inhibits glucagon secretion (1.3.2.2).

1.3.4 Glucagon secretion during hyperglycaemia

1.3.4.1 Reduced inhibition of glucagon secretion

Glucagon secretion is inhibited when glucose rises to 7-8mM [149, 150]. It has been suggested that this inhibition significantly decreases at higher concentrations of glucose in both mouse [150] and human [150] islets. Indeed, previous reports have indicated that glucagon secretion may in fact become accelerated at glucose concentrations above 20mM in mouse [125] and rat [151] islets. This diminished inhibition may be due to the presence of a strong stimulatory component which may overcome the inhibition at 7-8mM glucose as well as any additional inhibition from paracrine factors when β - and δ - cells become stimulated at high glucose concentrations. It is possible that the latter stimulation of glucagon secretion occurs via mechanisms that mirror β -cells [152, 153]. It has also been suggested that because 20-30mM glucose elevates cAMP levels in α -cells [154], cAMP acts as a stimulatory messenger for glucagon secretion [128, 136].

1.3.4.2 Pulsatile glucagon release during hyperglycaemia

During hyperglycaemia, β - and δ - cell secretion becomes stimulated and inhibitory paracrine factors are activated which affect glucagon secretion. Experiments looking at islet hormone secretion kinetics from both mouse [155] and human [156] islets have supported this. Insulin, glucagon and somatostatin secretion occur at stable rates at 3mM glucose. However, at 20mM glucose, hormone secretion becomes pulsatile with insulin and somatostatin in phase and glucagon in antiphase [155].

Though individual α - and δ -cells have been shown to display oscillations in $[Ca^{2+}]_{cyt}$ at 3mM glucose [157, 158], hormone secretion in whole islets occurs without pulsatility [155, 156]. This is due to the fact neither cell type are electrically coupled and show asynchronas behaviour within islets [159]. Pulsatile insulin, glucose and somatostatin secretion at high concentrations of glucose indicates that paracrine factors which originate from β -cells determine the pulsatility of somatostatin and glucagon secretion. A similar relationship between the pulsatile release of insulin and glucose was found in humans [160] where pulsatile delivery of insulin and glucagon have greater effects in controlling endogenous glucose production than continuous infusion [161]. Though an increase in glucose from 3 to 20mM inhibits time-average glucagon release from islets, the secretion peak exceeds secretion at lower concentrations of glucose. This highlights the stimulatory component and the effect of glucose. Because insulin and glucagon pulses in the opposite phase, the insulin/glucagon ratio (to determine the storage and release of glycogen from the liver) varies 20-fold [155, 156]. This results in the liver switching between the storage and release of glycogen during hyperglycaemia, the overall effect being storage. The reason why energy is spent on this cyclic mechanism remains unclear. This inverse pulsatile secretion of insulin and glucagon is not seen in patients with Type 2 Diabetes and hyperglucagonemia leading to the conclusion that loss of pulsatile secretion contributes to the impaired glucagon release found in diabetic patients [160].

1.3.5 *Influence of glucagon in Type 2 Diabetes*

In the 1970s it was suggested that glucagon is a pathophysiological factor in Type 2 Diabetes leading to the suggestion that Diabetes is a “bi-hormonal disease” [162]. Evidence for this was provided by the observation that patients with Type 2 Diabetes show higher baseline levels of glucagon both before and after a high in carbohydrate meal in comparison to non-diabetic participants [163]. Hyperglycaemia in patients with Type 2 Diabetes thus may be due to insulin resistance in α -cells, resulting in them being less responsive to the inhibitory action of insulin and/or due to defective suppression of glucagon secretion at high concentrations of glucose. However, the exact mechanisms underlying this remain uncertain [164]. More recently it has been demonstrated that impaired suppression of glucagon secretion is more evident after oral rather than intravenous glucose administration, suggesting that the mechanism is related to incretin hormones such as gastric

inhibitory polypeptide (GIP) and GLP-1 (discussed more in section 1.7) released after oral administration of glucose [165].

Glucagon action and α -cell number in Type 2 Diabetes appears to be unaffected [166, 167]. Of note, it has been suggested that glucagon plays an initial role in the development of Type 1 Diabetes, since glucagon receptor KO mice did not develop Diabetes after streptozotocin injection [168]. Questioning this view, diabetes ensues in man after surgical removal of the pancreas, whereas both insulin and glucagon are lost [169].

1.3.6 *Glucagon as a therapeutic target*

The roles of glucagon in controlling circulating glucose levels during both fasting and postprandial conditions and during hyperglycaemia have made glucagon a potential target for glucose-lowering therapies in healthy subjects and diabetic patients. The potential utility of this strategy is also supported by the improvement of hyperglycaemia resulting from GLP-1 receptor agonism and dipeptidyl peptidase (DPP)-4 inhibition (that prolong the incretin effect) [170, 171]. Treatment by suppressing the action of glucagon using immunoneutralization of glucagon, glucagon receptor antisense and glucagon receptor antagonists appears to be successful in the experimental settings however is yet to be used clinically [172]. Indeed important contraindications include hepatic lipid accumulation and steatosis [173]. The use of GLP-1 and its receptor as a therapeutic target is discussed in detail later in this chapter.

1.4 *Glutamate signalling*

Glutamate (L-glutamic acid) is a multifunctional amino acid present in food either in a free form or within peptides and de-protonated proteins. Despite being abundant in food, concentration of glutamate in the blood is low as glutamate becomes oxidised in the small intestines [174]. As well as being a building block for proteins [175], glutamate acts as a major neurotransmitter [176], a precursor of the citrate cycle [175], an intracellular messenger [177], and an inducer of taste [178].

In pancreatic β -cells and the inhibitory neuro-transmitting GABAergic neurones, intracellular glutamate can be transferred into γ -amino butyric acid (GABA) by the enzyme glutamic acid decarboxylase (GAD), also an autoantigen in T1D [179]. Gain-of-function mutations in glutamate dehydrogenase (GDH) leads to hyperinsulinemic syndrome [180] suggesting that in β -cells intracellular glutamate acts as a secondary messenger for GSIS [177].

There are three types of glutamate receptors: (i) ionotropic N-methyl-D-aspartate (NMDA); this receptor is doubly-gated and requires ligand binding and membrane depolarisation for activation [181], (ii) ionotropic α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor for rapid synaptic transmission; responses to the agonist kainite act through this receptor [182], [183], and (iii) the metabotropic glutamate synthetic receptors (mGluRs) which are G-protein coupled involved in slow synaptic transmissions [184]. Ionotropic and metabotropic glutamate receptors are found primarily in the brain but are also expressed in peripheral tissues such as pancreatic β -cells [185] and the liver. Glutamate signalling in these tissues play a key role in the control of metabolic homeostasis [186]. Glutamate influences both glucagon and insulin secretion from pancreatic α - and β -cells, respectively [187, 188].

1.4.1 *Glutamate-stimulated insulin secretion*

Early experiments performed of glutamate signalling in the β -cell failed to find an effect on insulin release from mouse pancreatic islets [183]. However, these studies involved long lasting static experiments may have other effects on secretion [183]. In studies performed on the isolated perfused rat pancreas, glutamate-dependent stimulation of insulin secretion via the AMPA receptor was observed at high concentrations of glucose, but not at low concentrations. This shows that glutamate acts as a potentiator of GSIS [183].

Ionotropic glutamate receptors in neuronal cells were found to be functionally expressed in MIN6 β -cells, suggesting that these receptors have a physiological role in islets [189]. RT-PCR revealed several functional subtypes of AMPA/kainite and NMDA expressed in β -cells [185].

mRNA encoding metabotropic receptor subtypes *mGluR3* and *mGluR5* was detected in rat and human islets as well as *mGluR2* in MIN6 β -cells [190]. In addition, *mGluR2* and *mGluR3* immunoreactivity was also displayed in islet cells [191] as well as genes encoding *mGlu8* [192].

Glutamate transport activity was detected in isolated rat islets [193] and glutamate transport has been found in human islets [194, 195], indicating that glutamate signalling plays a role in pancreatic islets.

Electrophysiology experiments demonstrated that glutamate receptors on β -cells initiate membrane depolarisation and an increase in $[Ca^{2+}]_{cyt}$ via AMPA/kainite and NMDA receptors [185]. As mentioned above, previous studies had found that kainite and AMPA receptors stimulate insulin secretion, however NMDA does not have this effect in the perfused pancreas [183]. This failure from NMDA receptors is possibly due to its low expression and response of $[Ca^{2+}]_{cyt}$ [185].

1.4.2 *Glutamate-induced activation of glucagon secretion*

L-glutamate has been found to stimulate glucagon secretion in the isolated rat pancreas mediated via AMPA receptors [188]. The competitive non-NMDA antagonist, CNQX, was found to completely prevent the glucagon response to glutamate [196] and NMDA stimulation of α -cells at very high concentrations (10^{-3}M) was found to be ineffective [188]. The three non-NMDA agonists AMPA, quisqualate and kainate also leads to a glucagon response [188]. Though glutamate stimulated insulin has a high efficacy (similar to kainate), efficacy of glucagon secretion is low [188]

α -cells express a high affinity Na^+ -dependent glutamate/aspartate transporter [195] and secrete glutamate via Ca^{2+} dependent exocytosis [197]. [188] IHC staining of α -cells have shown that glutamate is released from α -cells [185]. Due to the presence of the vesicular glutamate transporter, Na^+ -dependent inorganic phosphate (DNPI) co-transporter, it is thought that α -cells play a role in the storage and secretion of glutamate [198, 199].

mGlu8 immunoreactivity has been displayed in α -cells containing both glutamate and vesicular glutamate transporter 2 (VGLUT2), suggesting that mGlu8 may act as an autoreceptor and inhibit glucagon secretion [192].

The physiological role of glutamate in pancreatic islets remains unclear. However, it is clear that glutamate plays a role in the secretion of islet hormones by binding to both ionotropic and metabotropic receptors [192].

1.5 *AMP-activated protein kinase (AMPK)*

The AMP-activated protein kinase (AMPK) signalling cascade was first discovered in 1973 [200] and was only the second protein kinase-cascade discovered alongside the cAMP-dependent protein kinase/phosphorylase system [201]. AMPK is activated via elevation of intracellular 5-AMP from heat shock, fuel derivate and other stresses [202] and is regarded as a central energy sensor/switch in the regulation of metabolism [203]. It is one of the better known sensors involved in cellular energy metabolism [204] and plays roles in the regulation of cell growth, proliferation and polarity [205]. When activated, AMPK will activate ATP-generating pathways and inactivate ATP-consuming pathways restoring cellular energy balance [206].

AMPK is part of the serine/threonine family of protein kinases and forms a heterotrimeric complex as described in more detail below. AMPK phosphorylates enzymes involved in the control of lipid

metabolism, such as HMG-CoA reductase, acetyl-CoA carboxylase and hormone sensitive lipases [207]. Activation of AMPK has been shown to improve insulin sensitivity and glucose homeostasis making it a therapeutic target in the treatment of Type 2 Diabetes [208].

1.5.1 *The heterotrimeric structure of AMPK*

Mammalian AMPK is a heterotrimeric enzyme (containing several isoforms of each enzyme) comprising of a catalytic α -subunit ($\alpha1$ and $\alpha2$) the non-catalytic β ($\beta1$ and $\beta2$) and γ ($\gamma1$ - $\gamma3$) subunits [209, 210]. The α -subunit has a mass of 63kDa [211]. In skeletal and cardiac muscle, the $\alpha2$ isoform is the predominant form [212] whereas equal levels of $\alpha1$ and $\alpha2$ are present in the liver [213]. In β -cells, $\alpha1$ is the dominant isoform [214, 215]. The β - isoform is less well characterised is highly expressed in the liver whereas $\beta2$ is the dominant isoform in skeletal muscles. Both $\gamma1$ and $\gamma2$ isoforms are widely distributed throughout tissues though $\gamma3$ is specifically expressed in skeletal muscle [216, 217]. Co-expression of the catalytic and non-catalytic subunits is needed for optimal expression of the α -subunit. The structure of the AMPK heterotrimeric complex is illustrated in Figure 1.7.

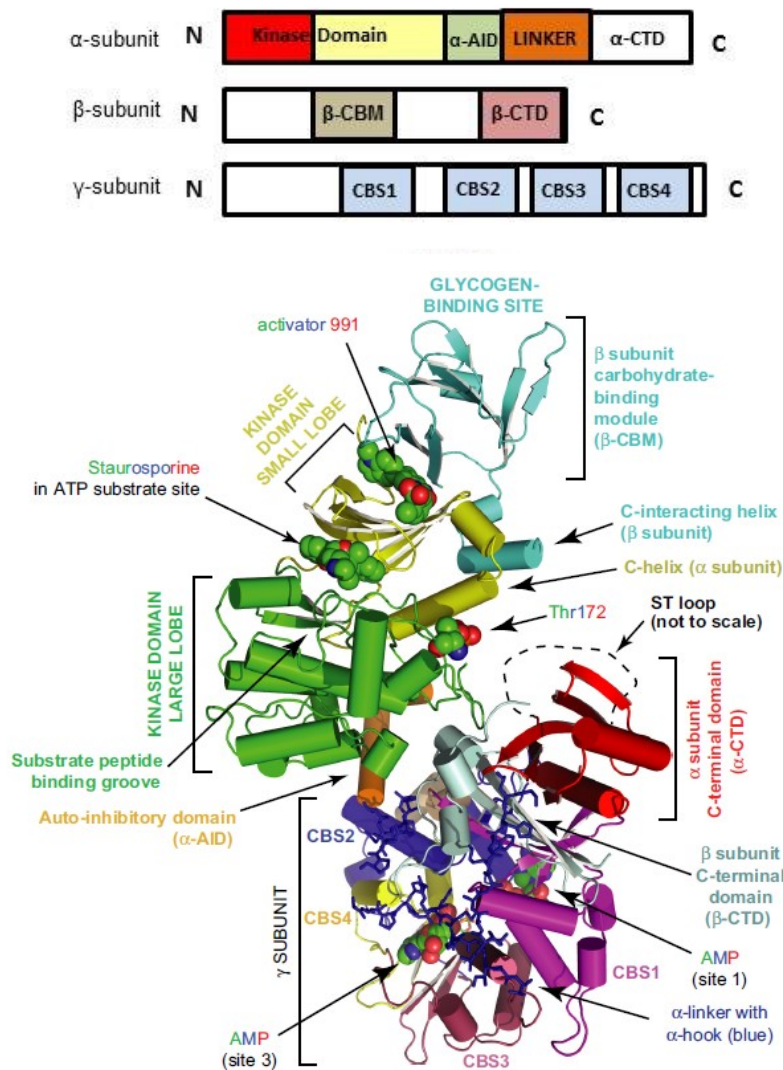


Figure 1.7 - Structure of the heterotrimeric AMPK complex

The top panel illustrates the linear layout of the three domains and the bottom a 3-D model of a human AMPK complex. The complex is divided into two separate regions, the catalytic and nucleotide binding modules. The extended linker connects the autoinhibitory domain (AID) and C-terminal domain (CTD), which wraps around one side of the C subunit [218].

1.5.2 Regulation of AMPK in the β -cell

AMPK activity is regulated via direct phosphorylation of upstream kinases such as liver kinase B (LKB) 1, calcium/calmodulin dependent protein kinase kinase (CAMKK) 2 and transforming growth factor β (TGFB) activated kinase (TAK) 1 [219, 220]. ATP, ADP and AMP modulate the activity of AMPK as well

as the phosphorylation via upstream kinase LKB1, which will be discussed in further detail later in this chapter. AMP and ADP bind to the γ -subunit resulting in AMPK phosphorylation and a conformational change of the kinase that prevents dephosphorylation and maintains AMPK activity. On the other hand, ATP binding will stimulate dephosphorylation [219, 220]. AMPK activation via CAMKK2 and TAK1 have been observed in HeLa and yeast cells, inhibition of the R6 regulatory subunit for protein phosphatase 1 (PP1) leads to a reduction in PP1-R6 activity causing preservation of P-AMPK levels in high glucose concentrations [221, 222] and inhibition of insulin secretion in MIN6 β -cells [223]. In β -cells, glucose phosphorylation by GK and its further metabolism is needed for dephosphorylation of AMPK. This indicates that AMPK activity is controlled via glucose during a regulated dephosphorylation step [223]. These studies target PP1-R6 as a possible therapeutic target to regulate activity of AMPK signalling [224].

1.5.3 Expression of AMPK in the β -cell

In MIN6 [214], INS1 cells and rat islets [225], AMPK α 1 was found to be the predominant subunit of AMPK. However, in insulin-sensitive tissues such as skeletal muscle [226] and the liver [227] the α 2 subunit (AMPK α 2) appears to be the predominant subunit in mouse. AMPK α 1 expression in MIN6 β -cells appears to be restricted to the cytosol whereas AMPK α 2 was found in both the cytosol and nucleus. Blockage of AMPK α 2 activity using antibody microinjection mimicked the effects of glucose by leading to transcription of pro-insulin [214]. These observations suggest a role for AMPK α 2 in the control of electrical activity and Ca^{2+} homeostasis in β -cells [228].

When islets are cultured with palmitate there is a decrease in AMPK α 1 expression as well as a decrease in GSIS [225] whereas, in MIN6 β -cells exposed to palmitate, increases in AMPK phosphorylation and insulin secretion occur [229].

Other members of the AMPK family present in the β -cell and phosphorylated by the up-stream kinase LKB1 include, Qin-induced kinase (QIK), QSK, salt-induced kinase (SIK), microtubule-affinity regulating kinase (MARK)-1, MARK2, MARK3, MARK4, AMPK-related kinase 5 (ARK5), SNF/AMPK related kinase (SNARK), brain specific kinase 1 (BRSK/SAD-1), BRSK2/SADB and sucrose non-fementing related kinase (SNRK) [204]. The roles of some of these kinases in the AMPK signalling pathway is illustrated in figure 1.8.

Another Ser/Thr kinase more distantly related to AMPK is PAS kinase (PASK) which has been shown to play a role in β -cell and proinsulin gene expression [230]. PASK becomes activated in β -cells in response to glucose by reversible phosphorylation at Thr-152 in the “T-loop” [230] and via up-regulated expression of the PASK gene. Inhibition of PASK using antibody injection blocked pre-

proinsulin and Pdx-1 induction by glucose [230]. PASK^{-/-} mice showed impaired GSIS although pre-proinsulin expression appeared normal [231]. These mice also showed improvement in insulin sensitivity after being exposed to a high-fat diet, indicating that lowered levels of PASK may protect against insulin resistance [231]. Other reports however, have suggested that PASK expression is insensitive to glucose regulation in islets [232].

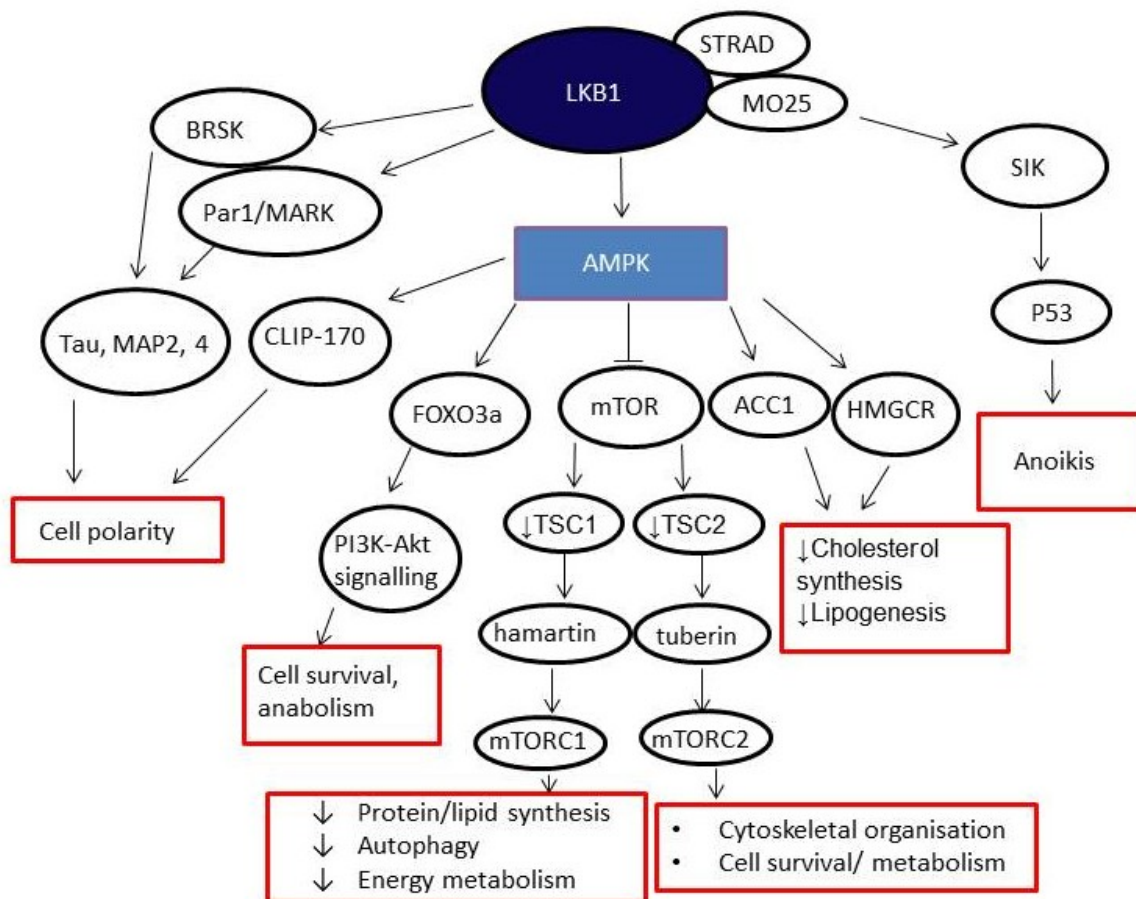


Figure 1.8 - The AMPK signalling pathway- controlled by LKB1 and AMPK intracellular signalling and metabolism

LKB1, in complex with the scaffold protein MO25 and pseudokinase STRAD, phosphorylates the AMPK family of proteins (13 members) [233], regulating diverse intracellular and extracellular biological processes. For example, AMPK regulates the central regulator of cellular energy metabolism mTORC as well as MARK/Par1 which plays a central role in cell polarity and glucose sensing

1.5.4 The role of AMPK in insulin secretion

There is contradictory evidence supporting both the positive and negative regulatory effects for AMPK in the control of insulin secretion. The relationship between AMPK and glucose homeostasis was first examined using the cell permanent analogue of AMP, 5-Aminoimidazole-4-carboxamide

ribonucleotide (AICAR) [215], AICAR is taken up by the cells and rapidly phosphorylated to form the AMP mimetic, AICAR monophosphate (ZMP), without changing the intracellular levels of ATP or AMP [234]. AICAR stimulates insulin release at basal glucose levels in mouse [235] and human islets [236] whilst secretion at high glucose was inhibited.

In addition, after INS1 and isolated rat β -cells are treated with AICAR, K_{ATP} channel trafficking to the cell surface under high glucose concentrations increases [237]. This suggests a link between AICAR, AMPK and the inhibition of insulin secretion through the K_{ATP} channel [237]. AMPK both inhibits and promotes insulin secretion, as discussed in detail below.

1.5.4.1 AMPK inhibits GSIS

Pharmacological activity of AMPK provides evidence which supports the view that AMPK activation inhibits insulin secretion. Thus, in a study monitoring the phosphorylation of the AMPK substrate acetyl CoA carboxylase (ACC)1 in INS1 cells [238], AICAR was found to both block GSIS and inactivate ACC1 through phosphorylation of Ser-79. Using INS1, MIN6 and HIT (clonal hamster β -cell line [239]) cell lines AMPK activity was found to be inversely related to insulin secretion when exposed to a variety of glucose concentrations [235, 240]. Acute treatment with AICAR was found to decrease insulin secretion triggered by glucose in both MIN6 cells and rat islets [241]. Additional evidence for the inhibitory effect of AMPK on GSIS was provided using the anti-diabetic drug thiazolidinedione (TZD), which reduced insulin secretion and activated AMPK [242, 243]. Long-term activation of AMPK using pharmacological agents has been shown to inhibit insulin secretion, whereas acute AMPK activation has a more varied effect [244, 245]. Inhibition of insulin secretion using AICAR was found to be restored when treating with the AMPK inhibitor compound C, suggesting GSIS inhibited by AICAR occurs via AMPK activation [246].

To further support the active view that AMPK inhibits GSIS, dominant negative (DN)-AMPK and constitutively active (CA)-AMPK constructs have been used. DN-AMPK overexpression at low concentrations of glucose reduced AMPK activity by 50% (similar to high glucose). By contrast, expression of CA-AMPK maintained AMPK activity at elevated levels even in the presence of high concentrations of glucose. In MIN6 cells and human islets, DN-AMPK was found to increase insulin secretion at low glucose concentrations whereas, CA-AMPK completely blocked GSIS [228, 235]. On the other hand, overexpression of DN-AMPK in INS1 cells slightly reduced AMPK activity but had no effect on GSIS [247]. At high glucose concentrations, DN-AMPK will decrease AMPK activity and increased GSIS in human islets, though this finding was not mirrored in cell lines or rat islets [235]. When CA-AMPK islets were transplanted into STZ-induced diabetic mice, restoration of glucose tolerance was blocked, whereas glucose tolerance was improved after transduction of DN-AMPK

expressing islets [236]. The impaired glucose clearance in mice that received CA-AMPK cells may have been due to an increase in apoptosis, suggesting that CA-AMPK plays a role in β -cell survival. This view has been supported in experiments using cell lines [248]. Moreover, CA-AMPK expression in MIN6 cells reduce the number of insulin granules, vesicle movement and actin reorganisation for exocytosis [249]. In addition to ATP and Ca^{2+} activity, expression of CA-AMPK leads to increases in response to glucose and inhibits sulfonylurea induced insulin secretion [228, 250].

1.5.4.2 AMPK promotes GSIS

Early studies showing AICAR mediated inhibition of gluconeogenesis in hepatocytes prompted to study of the impact of AICAR treatment on β -cells [251]. Perfusion of the pancreas at 6mM glucose whilst increasing the dosage of AICAR results in enhanced insulin secretion which indicates that activation of AMPK promotes GSIS [252]. However, the removal of AICAR results in further increases in insulin secretion, suggesting the possibility that AMPK inactivity may in fact promote insulin secretion [252]. Further studies showed that AICAR treatment increases insulin secretion in rat islets and cell lines when exposed to varied glucose concentrations [240, 247, 252, 253]. From the effect of AICAR on enzymatic activity on AMPK in these studies it was concluded that there is a correlation between AICAR treatment and an increase in AMPK activity. However, it is still unclear whether the effect of GSIS after AICAR treatment works independent from AMPK.

Experiments using Pre-diabetic Zucker rats showed that when AMPK activity became stimulated in both β -cells and in the periphery by AICAR administration, hyperglycaemia is prevented and β -cell mass preserved. However, in treated animals insulin sensitivity was greatly improved and β -cell “stress” decreased compared to wild type littermates [254].

1.5.5 *The role of AMPK in apoptosis*

The effect of long-term AMPK activation on β -cell survival has been studied using MIN6 cells cultured in high glucose concentrations and exposed to AICAR for 24 hours. Here AMPK was found to block glucose oxidation and insulin secretion as well as to increases in reactive oxygen species (ROS) production and cell death [255]. It possible this was due to changes in NADH/NAD⁺ ratio and effects on the oxidative state of mitochondrial flavin (FMN) in complex 1 [256]. There was an accumulation of BIM, BIP and BAX, which are pro-apoptotic, and Bcl-2 homology (BH) 3-domain containing members of the BCL2 family [245]. After overexpression of the anti-apoptotic BCL2 protein, β -cell survival was rescued. AICAR induced apoptosis treatment in wild-type (WT) islets (rather than AMPK α 2 KO islets) indicate that activation of AMPK may contribute to apoptosis [257]. Low glucose treatment in MIN6 cells results in apoptosis mediated via C-jun N-terminal kinase (JNK) and c-myc. This was mimicked by AICAR induced AMPK activation, indicating that prolonged AMPK activation may cause starvation

induced apoptosis in β -cells [258, 259]. On the other hand, INS1 cell treatment with AICAR in high glucose restored GSIS and reduced apoptosis indicating prolonged inhibition of AMPK which may underlie β -cell dysfunction in a glucotoxic environment [260].

There are other means by which active AMPK may prevent cell survival for example, via the inhibition of proliferative pathways. AMPK directly phosphorylates TSC2 [261], this complex acts as a GTP-activating protein on the mTOR activating Rheb, allowing transmission of growth factor receptor tyrosine-kinase derived signals to the P70 S6 kinase tumour suppressor [261]. Inhibition of Rheb leads to suppression of these proliferative signals and instead favours opposing cell death pathways. TSC2 KO mice have improved glucose tolerance, insulin secretion and β -cell mass which is also consistent with the suggestion that AMPK and its targets weaken insulin secretion and proliferation [262, 263]. Persistent activation of AMPK leads to phosphorylation of the tumour suppressor p53 at ser15 causing cellular senescence and eventual cell death [264]. It has been suggested that the inhibition of cyclin dependent kinases (CDK) p21^{Waf1/Cip1} is involved in this cell death pathway [265]. Suppression of AMPK activity appears to reduce cell death caused by proinflammatory cytokines and immunoreactive CD8 and T-cells [266], suggesting that AMPK activation may play a part in the immediate post-transplantation stage leading to cell death [267].

1.5.6 *Deletion of AMPK α 1 and α 2*

Knock-out models of the catalytic subunits AMPK α 1 and α 2 has demonstrated a role for AMPK α 2 in multiple tissues in regulating glucose homeostasis [268, 269]. These global AMPK α 2 KO mice became insulin resistant, have low levels of plasma insulin and raised blood glucose. However, normal islet insulin secretion is observed *in vitro* [269]. AMPK α 1KO mice displayed no phenotype, suggesting some compensation of insulin production and secretion from the α 2 subunit [270]. In comparison, the α 1 subunit is unable to compensate for the loss of α 2, suggesting tissue-specific roles for α 1, the α 1 subunit can compensate for α loss in β -cells.

Independently, two groups have generated β -cell and brain specific AMPK α 2 deficient mice using the RIP2 Cre promoter [271, 272]. Breeding of these animals to mice null globally for AMPK α 1 resulted in animals inactivated for both alleles in these tissues [271, 272]. In both studies, mice showed impaired glucose tolerance, lowered plasma insulin levels and no change in β -cell mass. The AMPK α 1 and - α 2 deleted strain using the rat insulin promoter (RIP2) Cre promoter studied in our group also noted in increase in β -cell proliferation and *in vitro* insulin secretion [271]. Whereas in the second study of AMPK α -subunit deletion, mice appeared to have some defect in GSIS [272].

Regarding these studies it has been argued that the loss of the α -subunits in the brain via the RIP2 Cre promoter may mask potential β -cell dependent defects in glucoregulation [273]. Deletion of the $\alpha 2$ -subunit in proopiomelanocortin (POMC) neurones of the brainstem (where RIP2 Cre expression occurs) results in an increase in body mass and fat, as well as loss of glucose responsiveness. In addition, these mice become more insulin sensitive, have lower plasma insulin levels and enhanced blood glucose levels. Therefore, the phenotype observed in the RIP2Cre AMPK $\alpha 1\alpha 2$ KO may be partially due to the effect of AMPK in peripheral tissues [274]. Further studies are required to assess the β -cell specific function of the AMPK α -subunits.

1.5.7 *Downstream mechanism of AMPK action in β -cells*

The most likely mechanism for AMPK action in the pancreatic β -cell is the phosphorylation and inhibition of acetyl CoA carboxylase-1 (ACC1) and subsequent down-regulation of malonyl-CoA synthesis. ACC1 catalyses the rate limiting step in fatty acid (FA) biosynthesis and becomes inactivated when phosphorylated by AMPK at the Ser residues 79, 1200 and 1215 [275]. Chronic inactivation and KO of ACC1 has been found to inhibit GSIS, glucose metabolism, ATP production and the synthesis of mitochondrial metabolites [276]. AMPK also plays a role in programming β -cell gene expression via SREBP, FOXO1a and HNF4 α [277]. Phosphorylation of cytoskeletal machinery components involved in secretory granule transport may also be involved in the mechanistic action of AMPK. For example, AMPK activation has been found to subdue granule movement whilst having no effect on vesicle fusion at the plasma membrane [249]. The exact mechanism by which AMPK suppresses glucose metabolism as well as Ca²⁺ signals [278] remain unclear.

1.5.8 *The role of AMPK in the control of food intake by the hypothalamus*

As mentioned above, AMPK acts through peripheral tissues to control food intake. Stereotactic injection of an adenovirus expressing activated AMPK into the hypothalamus of rats has been performed to study the role of AMPK in feeding centres of the ventromedial hypothalamus [279, 280]. These studies demonstrated that hypothalamic AMPK is activated by the orexigenic agent ghrelin and inhibition by intracerebrovascular (ICU) leptin. Recent studies show that changes in ACC activity and endogenous cannabinoid levels may be involved, implicating that AMPK plays a role in the control of body mass [281, 282]. Mice deficient for the AMPK α -subunits specifically in the hypothalamic nuclei became hypoglycaemic, demonstrating that AMPK activation plays a role in the sympathetic response to hypoglycaemia [283]. This suggests that AMPK is important in the control of glucose sensing neurones in the arcuate nucleus and paraventricular hypothalamus.

1.5.9 *AMPK and glucagon secretion*

Little is known of the roles of AMPK in glucagon secretion. In a study performed in our lab several years ago, the α -cell line α TC1-9 was used to find that activation of AMPK stimulates glucagon secretion, whereas inactivation prevents $[\text{Ca}^{2+}]_{\text{cyt}}$ elevation and glucagon release even at low glucose concentrations [284].

1.6 *Liver Kinase B 1(LKB1)*

LKB1 (also known as STK11), is known of as a tumour suppressor due to the polyp formation following heterozygous germline mutation. Thus, mutations in the STK11 gene increase the risk of developing Peutz-Jeghers Syndrome (PJS) which causes polyps in the GI tract, mucocutaneous pigmentations as well as leads to a predisposition for tumour development in the breast and gynaecological organs [285, 286]. The roles that LKB1 plays in the development PJS are discussed in more detail in section 1.6.6.

As mentioned previously, LKB1 acts as a major upstream kinase which phosphorylates and activates AMPK [287] leading to stimulation of its down-stream kinases and inhibition of protein synthesis, FA's and glucose metabolism as well as up-regulation of glucose transport [288]. AMPK inactivates mTOR (mammalian target of rapamycin) via the tuberous sclerosis (TSC) 1/2 tumour suppressor complex and by direct phosphorylation of the mTOR-binding partner raptor [289-291]. TSC2/TSC1 will inactivate Rheb which activates mTOR.

1.6.1 *Protein structure and localisation of LKB1*

LKB1 encodes a 433 amino acid ubiquitously-expressed protein (436 amino acids in mice) with N- and C- terminal domains and a central catalytic domain (residues 49-309). In the human genome LKB1 is part of single homolog and single ortholog in mouse. The N-terminal domain consists of a phosphorylation site (S31) and a nuclear localisation signal [292] whereas the C- terminal domain contains three phosphorylation sites (S325, T366 and S428). The latter two sites are targeted by PKA [293, 294]. LKB1 contains autophosphorylation sites (T185, T189, T336 and S404), a carboxylterminal prenylation motif and is farnesylated in cells [295], (indicated in Figure 1.9).

LKB1 forms a complex with Ste20-related adaptor (STRAD) and mouse protein 25 (MO25) which are needed for enzymatic activity [296, 297]. The overall LKB1-STRAD-MO25 complex is illustrated in Figure 1.9. When overexpressed, LKB1 is localised in the nucleus, though when co-expressed with STRAD and MO25, it is localised in the cytosol [298, 299]. Interaction with STRAD in the cytoplasm is

required for LKB1 to induce G1 arrest in a LKB1 KO melanoma cell line [299], indicating that interaction within this complex is required for the role LKB1 plays in cell proliferation and growth.

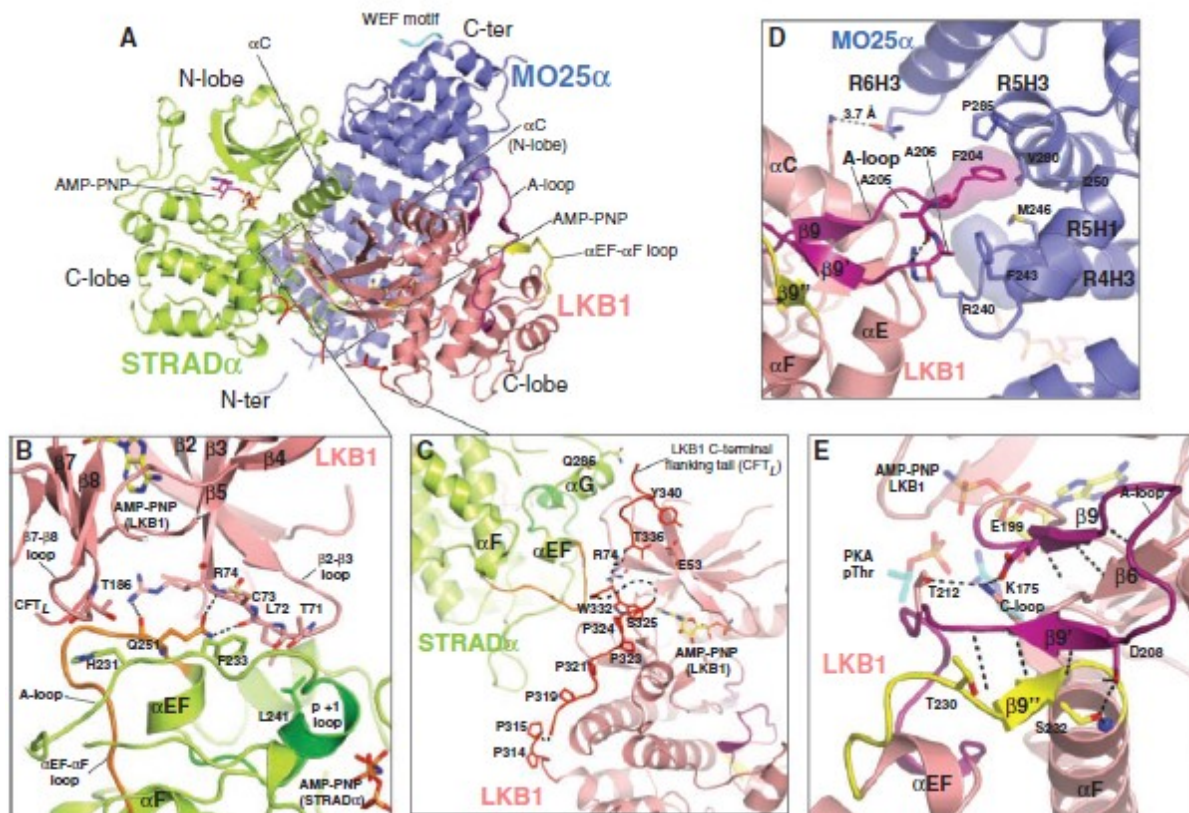


Figure 1.9 - Overall structure and interactions of the LKB1-STRADα-MO25α complex

(A) Representation of the hetero-trimeric complex and two bound AMP-PNP molecules; LKB1 shown as yellow carbons and STRADα as pink carbons. (B) LKB1-STRADα interaction; STRADα and αEF-αF loops are shown in green and orange, respectively. (C) Interaction of the LKB1 CTFL with STRADα and LKB1 N and C lobes, the proline rich CTFL is shown in red. In this panel we also see the phosphorylation sites, mentioned above, that act as autophosphorylation sites. (D) LKB1-MO25α interaction. The LKB1 activation loop is shown in pink. (E) The LKB1 A-loop interaction. Backbone interactions are shown as dashed lines. The residues Asp208, Thr230 and Ser232 which are normally mutated in PJS are labelled and side chains shown. Between Glu199 and Lys175, the dashed line represents a salt bridge and the interaction between the LKB1 activation segment and its catalytic loop (C-loop). The corresponding interaction in PKA between the phosphorylated Thr197 and Arg165 is shown and PKA residues are represented as transparent sticks [300].

1.6.2 The role of LKB1 in cell polarity

Using mammalian intestinal epithelial cells and *Drosophila* kinase-dead mutants of LKB1, LKB1 was discovered to play a role in cell polarity. These epithelial cells were unable to polarise, suggesting that the catalytic functions of LKB1 are needed for this process [301]. Furthermore, STRAD-mediated translocation of LKB1 is needed from the nucleus to cytoplasm [302]. It is likely that LKB1 plays a role

in polarity in a cell specific manner with different contributions from different members of the AMPK family [295]. For example, the phosphorylation of LKB1 on a C-terminal S431, via agonist activated cAMP dependent PKA is important for polarisation of follicular epithelial cells and *drosophila* oocytes [303]. Also, the brain-derived neurotrophic factor was found to promote axon differentiation in a manner that depends on PKA-mediated LKB1 phosphorylation, providing a link between extracellular signals and LKB1 mediated axon specification [303]. Finally, the farnesylation motif of LKB1 is needed for A-P polarity in *Drosophila* [304].

The regulatory subunit of myosin II, myosin regulatory light chain (MRLC), is important in AMPK-mediated changes in structure in certain cell types [305]. MRLC mutants in *drosophila* have similar defects as AMPK KO mice. MRLC acts as a direct target of AMPK phosphorylation at a site which initiates the MRLC polarity function [305]. Importantly, a phosphomimetic mutant MRLC transgene appears to rescue the AMPK KO phenotype in *Drosophila* which indicates the importance of the LKB1-AMPK-MRLC pathway in polarity control [295].

MARK 1, 2, 3 and 4, which are regulated by LKB1, are also linked to the control of cell polarity. Through the regulation of tubulin dynamics the MARK family promotes epithelial polarisation [306]. In mice, germ line deletion of MARK2 suggested a role for this enzyme in energy metabolism as these mice become hyperphagic and show a decrease in adiposity [307, 308]. To elucidate the relationship between the role of LKB1 in controlling cell polarity, and in its tumour suppressor function, mammary epithelial cells were grown in a 3D Matrigel culture system. Activation of c-Myc and inactivation of LKB1 caused abnormal cell-cycle progression of quiescent acinar cells resulting in structural defects indicating that polarity is needed to restrain cell proliferation after mitogenic stimuli [309]. Another suggestion is that defects in polarity via LKB1 inactivation causes tumorigenesis via defects in genomic stability, as shown in *Drosophila* [305, 310].

1.6.3 The role LKB1 in cell growth and proliferation

It has been suggested that LKB1 plays a role in phosphatase and tensin homolog (PTEN) signalling through upregulation of PTEN transcription or via direct protein binding and phosphorylation [311-313]. In human endothelial cells, LKB1 down-regulates protein kinase B (AKT) phosphorylation under oxidative stress, consistent with the existence of a LKB1-PTEN-AKT pathway [313]. By contrast, in other cell types AKT signalling does not appear to be regulated by LKB1 [290]. LKB1 also regulates Wnt/ β -catenin signalling [314, 315]. It has been suggested that LKB1 acts upstream of glycogen synthase kinase 3 (GSK3), up-regulating β -catenin activity [316]. KO models of the *Xenopus* LKB1 ortholog XEEK1, resulted in phenotypes similar to those following Wnt loss of function, indicating that they regulate a common pathway [314].

1.6.4 *LKB1 in diabetes*

A link between LKB1 and diabetes was uncovered by examining the effects of LKB1 inactivation in the liver. These studies revealed that LKB1 is essential for the beneficial effects of metformin [317]. Activation of LKB1 plays a role in gluconeogenesis via the regulation of AMPK and phosphorylation of the transcriptional co-activator target of rapamycin (TORC2). This results in inactivation of gluconeogenesis via sequestration in the cytoplasm which negatively regulates transcriptional events that promote synthesis of gluconeogenic enzymes. In the absence of LKB1, TORC2 is unable to become phosphorylated and gluconeogenesis may resume [317].

1.6.5 *Roles of LKB1 in the β -cell*

LKB1 has been deleted in adult mouse β -cells using a Pdx1-CreER transgene (PLB1) where LKB1 is deleted after tamoxifen injection [318, 319] as well as using the RIP2Cre transgene [320]. Both of these deleter strains show improved insulin content and secretion as well as β -cell size and polarity. This reveals the detrimental roles that LKB1 plays in the β -cell and in insulin secretion as discussed below.

1.6.5.1 LKB1 and β -cell polarity

β -cells, which are epithelial in nature, express the transmembrane protein E-cadherin [321] and also concentrate GLUT2 in microvilli in lateral surfaces [322]. In isolated islets, insulin is secreted to the lateral surfaces away from blood vessels [323]. However, as β -cells do not have a visible lumen and appear as symmetric spheres they are not regarded as polarised epithelium [323].

Ordinarily β -cells are organised as rosettes around islet capillaries [321] and monocillia localise at the lateral surfaces whereas, in the PLKB1 and RIP2Cre mice, β -cell cellular organisation undergoes dramatic changes. The nucleus becomes relocated towards the intrarosette capillary and the cilium moves to the opposite pole of the cell. This phenotype is similar to that occurring in the absence of Par1b [318-320, 324]. Both PLKB1 and par1b mutant mice show a shift toward columnar polarity. Par1b KO mice have similar redistributions of nuclei as these RIP2Cre LKB1 mice [318].

A loss of interaction between Par1b and E-cadherin may result in relocation of the latter to the apical pole, as found in RIP2Cre LKB1 β -cells. Par1b has been found to regulate glucose metabolism in adipose tissues [307], via syntaxin-4 interactions [325] but control GLUT4 trafficking to the plasma membrane. Syntaxin-4 is needed in the second phase of GSIS [326], therefore impaired phosphorylation of par1b when LKB1 is deleted may have an effect on β -cell trafficking of secretory granules to the cell surface [320].

1.6.5.2 LKB1 and β -cell size.

LKB1, independent of its effects on polarity, is known to restrict β -cell size through the mTOR pathway [289, 290]. In both the PLKB1 and RIP2Cre LKB1 mouse model, β -cell size became markedly enhanced, as assessed by optical projection tomography (OPT). This improvement seems to be due both to an increase in the volume of individual β -cells and a surge in proliferation. A similar phenotype was found in TSC1/2 deleted β -cells. Increased phosphorylation of ribosomal protein S6 and decreased phosphorylation of 4E-BP1 suggest that this increase is due to elevated mTOR signalling [320].

The rosette arrangements found in β -cells occurred twice as often in the RIP2Cre mice. This may be due to the increase in individual cell size. Arguing against this view, there was a 60% up-regulation in β -cell volume after mTORC overexpression, similar to that seen in the LKB1KO, whilst there was no increase in rosette number in mTORC overexpressing β -cells. Here there was an increase in β -cell mass but no change in the number of rosette structures [327-329]. With deletion of both AMPK α 1 and α 2 insulin release became impaired and there was no change in mass indicating the deletion of LKB1 does not reflect its requirement to activate AMPK [320]. The increase of islet acini may be due to kinases such as MARKs1-3 [320].

1.6.6 *Peutz Jeghers Syndrome*

Mutations in LKB1 are linked to PJS in humans [330-332], as discussed previously. Homozygous mutations of LKB1 are embryonically lethal in mice which die mid-gestation with vascular abnormalities and neural tube defects [333]. On the other hand, mice with heterozygous mutations develop hamartomatous GI polyps similar to those detected by human PJS patients [334]. These polyps appear at 4-6 months of age [335] and cause premature mortality at 11 months due to obstructions in the GI tract.

Studies of the coding regions and splice site mutations revealed that the LKB1 gene germline inactivating mutation occur in 35-70% of PJS patients. When scanning patients with PJS using multiplex ligation dependent probe amplification (MLPA), it was found that 94% of the patients that were screened had mutations in the LKB1 gene [336, 337]. Whereas no mutations were identified on the related genes STRAD α and MOD25 α [338].

Patients with PJS are predisposed to other cancers [339, 340]. Heterozygous LKB1 mice are also predisposed to cancer of the liver [341], bone [342] and endometrium [343]. Also mutations in p53 are detected in human PJS patients [344], whereas in heterozygous mice polyposis is enhanced when p53 becomes impaired [345, 346]. Examples of polyps from a patient with PJS are illustrated in Figure 1.10.

PJS polyps are thought to contain all cell types of surrounding tissue [335]. However, it has been noted that in LKB1^{+/-} mice, epithelial differentiation is disrupted in gastric and intestinal polyps [335]. Polyps in both human PJS patients and LKB1^{+/-} mice contain a large section of smooth muscle tissue. Additionally, in mice where LKB1 was deleted specifically in smooth muscle, polyps appeared in the stomach of mice with homo- and heterozygous mutations [347].

There are currently no drugs available for the treatment of PJS. Management of PJS depends mainly on endoscopies in the attempt to remove larger polyps [348]. The best short-term treatment for PJS patients is the enzyme cyclooxygenase-2 (COX-2) which is used for catalysing the rate-limiting step in prostaglandin synthesis as well as a role in tumour promotion [349]

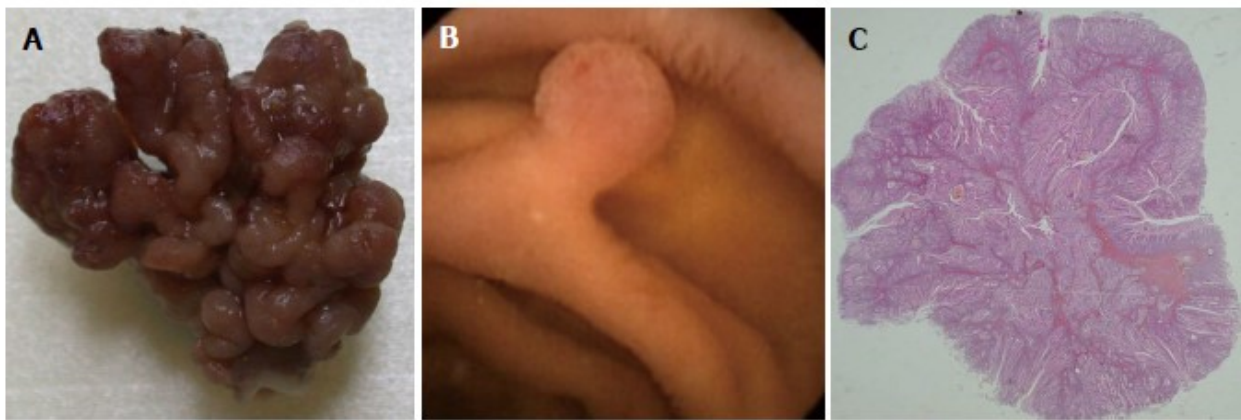


Figure 1.10 – Polyps extracted from a patient with PJS

A) Macroscopically lateral surface of a polyp from a 46 year old female with PJS, B) Capsule endoscopy image of polyp, C) Microscopically tree-like branching core derived from the muscularis mucosa covered by normal epithelium [350]

1.7 *Glucagon-Like Peptide 1*

Glucagon-like peptide 1 (GLP-1) is an insulinotropic incretin hormone expressed from the proglucagon gene. Preproglucagon is cleaved into mature peptides depending on the enzyme involved. In α -cell cleavage by PC2 and PC1/3 in intestinal L-cells, GLP-1 is generated [351].

1.7.1 *The GLP-1 receptor (GLP-1R) and its presence on the β -cell*

GLP-1 binds to and activates the membrane-bound GLP-1R in pancreatic β -cells, which is a ligand specific 7-transmembrane domain member of the G-protein coupled receptor family B [352, 353]. Members of this family contain a glucagon receptor subclass which includes receptors for glucagon, GLP-2, GIP and secretin. The human GLP-1R gene is located on the long arm of chromosome 6p21[354] encoding 463 amino acid containing a long extracellular N-terminal and 7 membrane spanning

hydrophobic motifs. Murine GLP-1R KO models display reduced glucose tolerance and impaired GSIS [355]. GLP-1 responsiveness has been found to decrease in β -cells of Type 2 diabetic patients due to hyperlipidemia [356] and/or hyperglycaemia [357] associated with reduced expression of GLP-1R.

On ligand binding, the GLP-1R becomes activated and couples to the trimeric G-protein complex releasing the G_{α_s} subunit. This results in an increase in the enzymatic activity of membrane bound adenylate cyclase (AC) and cAMP [352] production. Upon GLP-1R activation there is a rise in cAMP activity and the downstream signalling pathways protein kinase A (PKA) and exchange protein activated by cAMP (EPAC2) [358]. However, the roles of cAMP in the downstream pathways are unclear.

1.7.2 GLP-1 mediated regulation of insulin secretion

GSIS is activated when glucose enters the β -cell and undergoes glycolysis resulting in K_{ATP} channel closure, membrane depolarisation and Ca^{2+} influx which stimulates insulin exocytosis. Usually after feeding, GLP-1 released from the gut binds to GLP-1R, resulting in a rise in cAMP, and activation of PKA and EPAC2, as described above. This activation results in closure of K_{ATP} channels, depolarising the cell further.

Closure of the K_{ATP} channel is regulated through the pore forming $Kir_{6.2}$ and SUR regulatory subunits via the GLP-1R effector PKA inhibiting ADP binding [359, 360]. On the other hand, activation of EPAC2 results in the opening of ryanodine receptor (RyR) Ca^{2+} channels. Activated PKA and EPAC will also induce mobilisation of granules from the RP to the RRP. EPAC2 interact directly with SUR1, resulting in the acidification and priming of insulin granules [67]. EPAC2 may also activate proteins such as SNAP25 and Rim2, involved in the priming and fusion of insulin secretory granules with the plasma membrane [361, 362]. GLP-1R signalling inhibits compensatory repolarisation by opening K_{ATP} channels, this inhibition leads to an increase of glucose-initiated membrane depolarisation leading to the opening of VDCC [359]. EPAC2 also increases channel inhibition, increasing the effectiveness of K_{ATP} channel closure [359]. As illustrated in Figure 1.11.

GLP-1 also plays a role in insulin gene transcription and biosynthesis by activating key transcription factors such as cAMP response element-binding protein (CREB) and pancreatic and duodenal homeobox (PDX-1). When activated, CREB binds to cAMP response element (CRE) sites in promoter regions of genes such as insulin, insulin receptor substrate (IRS)-2, pro-hormone convertase-1 and CREB itself [363]. PDX1 plays a role in pancreatic development and β -cell function and its binding to the insulin promoter is increased upon GLP-1 treatment [364]. Studies examining glucose and GLP-1-stimulated RIN insulinoma cells demonstrated that the PKA inhibitor H89 led to a decrease in PDX1

mRNA, protein and nuclear translocation. This correlates with complete inhibition of GLP-1-induced insulin secretion under these conditions, indicating that PDX1 activation via GLP-1 is partially dependent on PKA [365]. Other studies have revealed a role for phosphoinositide 3-kinase (PI3K) in the effect of GLP-1 on the insulin gene promoter [364]. Thus, at low glucose concentrations, insulin mRNA levels decrease in β -cells, a consequence of reduced mRNA stability. However, mRNA stability was enhanced in the presence of elevated intracellular cAMP [365]. Finally, GLP-1 increases the expression of β -cell glucose transporters, GK, Kir_{6.2} and SUR1, improving the capability of β -cells to sense and respond to glucose [366].

1.7.3 Effect of GLP-1 in β -cell proliferation, differentiation and apoptosis

Chronic activation of GLP-1R signalling in both normal and diabetic animals causes an increase in β -cell mass due to an augmentation in β -cell proliferation and differentiation as well as a decrease in β -cell apoptosis. However, due to difficulties in measuring human islet mass the effect of human β -cell proliferation, differentiation and apoptosis is unclear [367].

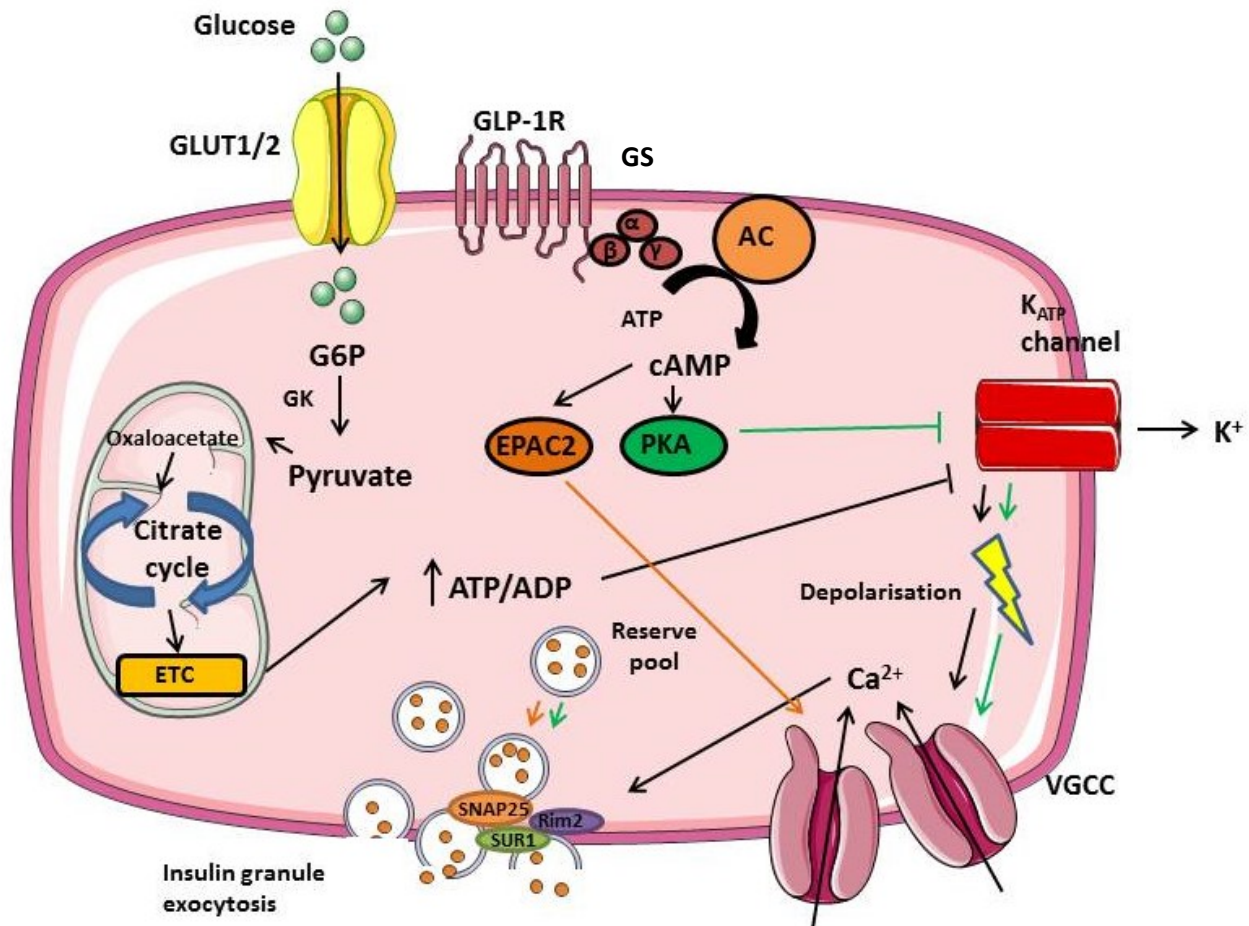


Figure 1.11- GLP-1 binding to and activating GLP-1R plays a role in GSIS

Whilst glucose enters the β -cell closing K_{ATP} channels during normal GSIS, GLP-1 binds to the GLP-1R resulting in cAMP up-regulation in the cell and subsequent PKA and EPAC₂ activation. This leads to the further closure of K_{ATP} channels and the opening of ryanodine receptor Ca^{2+} channels, as well as mobilisation of the RP to RRP. The Figure is generated using images from "Servier Medical Art". (<http://www.servier.com/servier-medical-art>).

1.7.3.1 GLP-1 stimulates β -cell proliferation

Studies of GLP-1 signalling in β -cell proliferation in rodents have shown the involvement of epidermal growth factor receptor (EGFR), PI3K [364] as well as components of the cAMP, PKA, Akt/PKB and MAPK signalling pathways [368]. PDX-1 has a central role in the stimulatory effect of GLP-1 on cell proliferation, resulting in improved effects on blood glucose in both diabetic and db/db mice [369]. However, this effect in humans remains unclear [370].

1.7.3.2 GLP-1 affects β -cell differentiation

Agonists of GLP-1R are able to induce differentiation of islet cell precursors and other exocrine cells into hormone-producing β -cells. Thus, long-term stimulation of GLP-1R on the acinar cell line AR42J and pancreatic ductal cells increases intracellular cAMP and Ca^{2+} levels as well as the PKA-dependent

expression of insulin, glucagon and somatostatin [371, 372]. Similarly, long-term treatment of immature islet precursors become differentiated into insulin-immunopositive cells and enhances GSIS [367].

1.7.3.3 GLP-1 on β -cell apoptosis

GLP-1 can also act as an anti-apoptotic agent. Thus, human islets incubated with GLP-1 were protected from glucolipotoxicity [109]. In Zucker diabetic mice, *db/db* mice and streptozotocin (STZ) induced diabetic mice, GLP-1 treatment was found to restore insulin secretion, lower blood glucose concentrations and decrease apoptosis [357]. Islets from GLP-1R KO mice treated with STZ were found to be more susceptible to apoptosis in comparison with islets from their WT littermates [373]. The anti-apoptotic properties of GLP-1R signalling indicate multiple signal transduction pathways including activating EGFR, PDX-1, PI3K/PKB signals and cAMP/PKA-dependent activation of CREB. Inhibition of β -cell apoptosis via GLP-1R is associated with anti-apoptotic proteins such as bcl-2, and reduced levels of pro-apoptotic proteins such as caspase-3 [374]. Reduction of ER stress-mediated expression of CHOP, Gadd34 (involved in growth arrest) and activating transcription factor 4, which inhibits dephosphorylation of eIF2 α , has also been observed. These transcription factors enhance recovery and protect cells from ER stress [375].

1.7.4 Actions of GLP-1 in pancreatic α -cells

GLP-1 and PC1/3 immunoreactivity has been found in α -cells [351, 376] indicating a role for GLP-1 in glucagon secretion [351]. GLP-1 inhibits glucose dependent-glucagon secretion *in vivo* [377] but *in vitro* has been found to stimulate glucagon release from α -cells [378]. GLP-1R expression in α -cells is unclear [379, 380]. Reports of expression of GLP-1R on α -cells have varied from zero [381], to little [379], to 20% of α -cells [380]. It has also been suggested that GLP-1R expression in α -cells is less than 0.2% to that of β -cells therefore, GLP-1-induced reduction of glucagon secretion does not rely on GSIS but is dependent on PKA [382]. Other studies have demonstrated that the inhibition of glucagon secretion via GLP-1 is mediated by somatostatin receptor substrate-2 (SSTR-2) [383].

It is possible that GLP-1 inhibition of glucagon secretion is initiated via regulation of insulin, GABA or somatostatin secretion. Though it is apparent that GLP-1 plays a role in the regulation of glucagon secretion, GLP-1R deleted mice have shown glucagon secretion is not dependent on GLP-1 [384].

In addition to L-cells, pancreatic α -cells are a source of GLP-1 [385]. When incubating the α -cell line α TC1-6, at high concentrations of glucose, PC2 transcription is unchanged. Secretion of GLP-1 is enhanced when exposed to high concentrations of glucose [386].

G-protein coupled receptor (GPR) 120, GPR119 and TGR5 expression are involved in the secretion of GLP-1 from L-cells. This is also observed in α TCI-6 cells. TGR5 agonist treatment was found to enhance GLP-1 secretion and PC1 expression [387], indicating that proglucagon can be processed to release GLP-1 from α -cells [388]. Therefore, intra islet GLP-1 may contribute to β -cell growth, survival and insulin secretion.

1.7.5 *The role of GLP-1 in Type 2 Diabetes*

In the 1980s it was recorded that patients with Type 2 Diabetes exhibited a reduction in incretin effects [389]. Insulinotropic incretin hormones GLP-1 and GIP account for over 50% of postprandial insulin response in non-diabetics [390] and only 20% in diabetic patients [389].

Several studies suggest that the reduction in GIP and GLP-1 secretion in Type 2 Diabetes doesn't significantly contribute to the loss of the incretin effect and that there is a decrease in insulinotropic action in GIP (whereas GLP-1 action remains preserved) secondarily to impairment in β -cell function. In addition, hyperglycemia further reduces the β -cell response to GIP and leads to the down-regulation of its receptor [391]. The reduction of the incretin effect in Type 2 Diabetic patients is a bi-product of chronic hyperglycemia, [391], as consistent with the fact that loss of insulinotropic efficacy in diabetic patients with other conditions such as secondary to chronic pancreatitis or autoimmune diabetes [392].

1.7.6 *GLP-1 treatment for Type 2 Diabetes*

A 'glucagonocentric' vision for the treatment of Type 2 Diabetes has been proposed relying on the fact that glucagon enhances the catabolic process which occurs without insulin present [393]. In poorly controlled diabetes hyperglucagonemia occurs however, glucagon receptor null mice following β -cell destruction do not develop diabetes [168]. The absence of hyperglycemia in this model may, as well as the lack of glucagon, be due to high concentrations of fibroblast growth factor 21 (FGF-21) and GLP-1 displayed by these mice [394].

The three drugs clinically used for the abnormalities of glucagon secretion in Type 2 Diabetes include; the GLP-1 receptor agonists (GLP-1RA), the inhibition of DPP-4i and the amylin agonist pramlintide. The first two exert insulinotropic effects, these drugs do not markedly increase insulin concentrations which is thought to be due to the GLP-1 signalling effect to lower glycemia, thereby decreasing β -cell stimulation [395]. It has also been suggested that GLP-1 treatment improves GSIS, causing secretion rates to remain unchanged, whilst glucagon secretion reduces [395]. These suggestions indicate that the glucagonostatic and insulinotropic effects of GLP-1 both equally contribute to its hypoglycaemia efficacy [396]. Patients with Type 2 Diabetes when treated with the DPP-4i, Vildagliptin showed a

reduction in post-meal glucagon levels after 4 weeks of treatment [397] whilst the GLP-1RA liraglutide reduces 24 hour glucagon levels [398]. Type 1 Diabetes patients also showed a reduction in glucagon levels with liraglutide treatment [399], these findings suggest that liraglutide inhibits glucagon irrespective of intra-islet insulin, via the GLP-1 receptor in α -cells [396].

Aims of thesis

This thesis is split into two main objectives.

1. To understand the role of LKB1 in the pancreatic β -cell by deleting this kinase selectively in this cell type and to examine the effects this deletion has on β -cell mass and insulin secretion.
2. To explore the roles, if any, of LKB1 and AMPK in proglucagon-expressing cells, including enteroendocrine L- and pancreatic α -cells and to determine if deletion of either of these kinases (in these cell types) improves GLP-1 production and secretion, thereby improving overall glucose homeostasis in the mouse.

Chapter 2

Materials and Methods

2.1 *In vivo mouse experiments*

2.1.1 *Generation of mice selectively lacking LKB1 in pancreatic β -cells*

Mice homozygous for the *floxed* allele of the LKB1/Stk11 gene were bred against mice expressing Cre under the mouse Ins1 promoter (Ins1Cre) [400]. The heterozygous offspring were inter-crossed to generate Ins1LKB1KO mice (LKB1^{f/f} Cre positive) and WT mice (LKB1^{f/f} Cre negative). All mice were kept on a mixed FVB/129S6 and C57BL/6 background, offspring genotypes were obtained in the expected Mendelian ratios.

2.1.2 *Generation of mice selectively lacking LKB1 in proglucagon-expressing cells*

Mice homozygous for the *floxed* alleles at exons 3-6 of the LKB1/Stk11 gene (Mouse Models of Human Cancer Consortium. <http://mouse.ncifcrf.gov/>) were first bred against mice expressing Rosa26tdRFP reporter mice [401]. The resulting double heterozygous LKB1^{f/+}, RFP mice were inter-crossed to generate LKB1^{f/f}RFP mice which were subsequently crossed with mice expressing iGlu Cre. The heterozygous LKB1^{f/+}iGlu CreRFP littermates were once again inter-crossed with LKB1^{f/+} mice to generate GluLKB1KO (LKB1^{f/f}, Cre and RFP positive), GluLKB1het (LKB1^{f/+}, Cre and RFP positive) and LKB1WT (LKB1^{+/+}, Cre and RFP negative). All mice were kept on a mixed FVB/129S6 and C57BL/6 background, offspring genotypes were obtained in the expected Mendelian ratios.

2.1.3 *Generation of mice selectively lacking AMPK α 1 and α 2 in proglucagon-expressing cells*

Mice homozygous for the *floxed* allele of the AMPK α 1 gene were first bred against mice with homozygous *floxed* allele for the AMPK α 2 gene (both provided by Dr Benoit Viollet, CNRS, Paris, France [402]), resulting in double heterozygous AMPK α 1^{f/+} α 2^{f/+} mice. These heterozygous mice were inter-crossed to generate homozygous AMPK α 1^{f/f} α 2^{f/f} mice which were subsequently crossed with mice expressing Cre-recombinase under the control of the proglucagon promoter (iGlu Cre) [403]. The heterozygous AMPK α 1^{f/+} α 2^{f/+}iGlu Cre littermates were then inter-crossed with AMPK α 1^{f/f} α 2^{f/f} mice to generate GluAMPKKO (AMPK α 1^{f/f}, AMPK α 2^{f/f}, iCre positive) and WT mice (AMPK α 1^{f/f}, AMPK α 2^{f/f}, iCre negative). All mice were kept on a mixed FVB/129S6 and C57BL/6 background, offspring genotypes were obtained in the expected Mendelian ratios.

2.1.4 Mice maintenance and diet

Mice were housed at up to five animals per cage in a pathogen free facility with a 12 hour light/dark cycle and unrestricted access to standard mouse chow diet (Research Diet, New Brunswick, NJ, USA). All *in vivo* procedures were conducted at the Imperial College Central Biomedical Service and approved by the UK Home Office Animals Scientific Procedures Act, 1986.

2.1.5 Body weight

Body weight of mice was monitored weekly after weaning as well as being assessed using the mouse body condition score [404].

2.1.6 Oral and Intraperitoneal glucose tolerance test

Mice were fasted for 15 hours, with free access to water, before being given 2g glucose/kg of glucose administered either through oral gavage (OGTT) or via intraperitoneal injection (IPGTT). Blood (2-4µl) from the tail vein was collected at 0, 15, 30, 60 and 120 mins. after injection. Blood glucose levels were measured using an automatic glucometer (Accucheck; Roche, Burgess Hill, UK).

2.1.7 Insulin tolerance test (ITT) and plasma glucagon measurement

Mice were fasted for 5 hours, with free access to water, before being given intraperitoneal injection with 0.75 U/kg bovine insulin (Sigma, Dorset, UK) between 14:00 and 15:00 hours. Blood glucose levels were measured at 0, 15, 30, 45 and 60 mins. after injection.

2.1.8 Plasma glucagon and insulin measurement

For measurement of plasma glucagon, 100µl of blood was collected from the mice tail veins into heparin coated tubes (Sarstedt, Beaumont Leys, UK) at 0, 15 and 30 mins. after insulin injection. Plasma was separated by centrifuging the blood at 4°C 13,200 r.p.m. for 20 mins. Blood serum (50µl) was used to measure plasma glucagon levels by radioimmunoassay (RIA) with a competitive ¹²⁵I labeled glucagon (Millipore, Watford, UK).

For measurement of plasma insulin levels after glucose injection, mice were fasted for 15 hours and given intraperitoneal injection of 2g glucose/kg mouse weight. 100µl blood from mice tail was collected and plasma was separated by centrifugation at 13,200 r.p.m. for 20 mins. As before, plasma insulin levels were measured using RIA with a competitive ¹²⁵I labelled insulin (Millipore, Watford, UK) and described in more detail in 2.2.3.

2.2 *In vitro cell experiments*

2.2.1 *Islet isolation and dissociation*

Mice were sacrificed via dislocation of the neck followed by decapitation. Collagenase NB8 from *Clostridium histolyticum* (SERVA Electrophoresis): (1mg/ml in RPMI-1640) was injected into the pancreatic duct (5ml/mouse). The inflated pancreas was then incubated at 37°C for 10 mins. The digested pancreas was washed 2-3 times with RPMI-1640 and separated from exocrine tissues by centrifuging through a Histopaque gradient (Sigma Aldrich, Dorset, UK) at the densities of 1.110, 1.083 and 1.077g/ml at 2,500 r.p.m for 20 mins. After washing in RPMI-1640, individual islets were picked and incubated further in RPMI-1640 medium treated with 100U/ml penicillin and streptomycin and 10% [vol./vol.] heat-inactivated fetal bovine serum (FBS) for 24 hours before further analysis.

Islets were dissociated after isolation to obtain single cells for imaging by washing the islets 2-3 times in PBS and incubating at 37°C for 10 mins. in Hanks Balanced Salt Solution. After a brief centrifugation, this solution was replaced by RPMI-1640, and the islets were disrupted by vigorous pipetting. Cells were incubated for over 4 hours on circular glass coverslips pre-treated with 0.01% [wt./vol.] polyLysine (Sigma, Dorset, UK).

2.2.2 *Treatment of islets for glucagon and insulin measurements*

To measure glucagon secretion from mouse islets, 12 size-matched islets were pre-incubated for 30 mins. at 37°C in Krebs-Ringer bicarbonate buffer (KRB) solution (in mmol/l: 130 NaCl, 3.6 KCl, 1.5 CaCl₂, 0.5 MgSO₄, 0.5 KH₂PO₄, 2NaHCO₃, 10 HEPES and 0.1% [wt./vol.] BSA, pH 7.4) containing 10mmol/l glucose, with gentle shaking (120 rev/min). Islets were further incubated in 500µl KRB solution containing 0.1, 1, 3 or 17 mmol/l glucose for 1 hour at 37°C. Total glucagon extracts were lysed in 500µl acid-ethanol-Triton solution (1.5% [vol./vol.] HCl, 75% [vol./vol.] ethanol, 0.1% [vol./vol.] Triton X-100).

To measure insulin secretion from pancreatic mouse islets, 6 size-matched mouse islets were pre-incubated in KRB solution containing 3mmol/l glucose, for 1 hour at 37°C with gently shaking (120 rev/min). Islets were further incubated in 500µl KRB solution containing 3 or 17 mmol/l glucose for 30 mins. at 37°C.

2.2.3 *Insulin and glucagon radioimmuno-assay*

To measure glucagon or insulin levels a RIA kit (Millipore, Watford, UK) with competitive ¹²⁵I-labelled glucagon or insulin was used according to the manufacturer's instructions.

To measure secreted and total glucagon levels, 50µl of supernatants from islets treated with KRB solution or 50µl of total islets (1:500 diluted in assay buffer (provided by the kit)) were incubated with glucagon antibody at 4°C overnight. After a further overnight incubation with ¹²⁵I labelled glucagon at 4°C, precipitation buffer was added. After 20 mins. incubation, the ¹²⁵I labelled glucagon bound to the antibody was precipitated by centrifuging at 2,000 r.p.m. for 20 mins. and measured using a gamma counter. The glucagon levels were calculated against the glucagon standards (20pg/ml-400 pg/ml) provided in the kit.

To measure secreted and total insulin levels, 100 µl of supernatants from islets treated with KRB solution or 100 µl of total islet lysates (1:1000 diluted in assay buffer) were incubated with insulin antibody and ¹²⁵I labelled insulin at 4°C overnight. After a further 20 mins. of incubation with precipitation buffer, ¹²⁵I labelled insulin bound to the antibody was precipitated by centrifuging at 2,000 r.p.m. for 20 mins. and measured using a gamma counter. The absolute insulin levels were calculated against the insulin standards (0.1 ng/ml- 10 ng/ml) provided.

2.2.4 *Ca²⁺, TMRE and ATP imaging using confocal microscopy*

Isolated islets were incubated (37°C, 90% O₂/ 5% CO₂) for 40 mins. in fluo2-AM (Cambridge Bioscience, UK) (10 µmol/L) diluted with a mixture of DMSO (0.01% [wt./vol.]) and pluronic acid (0.001% [wt./vol.]) (Invitrogen, Paisley, UK) in a bicarbonate buffer solution (120mmol/L NaCl, 4.8mmol/L KCl, 1.25mmol/L NaH₂PO₄, 24mmol/L NaHCO₃, 2.5mmol/L CaCl₂, 1.2 mmol/L MgCl₂ and 3mmol/L D-glucose, all Sigma Aldrich, Dorset, UK) adjusted to pH 7.4. Islets were placed in a custom-manufactured 36 C chamber (Digital Pixel) mounted on a Zeiss Axiovert coupled to a Nipkow spinning disk head and the resulting traces were normalised to minimum fluorescence (F_{min}). Whole-islet multicellular Ca²⁺ imaging was performed as described [405]. Briefly, Ca²⁺ dynamics were captured with cellular resolution from the 50-200 fluo-2-loaded cells residing within the first and second layers of the intact islets. A solid-state laser (CrystaLaser) controlled by a laser-merge module (Spectral Applied Physics) provided wavelengths of 491nm to excite fluo-2 (rate=0.5Hz; exposure time 260ms). Emitted light was filtered at 525/50nm, and images were captured by a highly sensitive 16-bit, 512 x 512 pixel back-illuminated EM-CCD camera (ImageEM 9100-13; Hamamatsu). Throughout the recordings islets were kept at 36°C and continuously perfused with the bicarbonate buffer at 95% O₂/CO₂.

Islets were imaged similarly using tetramethylrhodamine, ethyl ester (TMRE) to measure mitochondrial membrane potential. For cytosolic ATP/ADP imaging with Perceval [50], islets were infected for 48 hours with adenovirus at multiplicity of infection 10. Cells were imaged using the

bicarbonate solution (as mentioned above). Absorbance/ emission was 491/525nm and islets were captured as above.

2.2.5 *Detection of ATP*

Size-matched islets were separated into batches of 30 and treated in either 3 or 17mM of glucose for 30 mins. at 37°C before removal of supernatant and extraction of lysate using perchloric acid followed by sonication and storage on ice [406]. ATP concentration in the supernatant was measured using a luciferase-based detection kit according to the manufacturer's instructions (ATP Determination Kit, Life Technologies).

2.2.6 *Seahorse Bioscience XF24 assay*

Real-time measurements of mitochondrial oxygen consumption rates (OCR) was achieved using Seahorse XF analyser and the XF24 islet capture FluxPak and XF cell mito stress test kits available (Seahorse Bioscience, USA). Briefly, the XG sensor cartridge provided in the pack was calibrated by incubating overnight at 37°C without CO₂ in XF calibration buffer. 70 size-matched isolated islets were selected per well and washed once in Seahorse Bioscience MA media (8g/L Dulbecco's modified eagle's medium without additives (Sigma, Dorset, UK) and 1% FBS pH 7.4) and 500ul of MA media was added to each well. Islets were carefully positioned to the depression at the bottom of the well and the islet capture screen plates provided were added to the bottom of each well to ensure islets remained in the depression. Whilst the sensor cartridge was being prepared the microplate was incubated at 37°C without CO₂. The compounds added to each port of the XF sensor cartridge are listed in table 2.1 below.

Injection Port	Volume	Concentration in Port	Final Concentration in Well
A: Glucose	50ul	200mM	20mM
B: Oligomycin	55ul	50uM	5uM
C: FCCP	60ul	10uM	1uM
D: Rotenone	65ul	50uM	5uM
D: Antimycin	65ul	50uM	5uM

Table 2.1: Compounds added to each port of XF sensor cartridge

After the XF sensor cartridge is filled with compounds for injection the cartridge was calibrated in the XF analyser. After calibration was completed the XF24 islet capture plate was added to the XF24 analyser to start recordings, each port releasing its provided drug approximately every 30 minutes (going from A-B).

2.2.7 Metabolomic measurements

Islets were isolated and 200 islets per mouse were incubated for 30 mins. with gentle shaking in 17mM glucose. Islets were then centrifuged, the supernatant removed and was quenched with 70µl ice-cold Milli-Q water. Metabolites were extracted and derivatised as described in [407] and analysed on an Agilent 6890N gas chromatograph (Agilent Technologies, Atlanta, GA) prepared with an Agilent 7683B auto—sampler (Agilent Technologies) and joined to a LECO Pegasus III TOFMS electron impact time-of-flight mass spectrometer (LECO Corp., St Joseph, MI) as described in [408]. These experiments were performed by Peter Spiegel, *Unit of Molecular Metabolism, Lund University Diabetes Centre*.

2.2.8 Electron microscopy of islets

Approximatly 100 islets were isolated per mouse, spun at 500r.p.m. for 30 seconds and 1ml of fixative added to islets (2% formaldehyde, 2.5% glutaraldehyde, 3mM CaCl and 0.1M sodium cacodylate buffer (pH 7.4)). Islets were incubated for 30 mins. At 37°C and washed with fresh fixative for a further 2 hours at room temperature. Islets were once again washed in fresh fixative and left at 4°C overnight. Electron microscopy was performed as described in [409] and images were taken by Dr. Alejandra Tomas, *Department of Cell Biology and Functional Genomic, Imperial College London*.

2.3 Protein Experiments

2.3.1 Immunohistochemistry of whole pancreas and ileum sections

Isolated pancreata and ileum sections were fixed in 10% [vol./vol.] formalin, transferred into a tissue processing cassette and embedded in paraffin wax within 24 hours of being harvested from the mouse. Pancreas sections were cut at 5µm and gut sections were cut at 7µm and incubated overnight at 37°C on superfrost slides. Slides were submerged in histoclear (Sigma Aldrich, Dorset, UK) for 20 mins., followed by further sequential washes in decreasing concentrations of ethanol (100, 95, 75% and H₂O) for removal of paraffin wax. Slides were treated with vector antigen solution and subsequently blocked in 0.25% [vol./vol.] goat/donkey serum (Dako, ely, UK) in 2% BSA with 0.1% [vol./vol.] tween for 20 mins. at room temperature. The slides were then incubated in a mixture of the primary antibodies (see appendix 1 for a list of antibodies used) at 4°C in a moist box to ensure slides do not dry out. After washing in PBS, 0.25% bovine serum albumin (BSA) and 0.25% Triton X-100 three times for 10 mins. (each wash), slides were visualised with either Alexa Fluor 568 or 488 conjugated IgG (Invitrogen, Paisley, UK) (see appendix 1 for the full list of antibodies used) and DAPI (Vectalaboratory) under fluorescent microscopy using a Zeiss Axio Observer inverted

widefield microscope with LED illumination and VivaTome spinning disc unit and running Zen acquisition software.

2.3.2 *Optical Projection Tomography and determination of relative β -cell and L-cell mass*

Whole pancreatic and gut optical projection tomography (OPT), to 19 μ m resolution, was performed as described [410]. In short, whole pancreata fixed in 4% [wt./vol.] paraformaldehyde for 3 hours at 4°C were dehydrated and went through five freeze and thawing cycles (-80°C to room temperature). The pancreata were blocked overnight in TBS (pH 7.5), containing 10% goat/donkey serum (Dako, Ely, UK), 0.01% [wt./vol.] sodium azide and 0.05% [vol./vol.] Tween, before incubation with primary antibody (see appendix 1 for list antibodies) dissolved in the above buffer and further supplemented with 5% [vol./vol.] dimethylsulfoxide (DMSO) overnight for 48 hours at room temperature. Samples were visualised using either Alexa Fluor 594 or 568 conjugated IgG (Invitrogen, Paisley, UK), see appendix 1 for full list of antibodies used. The samples were mounted in 1% [wt./vol.] low melting temperature agarose, dehydrated in methanol and cleared in BABB solution (benzyl alcohol: benzyl benzoate [1:2] for optical tomographical scanning.

All specimens were scanned using two fluorescent channels (excitation: 580 $\pm$ 15nm and emission: 650 $\pm$ 25 nm for insulin and glucagon positive staining; excitation: 480 $\pm$ 9 nm and emission: 650 $\pm$ 25 nm for autofluorescence); performed in collaboration with Dr. James McGinty and Dr Chen Lingling, (*Department of Photonics, Imperial College London, UK*). The raw data for these two channels were reconstructed in a pair of 3D voxel data sets (voxel to μ m = 1:19) using Matlab software and cell volumes (μ m³) from each channel were measured using Volocity™ 5.0. Data from Volocity™ 5.0 were further analysed in Microsoft Excel to provide total volume graphs and size frequency histograms. Relative β and L-cell mass was calculated by dividing the volume of the insulin/ glucagon positive area (“total beta/L cell mass”) by that of the autofluorescence area (“total pancreatic/ gut mass”).

2.4 *Molecular Biology experiments*

2.4.1 *DNA extraction and polymerase chain reaction (PCR) for genotyping studies*

Genomic DNA from ear extracts of mice was obtained by lysing samples in 1.25 μ l proteinaseK (Sigma) (20mg/ml in ddH₂O) with 5 μ l 10X PCR-buffer (New England Biolab, Hitchin, UK) and 43.75 μ l ddH₂O at 55°C for 40 mins. and 95°C for 10 mins.

For genotyping using LKB1 and Ins1 Cre primers, the PCR reaction was performed in 20µl reaction mixture containing: 5x Phire™ reaction buffer, 0.2 mmol/l dNTP (Qiagen, Hilden, Germany), 0.5 µmol/l forward and reverse primers (see appendix 2 for list of primers used for genotyping), 0.4 µl Phire™ hot start DNA polymerase (Thermo Scientific) and 1µl DNA. The reaction consists of a 30s 98°C pre-heating step, followed by an amplification step of 30 cycles: 98°C for 5 seconds, T_m 55-60°C for 5 seconds and 72°C for 10 seconds. After the last cycle, a prolonged extension step was carried out for 1 min. at 72°C.

For genotyping iGlu Cre, the PCR reaction was performed in a 25µl reaction mixture containing: 12.5µl 5x GoTaq Green master mix (Promega, UK), 2.5µl primer (see appendix 2), 1.25µl DMSO and 5.75µl H₂O. The reaction consists of a 3 min. 95°C pre-heating step, followed by an amplification step of 40 cycles: 95°C for 1 min., T_m 58°C for 1 min. and 72°C for 1 min.. After the last cycle, a prolonged extension step was carried out for 5 min. at 72°C.

2.4.2 RNA extraction and real-time (RT) PCR

Total cellular RNA from mouse islets was obtained using Trizol reagent (Invitrogen, Paisley, UK), after 10 mins. incubation 0.2 volume of Chloroform (Sigma) was added which was centrifuged at 12000r.c.f. for 15 mins. at 4°C and the supernatant removed and mixed with 2µl glycogen. 0.8 volume of isopropanol was added and incubated at 4°C for 15 mins. The RNA was then centrifuged for 15 mins. and the supernatant removed, the RNA was washed in 70% ethanol and centrifuged at 12000r.c.f. for 5 mins., this step was repeated twice. Total RNA (2µg) was then reversed transcribed into cDNA with a high-capacity reverse transcription kit (Applied Biosystems). The PCR reaction was performed in 20µl of reaction mixture containing: 2µl 10x RT buffer, 0.8µl 25x dNTP mix (100mM), 2µl 10x RT random primers, 1µl MultiScribe™ reverse transcriptase, 4.2µl H₂O and 10µl 500ng/µl DNA. The PCR was heated at 25°C for 10 mins., 37°C for 2 hours and 85°C for 5 mins.

2.4.3 Quantitative Real-Time PCR (qRT-PCR)

The expression levels of genes were quantified by real-time PCR using SYBR green. In 12µl final volume, the reaction contained: 6µl fast SYBR green master mix (Invitrogen, Paisley, UK), 0.35µmol/l forward and reverse primers, and 20ng cDNA (see Appendix 3 for the list of genes and primers). The PCR reaction was preheated at 60°C for 2 mins. and 95°C for 10 mins. PCR amplification was performed for 40 cycles of 95°C for 15 seconds and 60°C for 1 min. At the end of the last cycle, a deissociation step consisting of 95°C for 15 seconds, 60°C for 1 min. and 95°C for 15 seconds was performed.

The PCR cycle numbers required to generate fluorescent signals for each gene were plotted in the equipment software (Fast 7500 v2.0.4, Applied biosystems) showing a shape of a sigmoidal curve (the amplification curve). A horizontal line intersecting the exponential part of the amplification curve above the background was drawn and the corresponding cycle number (Ct) was selected. The relative gene expression against the house keeping gene cyclophilin was calculated by using the equation below [411]:

$$\text{Relative gene expression} = 2^{\text{Ct}(\text{cyclophilin}) - \text{Ct}(\text{gene})}$$

2.4.4 Amplification and purification of high titre adenovirus

Approximately 80% confluent HEK 293 cells in a 25cm² tissue culture flask were infected with 40-50% of primary transfection viral supernatant. After 3-5 days, when 90% cells were expressing green fluorescent protein (GFP) and 30-50% of the cells had detached from the flask, the remaining cells were scraped from the flask and collected into a 15ml conical tube. Cells were centrifuged at 500g at 4°C for 10 mins., resuspended in 15ml sterile PBS and after freezing in a dry ice/methanol bath and thawing in 37°C water bath five times, cells were used to re-infect 90% confluent HEK 293 cells in a 75cm² tissue culture flask. After 3-5 days this process was repeated re-infecting into 3-5 75cm² tissue culture flasks and finally in a final round of large-scale amplification, re-infecting into 20 75cm² tissue culture flasks. One pellet was generated from cells collected from the 20 flasks by centrifuging cell suspension at 500g for 10 mins. After the pellet had been re-suspended in 8ml PBS, another 4 rounds of freeze-thaw cycles were applied. The pellet suspension was mixed thoroughly with caesium chloride -0.1M Tris-HCl (Sigma) (pH 8.0) solution at a ratio of 5:3 and centrifuged at 65,000 r.p.m, 4°C overnight. The virus was collected from the middle layer of the ultra-centrifuge tube and further washed in dialysis buffer (10% [vol./vol.] glycerol, 10 mmol/l Tris (pH 8.0), 10 mmol/l MgCl₂.6H₂O and 150mM NaCl) and resuspended in the same volume of storage buffer (70% [vol./vol.] glycerol, 0.1333% [wt./vol.] BSA, 0.013 mmol/l NaCl and 1.3 mol/l MgCl₂).

2.5 Statistical analysis

Data are expressed as means ± SEM. Significance was tested using two tailed paired or unpaired Student's t test or by ANOVA, unless stated otherwise, using GraphPad Prism 6.0. A value of p<0.05 was considered significant.

Chapter 3

LKB1 deletion in β -cells enhances β -cell mass despite impaired Ca^{2+} and ATP flux

Abstract

Background and Aims: Regulation of glucose-stimulated insulin secretion (GSIS) by the pancreatic β -cell is vital for the preservation of glucose homeostasis and when compromised can lead to some forms of diabetes. The tumour suppressor Liver Kinase B1 (LKB1) exerts its biological effects largely via direct phosphorylation of AMP-activated protein kinase (AMPK) and related kinases. Here, we delete LKB1 in β -cells and examine insulin secretory function in these mice.

Methods: Mice deleted selectively in the β -cell for LKB1 were generated by breeding animals bearing *floxed* alleles at exons 3-6 with the β -cell-selective Ins1Cre deleter line. Optical projection tomography of whole pancreata was performed to assess β -cell mass and radioimmunoassay to assess insulin secretion of isolated islets. Changes in cytosolic ATP/ADP and Ca^{2+} were measured by confocal microscopy of whole islets expressing the recombinant GFP-based probe *Perceval*, or loaded with the fluorescent Ca^{2+} dye, fluo-2, respectively. Seahorse XF24 analysis was used to measure oxygen consumption rate and the fluorescent dye TMRE to measure mitochondrial membrane potential in islets. Metabolomics profiling studies were performed using gas chromatography mass spectroscopy.

Results: β -cell mass increased in LKB1 deleted mice ($P < 0.05$). Despite this, insulin secretion was not affected by LKB1 deletion *in vitro*. We observed significant impairment in $[\text{Ca}^{2+}]_{\text{cyt}}$ and $[\text{ATP/ADP}]_{\text{cyt}}$ increases ($P < 0.01$, 0.05 , respectively) in response to high glucose. Moreover, enhanced signalling by the glutamate receptor agonist kainite lead to intracellular Ca^{2+} changes and insulin secretion being significantly enhanced in these mice ($P < 0.05$).

Conclusions: LKB1 is vital for normal glucose signalling and mitochondrial function in β -cells. However, when deleted other signalling pathways become amplified, such as glutamate signalling, to maintain normal GSIS. These findings thus reveal a new mechanism which may be targeted to increase insulin release in some forms of Type 2 Diabetes.

3.1 Introduction

Regulation of insulin secretion from the pancreatic β -cell is vital for the maintenance of glucose homeostasis, as discussed in chapter 1. GSIS is initiated by the breakdown of glucose to pyruvate through glycolysis and pyruvate is in turn oxidised in the mitochondria leading to increased ATP/ADP ratio. ATP-sensitive potassium ($\text{K}^{+}_{\text{ATP}}$) channels on the plasma membrane of pancreatic β -cells detect an increase in ATP/ADP ratio in the cytoplasm and the closure of this channel initiates the depolarisation of the cell [55, 412]. This results in the opening of voltage-sensitive L-type Ca^{2+} channels and consequently the release of insulin granules from a readily releasable pool (RRP) [60, 69]. The insulin secretory response is biphasic and after secretion from the RRP a slower and more prolonged second phase occurs which requires the recruitment and trafficking of insulin granules to the plasma membrane of the β -cell [35]. Thus, GSIS requires normal mitochondrial function as well as an increase in the ATP/ADP ratio and influx of Ca^{2+} into the cytoplasm of β -cells [413-415]. β -cell dysfunction will result in impairment of GSIS and contributes to some forms of diabetes [416].

As mentioned in chapter 1, LKB1 is an upstream kinase for AMPK and other members of the AMPK family [204] that has previously been implicated in β -cell glucose sensing [228, 417]. LKB1 plays a central role in the determination and maintenance of cell polarity [418-420], cellular metabolism [421] and genome integrity [93]. A link between LKB1 and diabetes was uncovered upon LKB1 deletion in the liver, demonstrating that LKB1 is essential for the beneficial effects of metformin [317]. Additionally, activation of LKB1 via the regulation of AMPK and phosphorylation of TORC2 will prevent gluconeogenesis by preventing synthesis of gluconeogenic enzymes [319].

LKB1 has been previously deleted in pancreatic β -cells of adult mice carrying the Pdx1 CreER promoter as well as in LKB1 *floxed* mice expressing the RIP2 Cre deleter [318-320]. *In vivo* insulin secretion, β -cell proliferation and mass were improved in these mice (~20-65% increase) and MARK became inactivated resulting in alterations in cell polarity [318-320]. These studies indicate that the deletion of LKB1 in β -cells may work as an effective therapy for the treatment of Type 1 and Type 2 Diabetes. However, the precise mechanisms underlying the enhanced insulin secretion in these mice remained unclear.

Several phosphorylation targets for LKB1 have been suggested as positive regulators of GSIS such as SIK2, which improves insulin secretion via the degradation and phosphorylation of CDK5RI/p53 [422] and SAD-A, which regulates GSIS and β -cell size [423]. Nevertheless, deletion of these genes results in impairment of GSIS [422, 423]. Deletion of LKB1 prevents phosphorylation of these proteins,

despite this GSIS becomes enhanced overall. Therefore, it is thought that an alternative pathway may be present for normal GSIS to occur.

The neurotransmitter glutamate is thought to play a role in GSIS [185, 187, 189, 190, 194] and though primarily expressed in the brain, both ionotropic and metabotropic glutamate receptors are present in the pancreatic β -cell [185]. The precise mechanism by which glutamate affects insulin secretion remains unclear. RNASeq analysis in islets of mice deleted for LKB1 revealed an up-regulation of the genes *Nptx2*, *Gria3* and *Dlgap2*, involved in glutamate signalling. Thus, *Gria3* encodes for the ionotropic glutamate receptor GluR1 [424], *Dlgap2* encodes large homologue-associated protein 2 which is a membrane-associated protein which makes up part of the postsynaptic density scaffold [425] and *Nptx2* is involved in synaptic formation and in the clustering of AMPA receptors.

In this chapter we use Ins1Cre recombinase to delete LKB1 *floxed* alleles and so generate β -cell selective null alleles. Ins1Cre induces deletion in approximately 94% of pancreatic β -cells from the immediate postnatal period to adulthood therefore differing from the previously used Pdx1CreER transgene which was induced in 8-10 week old mice [426] and the RIP2Cre promoter which deletes in the hypothalamus as well as the β -cell [320]. Thus, we aimed to determine the role of LKB1 in the β -cell, GSIS and whether alterations of glutamate signalling contributes to the LKB1 phenotype.

3.2 Results

3.2.1 *Ins1LKB1KO improves β -cell mass and has no effect on insulin secretion in vitro*

As shown by Kone *et al*, 2014 [427], glucose tolerance and plasma insulin measurements are significantly improved in Ins1LKB1KO mice *versus* littermate controls (Appendix 4). To study β -cell mass in these mice we used OPT, allowing us to analyse (alongside conventional immunohistochemistry) mean, average, total volume, size and number of islets within the whole pancreas, see materials and methods 2.3.2 (Figure 3.1a-e). Relative β -cell mass was increased in Ins1LKB1KO versus WT mice, $P < 0.05$ ($1.50 \pm 0.30\%$, $0.80 \pm 0.09\%$, respectively). Overall, islet number was greater in Ins1LKB1KO mice and in addition, the number of larger islets ($3000\text{--}40000\mu\text{M}^3 \times 10^3$) was also higher (Figure 3.1e). Expression of the insulin gene *Ins2* was up-regulated in islets of Ins1LKB1KO mice (WT: 1.297 ± 0.137 , KO: 2.462 ± 0.045 normalised to cyclophilin, $P < 0.01$) as was, to a lesser extent *Ins1* expression, NS (WT: 1.13 ± 0.25 , KO: 1.69 ± 0.10 normalised to cyclophilin, Figure 3.2a-b). This suggests that insulin production is up-regulated in Ins1LKB1KO mice.

As glucose tolerance and β -cell mass (Figure 3.1) were exaggerated in Ins1LKB1KO in comparison to WT mice, we anticipated an improvement in GSIS *in vitro*. Six size-matched islets per well were incubated in either 3mM or 17mM glucose and secreted and total insulin were measured using radioimmunoassay. As expected, in both Ins1LKB1 KO and WT islets insulin secretion was enhanced when islets were incubated in 17mM glucose in comparison to 3mM (WT: $P < 0.01$, KO: $P < 0.05$, Figure 3.3). Surprisingly, when comparing the rate of insulin secretion between Ins1LKB1KO and WT islets, results were similar (3mM WT: $0.78 \pm 0.27\%$, KO: $1.02 \pm 0.22\%$; 17mM WT: $2.17 \pm 0.60\%$, KO: $2.81 \pm 0.50\%$).

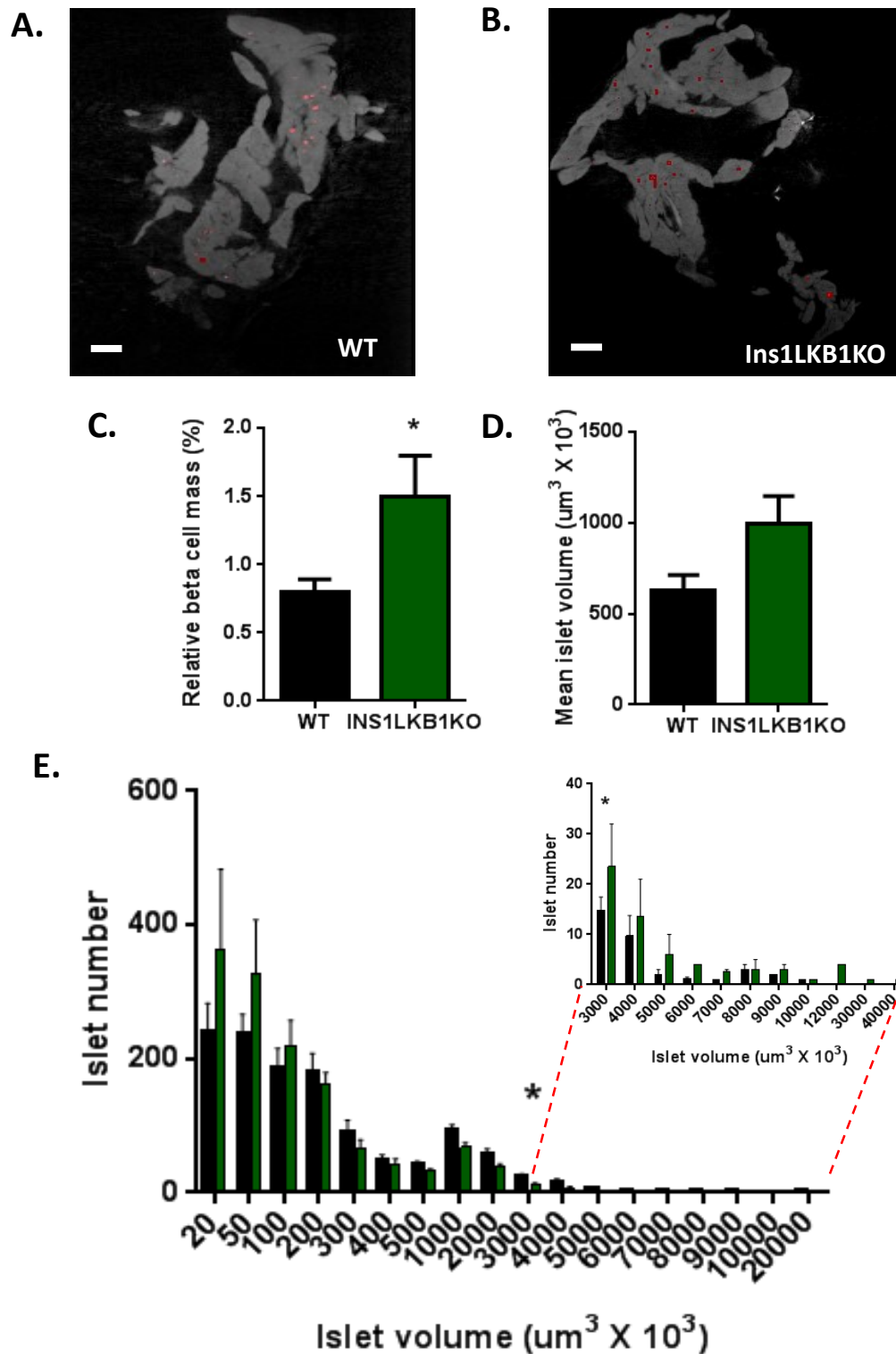


Figure 3.1 - β -cell mass is enhanced in Ins1LKB1KO mouse pancreata

(A,B) Representative OPT images of fixed and permeabilised pancreatic tissue. Red, insulin-positive structures within the pancreas. The overall shape of the pancreas was visualised as autofluorescence and is apparent as white/grey shading. Scale bar, 500 μm . (C) Relative β -cell mass and (D) mean β -cell volume. (E) Distribution of pancreatic volume with dashed lines representing the magnified section. Data are from $n=4$ pancreatic tissue per genotype and expressed as means $\pm$; * $p < 0.05$, by Student's unpaired t-test.

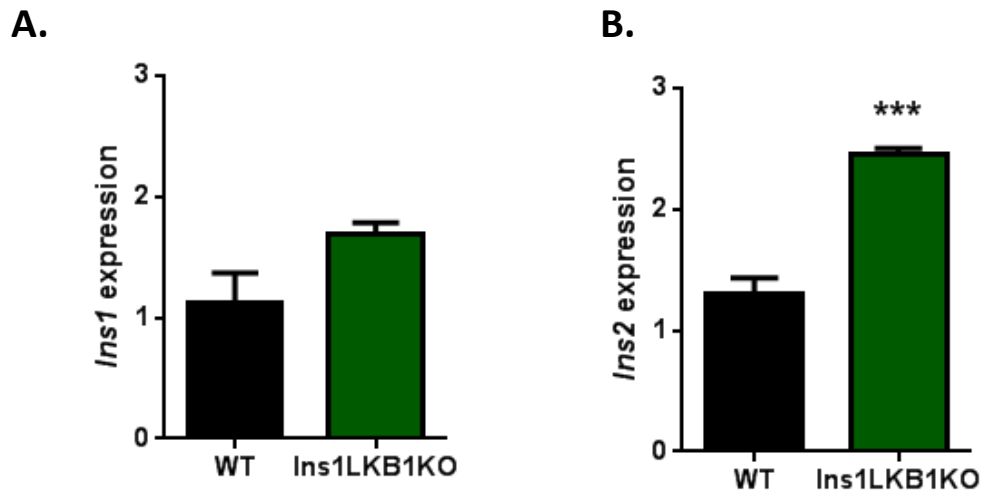


Figure 3.2 - Gene expression of *Ins1* and *Ins2* are up-regulated in Ins1LKB1KO islets

Levels of gene expression of (A) *Ins1* and (B) *Ins2* genes were measured in WT and Ins1LKB1KO mice normalising with β -actin. Data are expressed as means $\pm$ SEM, $n=3$ mice/genotype, *** $P<0.001$, by Student's unpaired t-test.

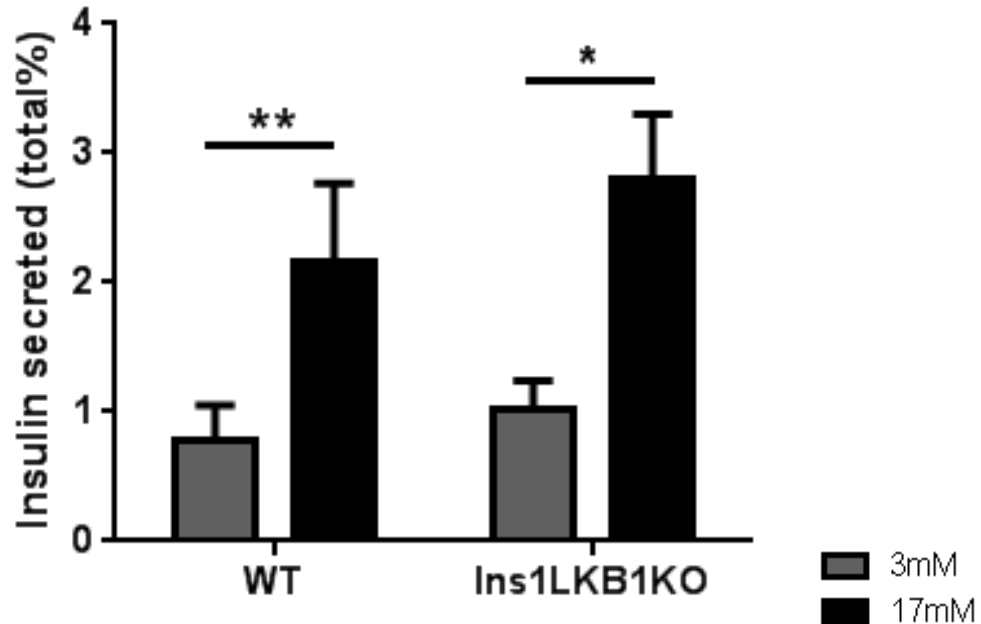


Figure 3.3 - Insulin secretion is unaffected in Ins1LKB1KO islets

Insulin secretion was measured in 6 size-matched islets isolated from WT and Ins1LKB1KO mice and incubated in either basal (3mM) or 17mM glucose. Data are expressed as means $\pm$ SEM, $n=4$ mice/genotype, * $P<0.05$, ** $P<0.01$, by 2-way ANOVA.

3.2.2 Defects in ATP and Ca^{2+} in Ins1LKB1KO occur independently of insulin secretion

As mentioned earlier and shown in the previous LKB1 KO mouse models [318-320] *in vivo* insulin secretion and β -cell mass are increased in Ins1LKB1KO *versus* WT mice (Figures 3.1, 3.2). However, *in vitro* insulin secretion is unchanged in Ins1LKB1KO *versus* WT islets (Figure 3.3). To further investigate the functionality of these islets *in vitro*, islets from Ins1LKB1KO and WT mice were isolated and cytosolic ATP/ADP ($[\text{ATP/ADP}]_{\text{cyt}}$) and Ca^{2+} ($[\text{Ca}^{2+}]_{\text{cyt}}$) levels measured after glucose stimulation (11mM) using confocal microscopy (Figures 3.4 and 3.6).

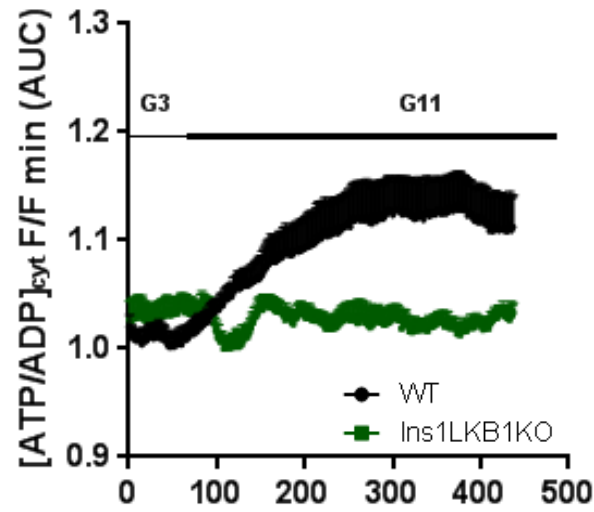
For normal insulin secretion, increasing the concentration of glucose in the β -cell results in elevated cytosolic ATP/ADP levels; this is vital for closure of K_{ATP} channels and subsequent depolarisation of the plasma membrane. $[\text{ATP/ADP}]_{\text{cyt}}$ was measured in isolated islets after 48 hours expression of the ATP sensor *Perceval* (chapter 2.2.4) and response to glucose was found to be impaired in islets of Ins1LKB1KO *versus* WT mice ($P < 0.05$, WT: $116.2 \pm 3.46\%$, KO: $106.6 \pm 1.12\%$, Figure 3.4b). In addition, the percentage of cells responsive to rising glucose concentrations was also greatly reduced in these Ins1LKB1KO islets ($P < 0.05$ WT: $37.64 \pm 4.51\%$, KO: $22.75 \pm 2.90\%$, Figure 3.4c). We further demonstrate that ATP production is down-regulated in the islets of these mice by the use of a luciferase based ATP kit which, though not significant, demonstrated a decreasing trend of ATP detection in Ins1LKB1KO mice (NS, 3mM WT: $52.59 \pm 11.84\text{pmol/islet}$, KO: $38.47 \pm 13.61\text{pmol/islet}$; 17mM WT: $238 \pm 36.34\text{pmol/islet}$, KO: $168.4 \pm 60.51\text{pmol/islet}$, Figure 3.5).

These results suggest that surprisingly normal cytosolic signalling is disrupted, despite normal insulin secretion. To further investigate the glucose-ATP- Ca^{2+} response, $[\text{Ca}^{2+}]_{\text{cyt}}$ was measured using Fluo-2AM, a sensitive indicator for intracellular free ions. In stimulation of normal insulin secretion the surge in $[\text{Ca}^{2+}]_{\text{cyt}}$ is the main trigger for insulin exocytosis [57]. By measuring $[\text{Ca}^{2+}]_{\text{cyt}}$ we further confirmed that the fraction of responding β -cells is smaller in Ins1LKB1KO islets ($P < 0.001$, WT: $66.88 \pm 6.07\%$, KO: $28.67 \pm 6.13\%$, Figure 3.6c), as is the amplitude of $[\text{Ca}^{2+}]_{\text{cyt}}$ response ($P < 0.01$, WT: 180.1 ± 5.03 , KO: 142.9 ± 5.93 , Figure 3.6a-b). Thus Ins1LKB1KO islets have a defect in glucose-stimulated Ca^{2+} entry which remarkably does not interfere with normal GSIS.

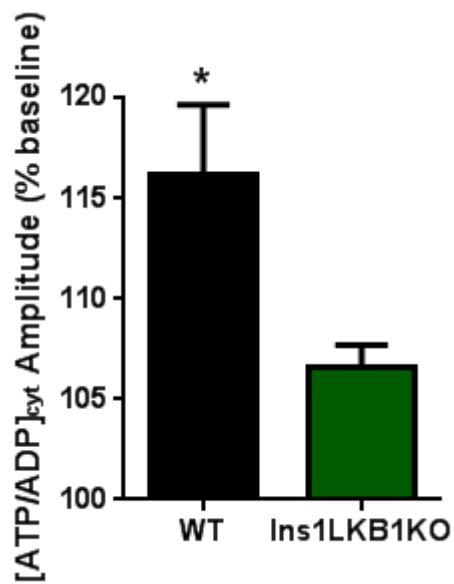
To examine whether this defective cytoplasmic signalling function in Ins1LKB1KO islets acts in a cell autonomous manner, we re-introduced WT LKB1 by adenoviral infection (Ad-STK11, Vector Biolabs) into cultured Ins1LKB1KO islets. We found that within 48 hours of infection, levels of LKB1 in Ins1LKB1KO islets were restored (Figure 3.7a). In these islets, both cytosolic ATP and Ca^{2+} fluxes were restored to near normal levels ($[\text{ATP/ADP}]_{\text{cyt}}$ WT: $118.2 \pm 15.09\%$, KO: 74.91 ± 7.28 , LKB1 adeno:

113.8 ± 13.86 , $P < 0.05$ WT *versus* Ins1LKB1KO, Figure 3.7b; $[\text{Ca}^{2+}]_{\text{cyt}}$ WT: $147.4 \pm 6.10\%$, KO: $78.90 \pm 15.72\%$, LKB1 adeno: $135.4 \pm 2.03\%$, $P < 0.001$ WT *versus* Ins1LKB1KO, $P < 0.01$ Ins1LKB1KO *versus* LKB1 adeno). The percentage of cells responding to glucose stimulation was also restored to near normal levels ($[\text{ATP}/\text{ADP}]_{\text{cyt}}$ WT: $62.90 \pm 2.22\%$, KO: $47.71 \pm 6.39\%$, LKB1 adeno: $73.80 \pm 4.50\%$, $P < 0.01$ Ins1LKB1KO *versus* LKB1 adeno, Figure 3.7d; $[\text{Ca}^{2+}]_{\text{cyt}}$ WT: $75.20 \pm 6.24\%$, Ins1LKB1KO: $53.50 \pm 3.62\%$, LKB1 adeno: $64.33 \pm 7.17\%$, $P < 0.05$ WT *versus* Ins1LKB1KO, Figure 3.7g). These experiments indicate that defects in Ins1LKB1KO cell signalling are a direct effect of LKB1 loss in β -cells, rather than an indirect compensatory mechanism.

A.



B.



C.

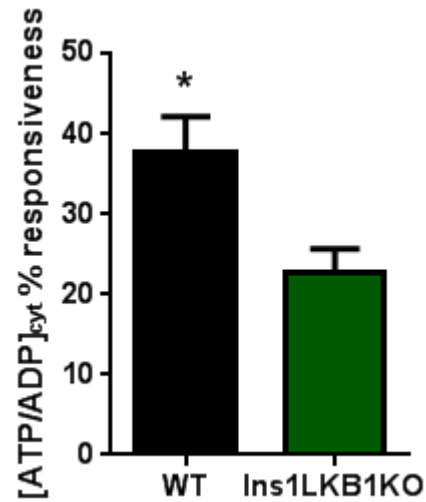


Figure 3.4 - Glucose-induced $[\text{ATP}/\text{ADP}]_{\text{cyt}}$ changes are drastically impaired in Ins1LKB1KO versus WT mouse islets

(A) $[\text{ATP}/\text{ADP}]_{\text{cyt}}$ changes in WT versus Ins1LKB1KO islets (B) quantitation of amplitude and (C) % of islets or cells responsive to glucose. Data are expressed as means $\pm$ SEM, $n=3$ mice/genotype and 12 islets/genotype, * $P<0.05$, by Student's unpaired t-test.

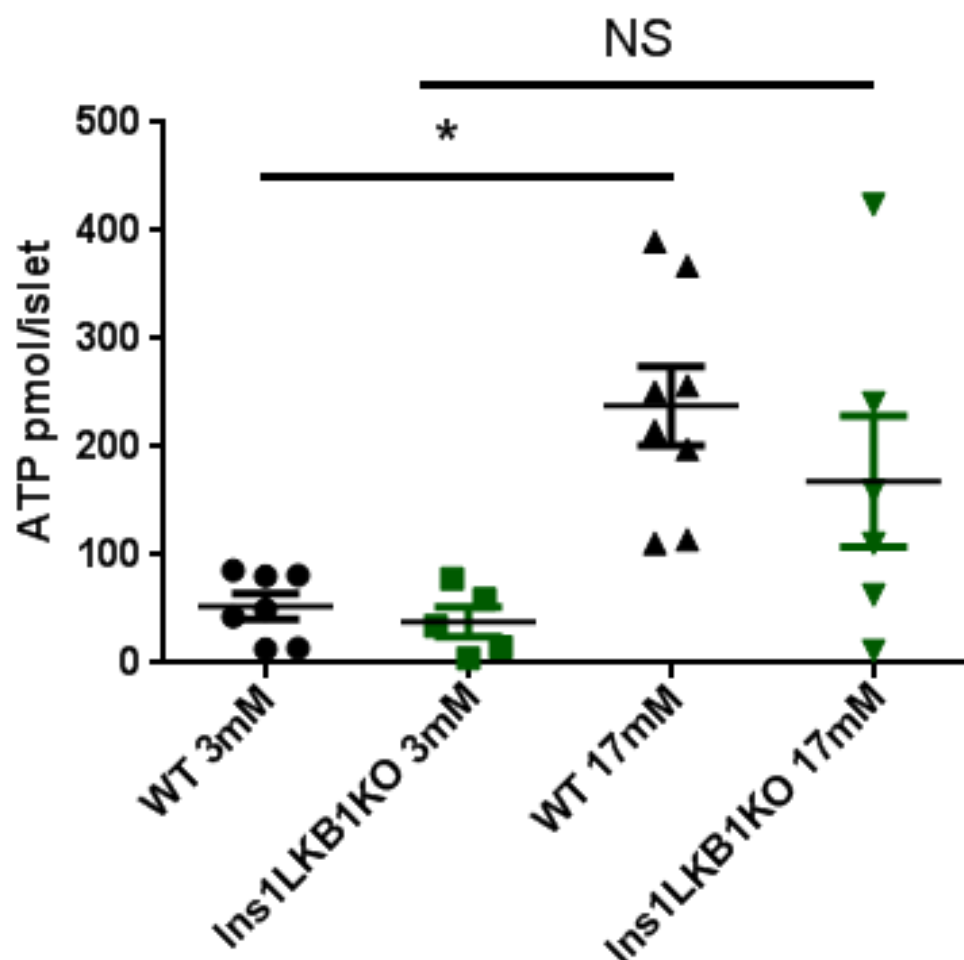


Figure 3.5 - Luciferase-based measurements of ATP concentrations in glucose-treated islets
Islets from WT and Ins1LKB1KO mice were incubated in 3 or 17mM glucose and ATP levels measured in the luciferase based ATP assay after 30 mins. Data are expressed as means $\pm$ SEM, $n=3$ mice/genotype, * $P<0.05$, by 2-way ANOVA.

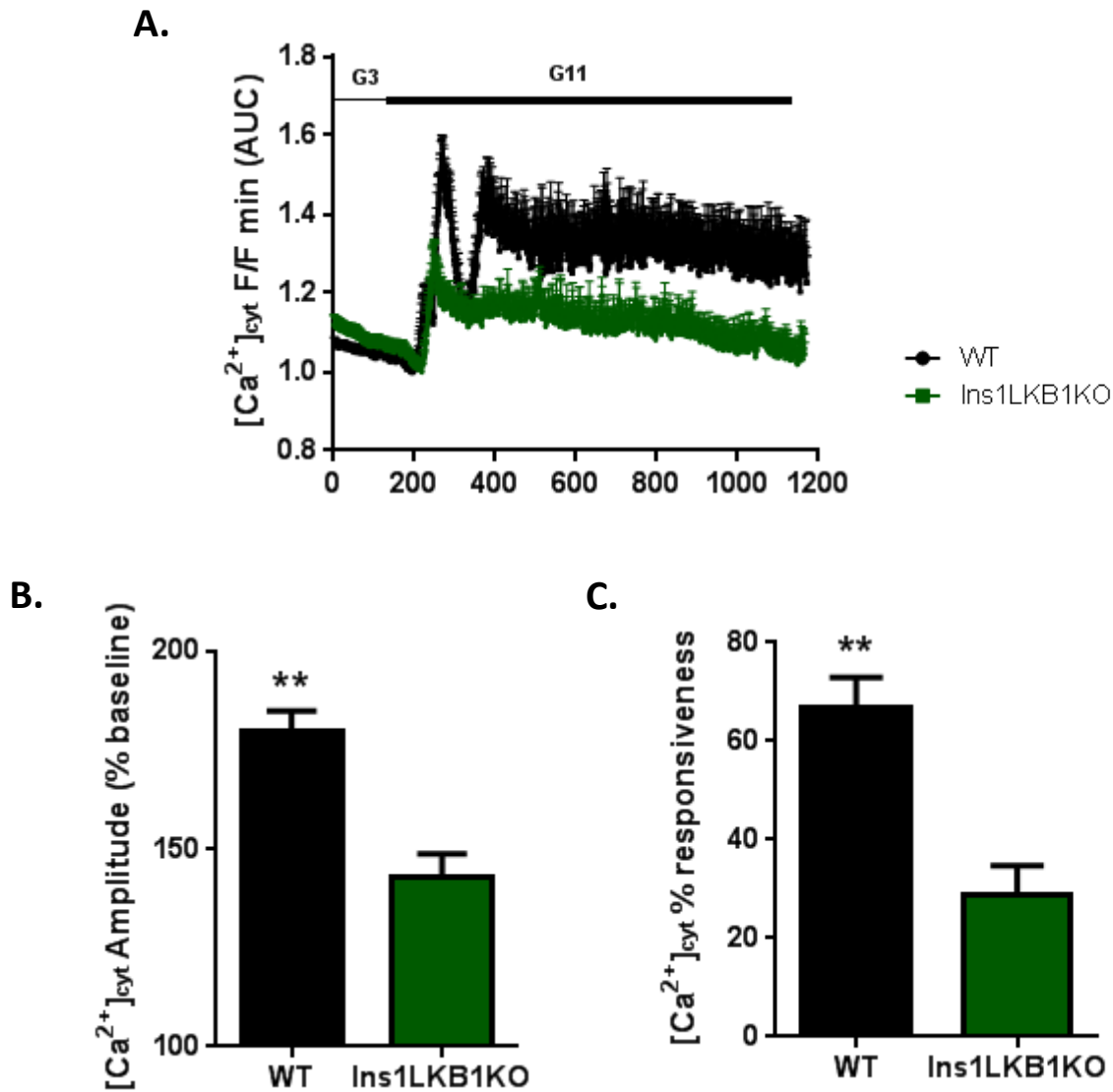


Figure 3.6 - $[\text{Ca}^{2+}]_{\text{cyt}}$ changes are drastically impaired in Ins1LKB1KO islets

(A) Glucose-induced $[\text{Ca}^{2+}]_{\text{cyt}}$ dynamics in islets from WT and Ins1LKB1KO mice, (B) quantitation of amplitude and (C) % of islets or cells responding to glucose. Data are expressed as means $\pm$ SEM, $n=3$ mice/genotype and 12 islets/genotype, ** $P<0.01$, by Student's unpaired t-test.

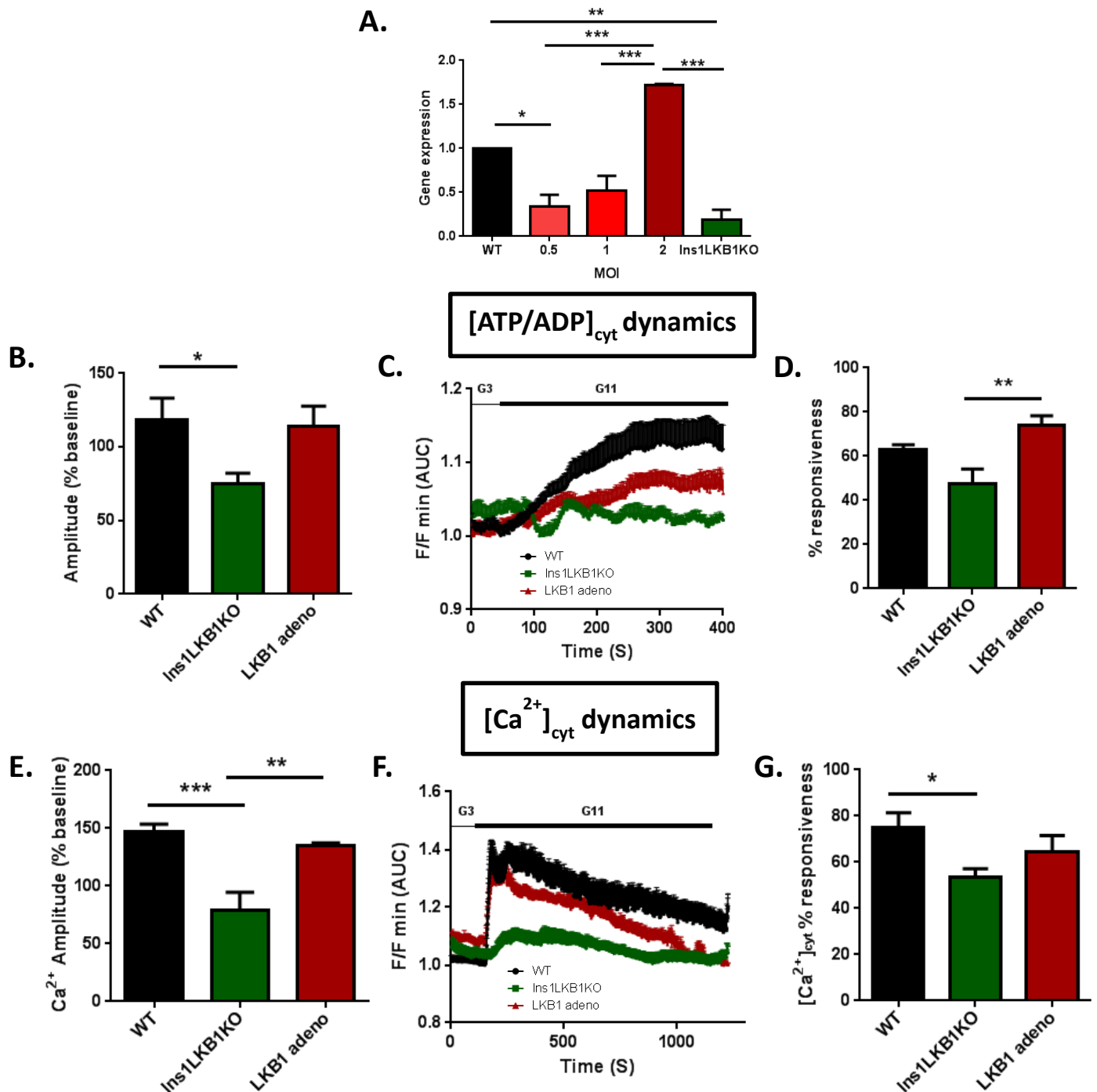


Figure 3.7 - *In vitro* rescue of LKB1 expression results in restoration of $[\text{ATP/ADP}]_{\text{cyt}}$ and $[\text{Ca}^{2+}]_{\text{cyt}}$ signalling in Ins1LKB1KO mice

(A) Expression of LKB1 was measured in WT and Ins1LKB1KO islets infected with the adenoviruses encoding WT LKB1 at the multiplicity of infection (MOI) indicated, $n=3$ mice/genotype (B) Rescue of glucose-stimulated calcium influx in Ins1LKB1KO islets. Both the amplitude (left) and the fraction of responding cells (right) returned to normal upon LKB1 re-expression, $n=3$ mice/genotype, 9-12 mice/genotype (C) Rescue of glucose-stimulated $[\text{ATP/ADP}]_{\text{cyt}}$ rise in Ins1LKB1KO islets, both amplitude (left) and the % of responding islets (right) are improved upon LKB1 overexpression $n=5$ mice/genotype, 9-15 islets/genotype. Data are expressed as means $\pm$ SEM, * $P<0.05$, ** $P<0.01$, *** $P<0.001$, by one-way ANOVA.

3.2.3 **LKB1 is required for normal mitochondrial function**

Glucose entry into β -cells triggers Ca^{2+} influx and insulin exocytosis through oxidative phosphorylation in the mitochondria and generation of ATP, resulting in K_{ATP} channel closure. Given the defects found in $[\text{Ca}^{2+}]_{\text{cyt}}$ and $[\text{ATP}/\text{ADP}]_{\text{cyt}}$ KO islets, we predicted that mitochondrial metabolism may also be impaired in islets of Ins1LKB1KO mice.

We first examined mitochondrial membrane potential in isolated islets using the mitochondrial membrane potential sensitive dye TMRE by confocal imaging (Figure 3.8). Ins1LKB1KO islets showed a reduction in TMRE staining in response to high glucose ($P < 0.01$, WT: $119.3 \pm 4.14\%$, KO: $96.09 \pm 6.38\%$, Figure 3.8a-b), though the % of cell responsive was not affected (NS, WT: $70.91 \pm 7.30\%$, KO: $60.14 \pm 9.76\%$, Figure 3.8c). These results indicate some degree of mitochondrial dysfunction in Ins1LKB1KO islets.

To further assess metabolic regulation in the absence of LKB1 we performed metabolite profiling to determine which metabolites were affected in KO isolated islets after incubation in 17mM glucose (Figure 3.9). Data was analysed using orthogonal projections to latent structures- discriminant analysis (OPLS-DA) (data not shown). A shared-and-unique-structure (SUS)-like plot, revealed differences in metabolite levels in KO in comparison to WT islets (Figure 3.9a). This showed that fructose-6-phosphate was higher in Ins1LKB1KO islets whereas TCA-metabolites and FFAs were all down-regulated in these islets. Several FA derivatives levels were statistically higher in WT *versus* Ins1LKB1KO islets ($P < 0.05$, Figure 3.9b). Other metabolites which tended to be up-regulated included citrate (NS, $P < 0.092$), succinate (NS, $P < 0.096$) and fumerate (NS, $P < 0.079$) as well as glycerol (NS, $P < 0.066$). On the other hand, fructose-6-phosphate was significantly increased in Ins1LKB1KO islets *versus* WT islets, $P < 0.001$. Metabolite measurements were performed by Peter Spiegel, *Unit of Molecular Metabolism, Lund University Diabetes Centre*.

To further assess mitochondrial flux, we also measured oxygen consumption rate (OCR) in mitochondria of Ins1LKB1KO islets using a XF24 seahorse analyser (Figure 3.10 and 3.11). To determine the optimal concentrations of drugs to use for this experiment, islets were stimulated with varying concentrations of glucose, oligomycin and mesoxalonnitrile 4-trifluoromethoxyphenylhydrazon (FCCP) (Figure 3.10).

Oligomycin was added to islets to block ATP synthesis by inhibiting ATP synthase (complex V), thus allowing us to distinguish oxygen consumption dedicated to ATP synthesis and the proportion of oxygen consumption necessary to overcome the natural protein leak through the inner

mitochondrial membrane. ATP production can be calculated by comparing the decrease in OCR after oligomycin is added to islets with basal respiration. Proton leak can also be quantified by comparing the OCR in islets after oligomycin is added with non-mitochondrial respiration. We determined that islets responded best to oligomycin at 1 μ M Figure 3.10b.

The uncoupling agent FCCP disrupts ATP synthesis by carrying hydrogen ions across the mitochondrial membrane thereby collapsing mitochondrial membrane potential and resulting in the rapid consumption of oxygen without the generation of ATP. Exposing islets to FCCP allows us to calculate the “spare respiratory capacity” of islets, which is the quantitative changes between maximal uncontrolled OCR, and the original basal OCR. FCCP was found to work most efficiently at 1 μ M. As concentrations of FCCP increased OCR dropped as islets appear to die (figure 3.10c).

Rotenone (a complex I inhibitor) and antimycin (a complex III inhibitor) prevents mitochondrial respiration allowing the calculation of both the mitochondrial and non-mitochondrial fractions that contribute to respiration.

Following optimisation, Seahorse experiments in islets from Ins1LKB1KO mice and their WT littermates were conducted by injecting islets with these above four drugs (including antimycin and rotenone) at different stages throughout the recordings as explained in 2.2.8, Figure 3.11a and table 2.1. As expected OCR increased after glucose stimulation (port A) but was unaltered in Ins1LKB1KO *versus* WT islets.

OCR was unaltered between Ins1LKB1KO and WT islets after oligomycin was added, Figure 3.11a. There also appears to be no significant changes in proton leak between these islets (NS, WT: 35.25 $\pm$ 1.53%, KO: 40.45 $\pm$ 2.85%, Figure 3.11d), or in the rate of ATP production (NS, WT: 24.40 $\pm$ 5.76%, KO: 27.15 $\pm$ 6.01%, Figure 3.11b).

Interestingly, we found that the % OCR was significantly higher in Ins1LKB1KO *versus* WT islets after FCCP injection, $P < 0.05$ (Figure 3.11a). Thus, the maximal respiration of KO islets was elucidated *versus* WT, (NS, WT: 95.80 $\pm$ 7.20%, KO: 130.5 $\pm$ 14.89%, Figure 3.11c) as well as the spare respiratory capacity ($P < 0.05$, WT: 47.20 $\pm$ 12.53%, KO: 102.9 $\pm$ 17.13%, Figure 3.11e). When we look at OCR at 20mM glucose *versus* FCCP we see a significant difference between Ins1LKB1KO and WT islets ($P < 0.01$, WT: 0.88 $\pm$ 0.04%, KO: 0.64 $\pm$ 0.04%, Figure 3.11g).

Finally, to assess any effect LKB1 has on mitochondrial structure, EM was carried out on pancreatic sections of WT *versus* Ins1LKB1KO mice (Figure 3.12). These images show no changes in mitochondrial structure.

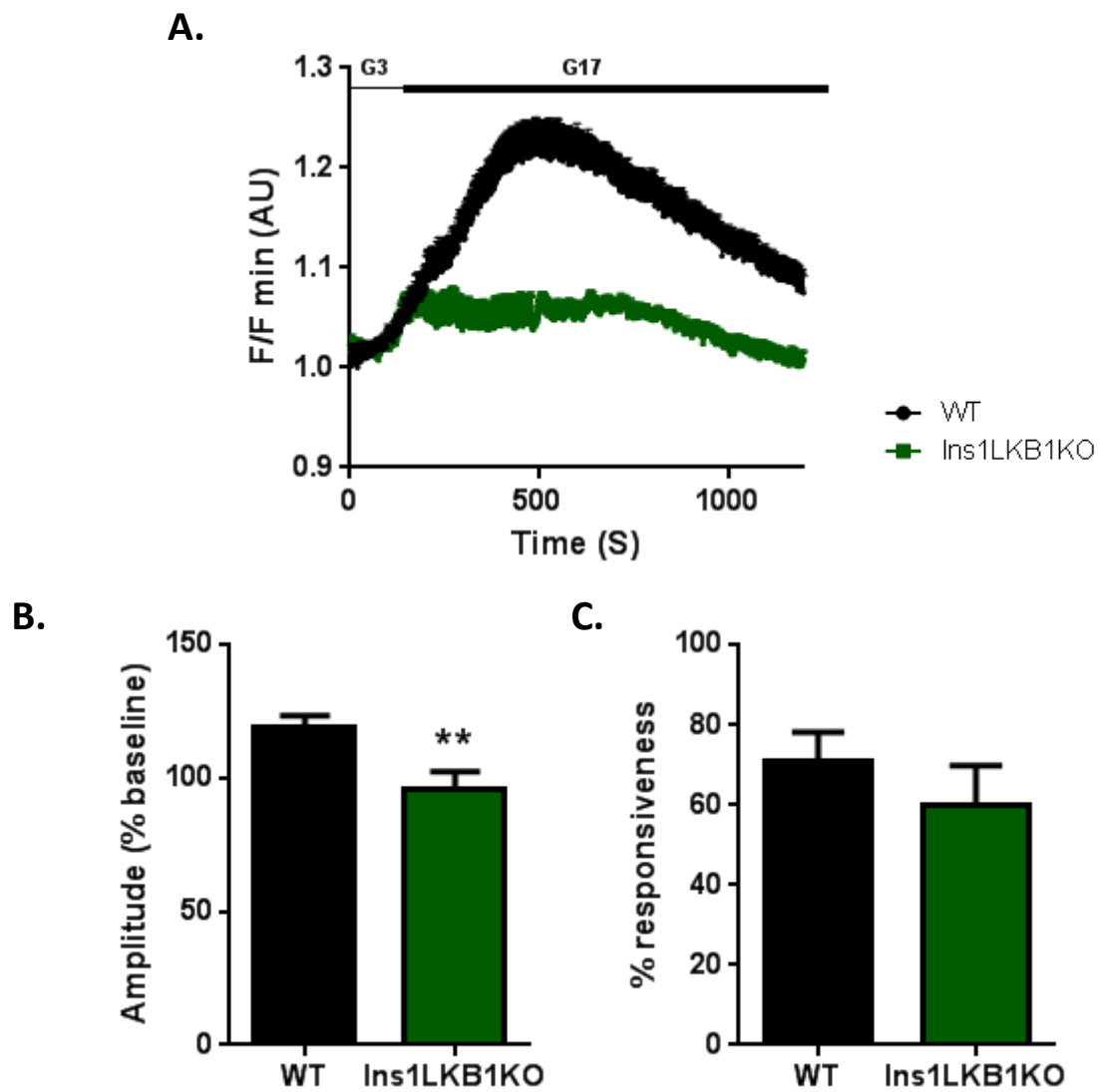


Figure 3.8 - Mitochondrial defects are present in Ins1LKB1KO mice

Mitochondrial membrane potential was measured in WT and Ins1LKB1KO islets. (A) Quantification of fluorescence intensity of TMRE, (B) amplitude and (C) % of islets responsive to stimulation. Data are expressed as means $\pm$ SEM, $n=3$ mice/genotype, 9-12 islets/genotype, ** $P<0.01$, by Student's unpaired t-test.

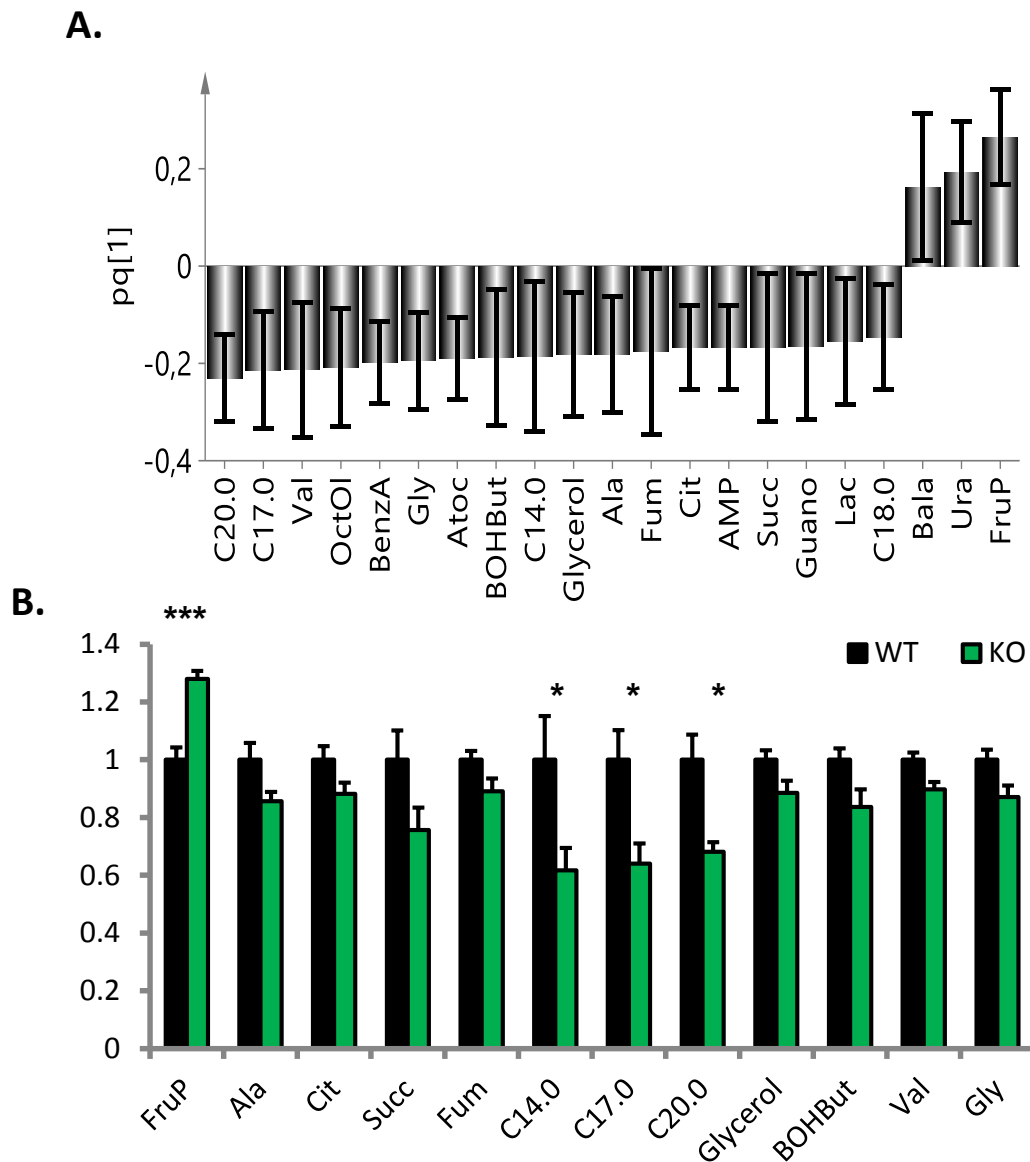


Figure 3.9 - Metabolomic profiling data show that several citrate cycle intermediates and FA's are down-regulated in Ins1LKB1KO islets whereas fructose-6-phosphate is up-regulated

(A) Significant loadings, fold change of metabolites in KO islets in comparison to WT islets. (B) Student's unpaired t-tests confirming this fold change. C14.0, C17.0 and C20.0 represent FA derivatives. Data are expressed as means $\pm$ SEM, $n=4$ mice/genotype (200 islets per mouse), * $P<0.05$, ** $P<0.01$.

Metabolomic experiments were performed by Peter Spiegel, *Unit of Molecular Metabolism, Lund. University Diabetes Centre.*

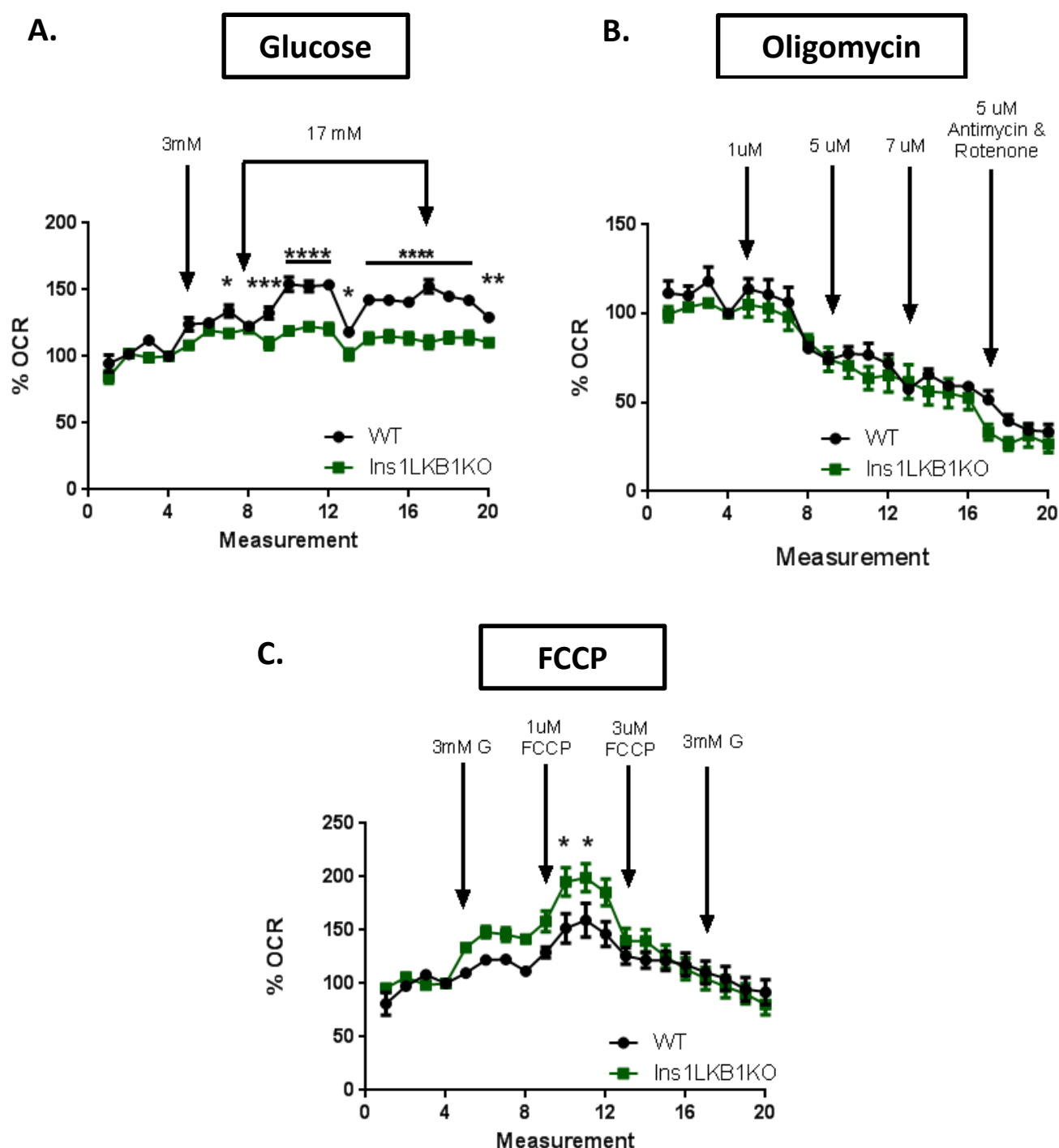


Figure 3.10 - OCR measured by Seahorse XF24 analyser in the presence of glucose, oligomycin, FCCP, rotenone and antimycin

Each plot represents the mean of 6 wells with 70 islets from WT and Ins1LKB1KO mice, (A) Port A injecting 3mM of glucose and ports B, C and D injecting 17mM glucose, (B) Ports A, B and C injected increasing concentrations of oligomycin and port D injecting 5uM rotenone and antimycin, (C) Ports B and C injecting 1 and 3uM (respectively) FCCP. Data are expressed as means $\pm$ SEM, $n = 6$ mice/genotype, * $P < 0.05$, *** $P < 0.01$, **** $P < 0.001$, by 2-way ANOVA.

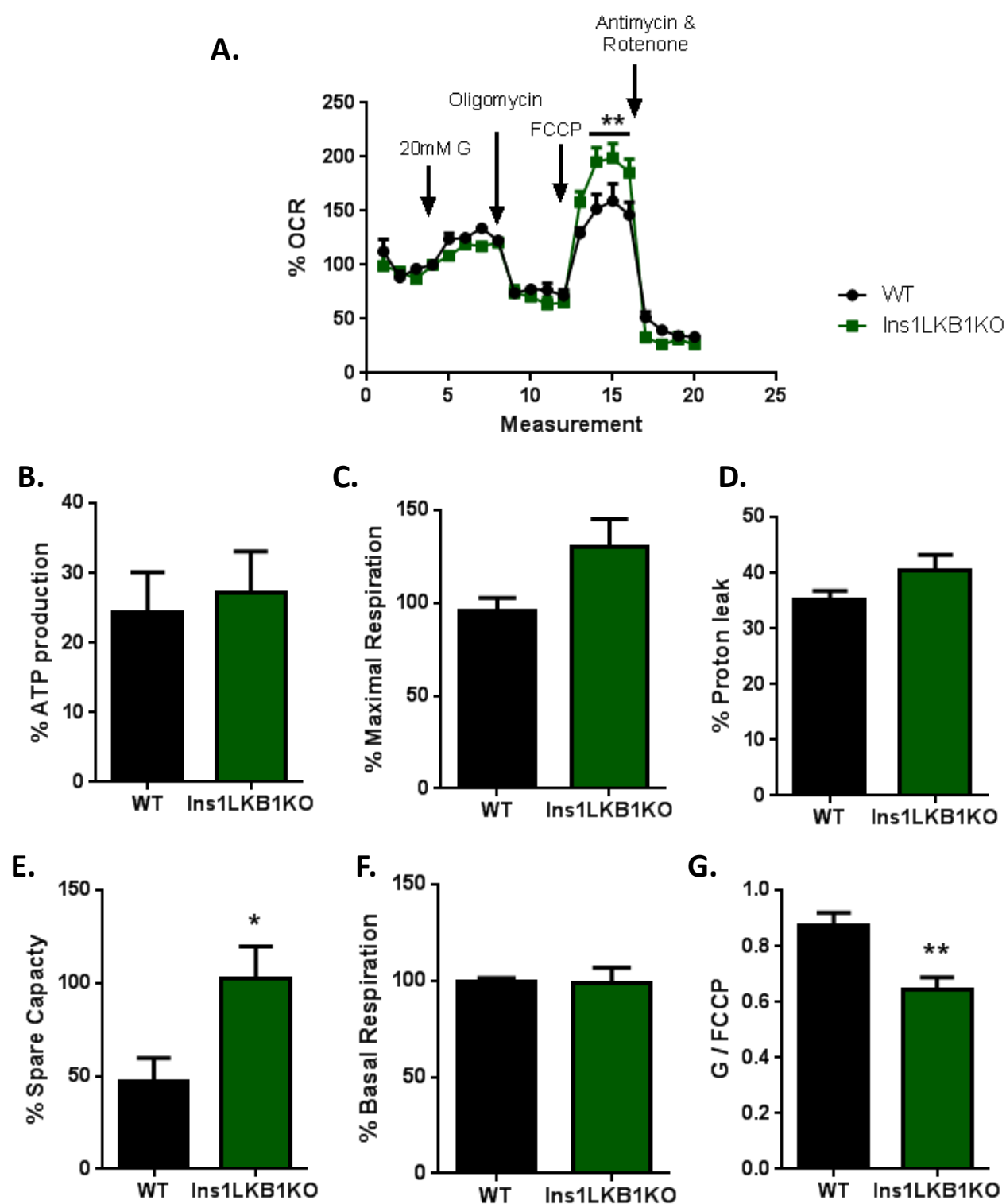


Figure 3.11 - Respiratory capacity is up-regulated in Ins1LKB1KO islets

OCR was measured in Ins1LKB1KO and WT islets using 70 islets/well/mouse. (A) % OCR measured over time, data is presented as % change from measurement 4, (B) ATP production, (C) proton leak, (D) maximal respiration and (E) spare capacity, (F) % Basal respiration, (G) OCR of glucose/ FCCP stimulated islets. Data are expressed as means $\pm$ SEM, $n = 5$ mice/genotype, * $P < 0.05$, ** $P < 0.01$, by 2-way ANOVA and Student's unpaired t-test.

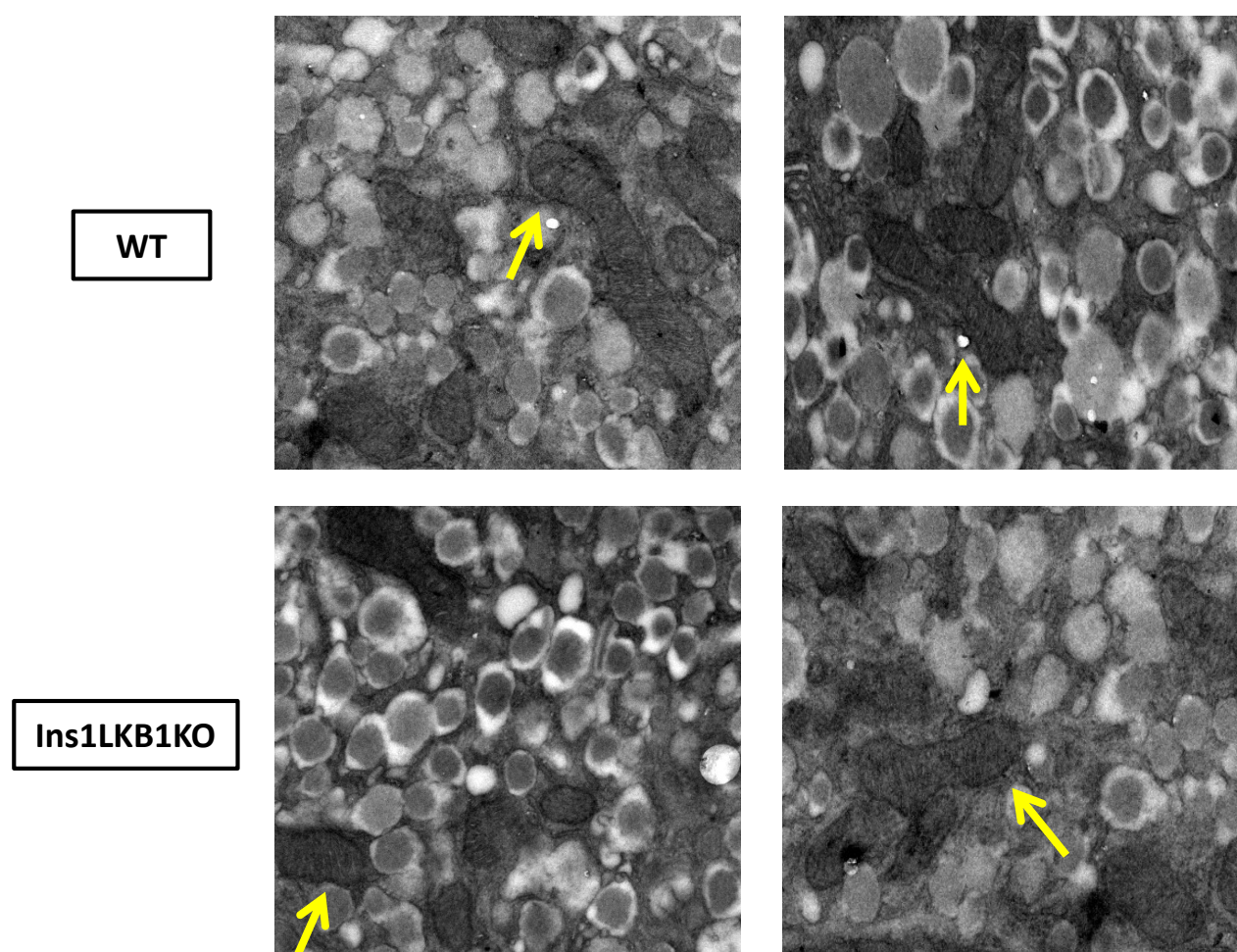


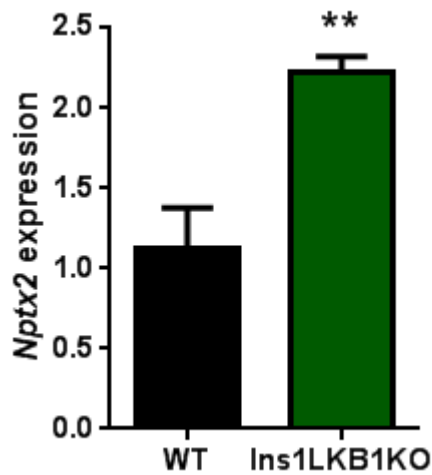
Figure 3.12 - LKB1 is not essential for normal mitochondrial integrity in the β -cell
Representative EM traces of pancreatic sections showing mitochondria (yellow arrows) in Ins1LKB1KO and WT mice, mitochondria are found intact in both mouse models.

3.2.4 Glutamate signalling is up-regulated in *Ins1LKB1KO* mice

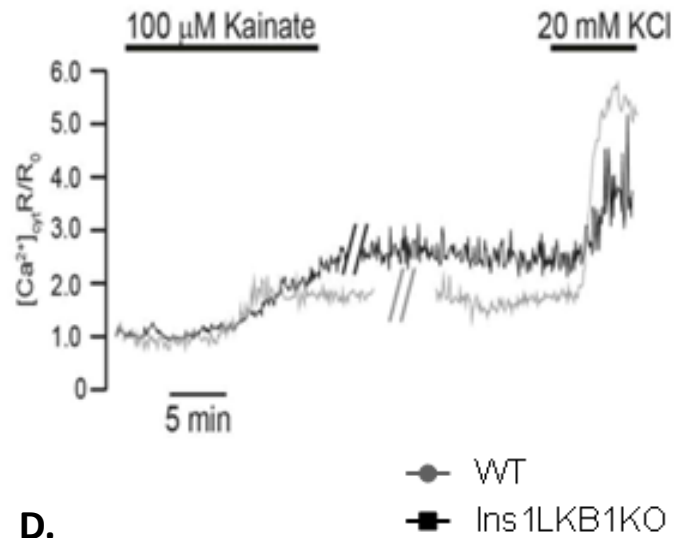
As mentioned previously, RNAseq experiments performed on islets from *Ins1LKB1KO* versus WT mice revealed an up-regulation of certain genes involved in glutamate signalling [427]. These genes include *Dlgap2*, *Nptx2* and *Gria3* (3.60, 4.48 and 1.12-fold increase, respectively) [427]. We further confirmed the increase of *Nptx2* expression in islets at the RNA level using qPCR ($P < 0.01$, WT: 1.12 ± 0.36 , KO: 2.22 ± 10 , normalised to cyclophilin, Figure 3.13a) and at the protein level using immunocytochemistry on whole pancreatic paraffin embedded slices (these experiments were performed by Dr Marie-Sophie Nguyen Tu, Appendix 5).

To investigate glutamate signalling, β -cells were dissociated from isolated islets and perforated patch clamping studies were performed where *Ins1LKB1KO* and WT β -cells were perfused with the glutamate receptor agonist kainite, binding to and activating ionotropic glutamate receptors [424] (Figure 3.13b-d). Perforated patch traces (Figure 3.12b) revealed that kainite stimulation leads to a more pronounced rise in cytosolic free Ca^{2+} ($[\text{Ca}^{2+}]_{\text{cyt}}$) in β -cells from *Ins1LKB1KO* mice versus WT ($P < 0.001$, WT: $203.3 \pm 14.53\%$, KO: $288.8 \pm 21.99\%$, Figure 3.13c). Similarly, the peak response to kainite and area under the curve were higher in *Ins1LKB1KO* β -cells ($P < 0.01$, WT: $5.17 \pm 0.86\text{AU}$, KO: $10.75 \pm 2.18\text{AU}$, Figure 3.13c). These experiments were performed alongside Dr Chase Kantor. Consistent with increased glutaminergic signalling in these mice, kainite-stimulated insulin secretion was significantly increased in *Ins1LKB1KO* versus WT islets ($P < 0.05$, WT: $0.57 \pm 0.16\%$, KO: $1.25 \pm 0.24\%$, Figure 3.14). Insulin secretion also increases in *Ins1LKB1KO* versus WT islets when incubated in glutamate, though not significantly (NS, WT: $2.57 \pm 0.80\%$, KO: $3.50 \pm 0.56\%$, Figure 3.14).

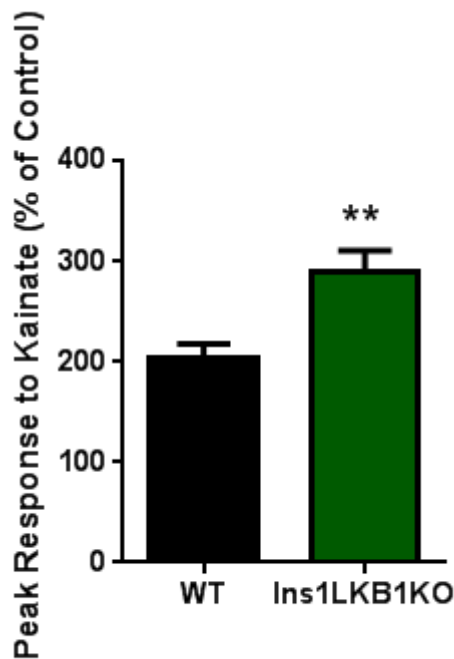
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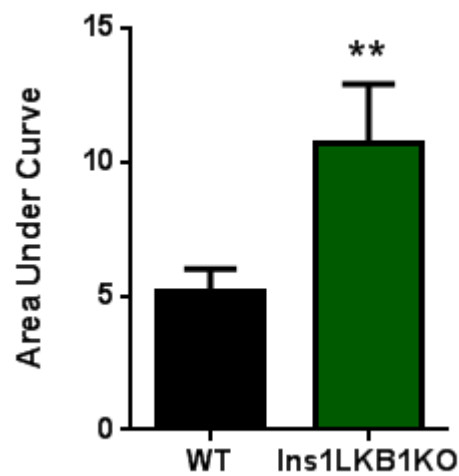


Figure 3.13 - Up-regulation of *Nptx2* expression and Ca^{2+} signalling in Ins1LKB1KO mice using the glutamate receptor agonist kainite

(A) Gene expression of *Nptx2* was measured in Ins1LKB1KO and WT islets, normalising with β -actin. (B) Glutamate receptor signalling increases intracellular [Ca^{2+}]_{cyt} (C) mean amplitude of Ca^{2+} peak and (D) AUC in response to kainite. $N = 4$ mice/genotype. Data are expressed as means $\pm$ SEM, ** $P < 0.01$, by Student's unpaired t-test.

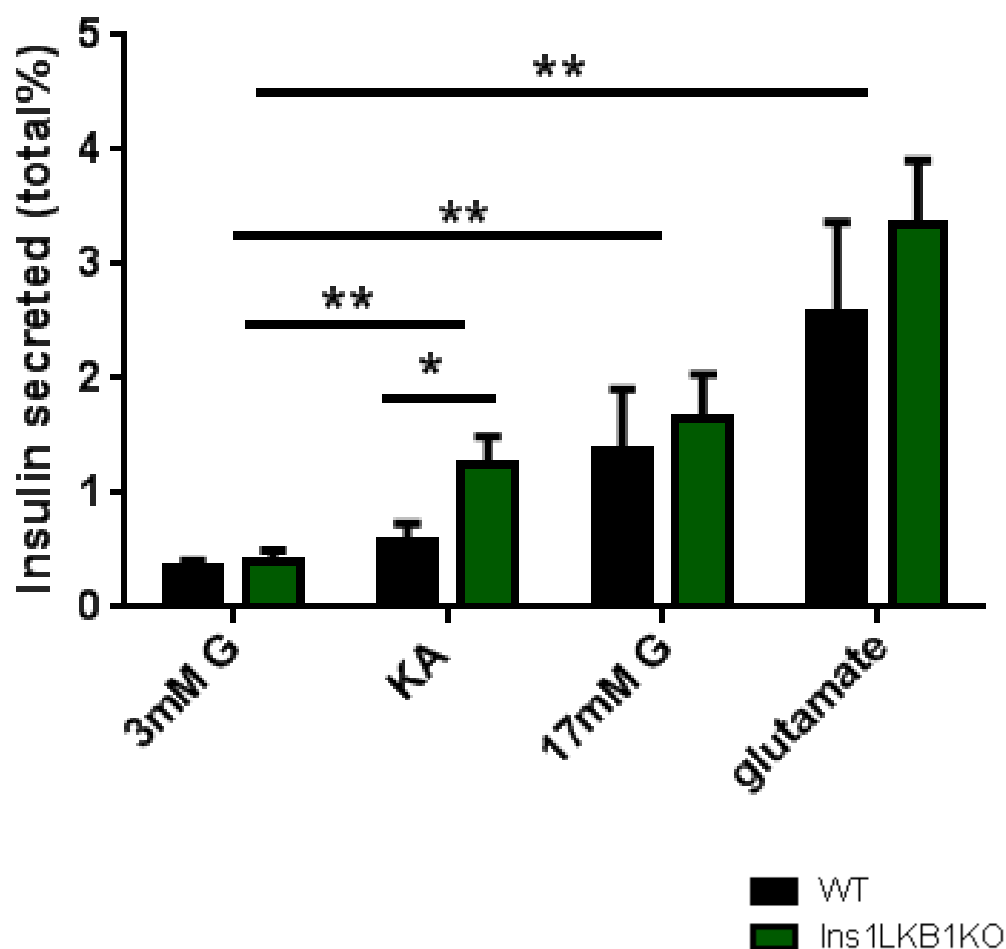


Figure 3.14 - Glutamate signalling enhances insulin secretion in Ins1LKB1KO mice

Insulin secretion was measured in 6 size-matched islets incubated in 3 and 17mM of glucose, kainite and glutamate. Data are expressed as means $\pm$ SEM, $n=4$ mice/genotype, * $P<0.05$, ** $P<0.01$, by 2-way ANOVA.

3.3 Discussion

In this chapter, we show two contrasting effects of LKB1 deletion in the pancreatic β -cell. Differing to other studies [318-320] we used the highly β -cell selective Ins1Cre promoter that deletes at E11.5. The absence of LKB1 resulted in marked improvements in glucose tolerance, *in vivo* insulin secretion [427] and β -cell mass on one hand, whilst on the other hand caused dramatic defects in Ca^{2+} and ATP/ADP signalling within the cytosol of β -cells whereas, *in vitro* GSIS remained unchanged. Moreover, islets of mice deleted for LKB1 showed an up-regulation in the expression of genes involved in glutamate signalling (*Nptx2*, *Gria3* and *Dlgap2*) and glutamate signalling was enhanced, suggesting that LKB1 deletion stimulates the amplification of glutamate production and signalling resulting, overall, in normal GSIS.

3.3.1 The Ins1Cre promoter deletes selectively in the β -cell at E11.5

It should be noted that LKB1 *floxed* mice crossed with Ins1Cre recombinase differ from mice deleted for LKB1 using RIP2Cre recombinase and the Pdx1 CreER transgene. As well as the Ins1Cre model being the more effective deleter [426], deletion occurs during the post-natal period and lasts until adulthood (also observed in the RIP2Cre model) whereas, Pdx1 CreER transgenic mice deletes from 8-10 weeks of age. Thus, experiments conducted on our Ins1LKB1KO mice occur at a much earlier stage in life than the Pdx1 CreER model. The RIP2Cre promoter deletes both in β -cells and a small population of cell within the hypothalamus. On the other hand, the Ins1Cre promoter deletes in a high population of β -cells (94%) specifically. RIP2Cre LKB1 deficient mice developed hind-limb paralysis and axon degeneration and died at approximately 20 weeks of age. Comparison of timelines between these three models is illustrated in Figure 3.15.

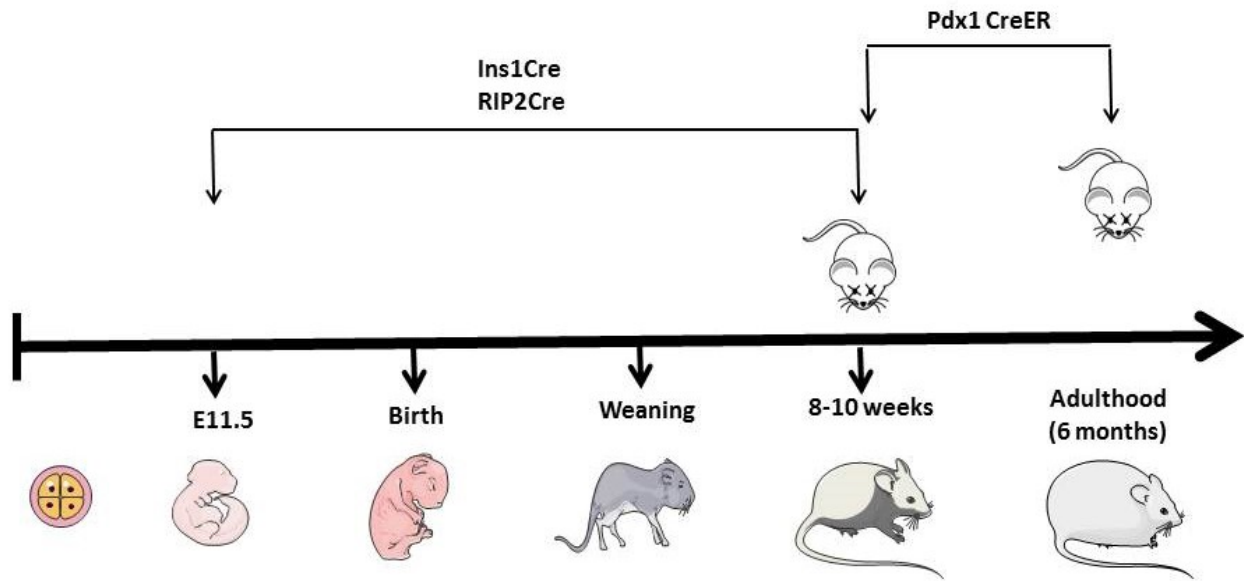


Figure 3.15 - Timeline of Ins1Cre, RIP2Cre and Pdx1 CreER mouse models floxed for LKB1

The horizontal arrow represents the timeline of these mice, starting from development. The vertical arrow at E11.5 represents the point when Ins1 and RIP2 Cre recombinase deletes in the β -cell and hypothalamus. *In vivo* experiments were performed in these mice from 8-10 weeks of age followed by sacrifice for islet extraction whereas deletion of LKB1 in Pdx1 CreER mice occurs at 8-10 weeks. *In vivo* experiments with these mice take place at 5-6 months, followed by sacrifice for islet isolation. Due to axon degeneration in the spinal cord of RIP2Cre LKB1 deleted mice, mice died prematurely at approximately 20 weeks of age. The Figure is generated using images from “Servier Medical Art”. (<http://www.servier.com/servier-medical-art>).

3.3.2 ***LKB1 deletion improves glucose tolerance and β -cell mass however, GSIS is unaffected***

Our findings [427], together with those of previous studies [318-320] show that deletion of LKB1 in pancreatic β -cells improves both glucose tolerance, β -cell mass (Figure 3.1) and insulin expression (Figure 3.2). Whilst the mechanisms involved were not explored here they may reflect loss of the activity of LKB1 to restrict cell phosphorylation and consequent inactivation of the mTOR signalling pathway [428, 429].

However, when comparing our findings to that of these previous models, *in vitro* GSIS did not appear to improve in these Ins1LKB1KO mice *versus* their WT littermates (Figure 3.3) [430, 431], despite the fact that glucose tolerance and β -cell mass were improved. The absence of GSIS improvement in Ins1LKB1KO mice may be due to other roles that LKB1 appear to play in insulin

secretion. For example, previous studies show that two targets of LKB1 phosphorylation, SIK2 and SAD-A, up-regulate insulin secretion and thus their deletion impairs insulin secretion [422, 423]. In contrast, other targets of LKB1 are known to restrict insulin secretion. For example, MARK2 phosphorylates and prevents translocation of TORC2 (a co-activator of CREB), which contributes to restoring β -cell metabolism after insulin secretion [432, 433]. Thus, MARK2 could mediate LKB1 action in β -cells as suggested in previous studies [318, 319].

3.3.3 Cytosolic signalling and mitochondrial defects are present in *Ins1lkb1ko* islets

Despite normal GSIS, enhanced β -cell mass and glucose tolerance in *Ins1LKB1KO* mice, Ca^{2+} and ATP responses to high glucose were drastically impaired (Figure 3.4 and 3.6). We confirmed that this defect is due to the deletion of LKB1 as re-introduction of LKB1 via adenovirus infection resulted in the recovery of normal $[\text{Ca}^{2+}]_{\text{cyt}}$ and $[\text{ATP/ADP}]_{\text{cyt}}$ signalling (Figure 3.7)[430]. Similar results were found in the LKB1 *floxed* Pdx1-Cre mouse strain by Fu *et al*, 2015 [431], where despite enhanced GSIS, mitochondrial function was impaired.

We also assessed the rate of oxygen consumption in *Ins1LKB1KO* islets in response to high glucose, oligomycin to measure ATP production, the uncoupler FCCP to measure the maximal respiration and spare respiratory capacity of mitochondria and antimycin and rotenone to measure non-mitochondrial respiration (Figure 3.11). Overall, we found that there was no difference in oxygen consumption between *Ins1LKB1KO* and WT islets when stimulated with glucose. However, after FCCP was added OCR was higher in *Ins1LKB1KO* *versus* WT islets, indicating higher spare respiratory capacity. It is thought that preservation of the spare respiratory capacity is a hint of the vitality and survival of cells and is influenced by the bioenergetic capacity of mitochondria that is determined by factors such as the functional capacity of enzymes in the electron transport chain and the cell's ability to deliver substrates to the mitochondria. Our results differs to other studies performed on LKB1 deficient islets generated using Pdx1 CreER and studies in older animals where OCR was found to be impaired [430, 431]. Additionally, and as repeated in Swisa *et al*, 2015 [430], it was found by electron microscopy that LKB1 deletion using the Pdx1 CreER transgene in β -cells leads to mitochondrial defects as well as impairments in mitochondrial metabolism and its downstream signalling. These findings indicate that LKB1 is vital for normal mitochondrial function. However, mitochondrial integrity does not appear to be affected in islets of *Ins1LKB1KO* mice (Figure 3.12).

We show that LKB1 is vital for normal mitochondrial function in β -cells. Mitochondrial membrane potential was measured in these islets using the dye TMRE (Figure 3.8), and found to be significantly

impaired in KO islets. Previous studies in LKB1 KO mice find that mitochondrial defects were also present [430, 431]. Metabolic profiling experiments show that several citrate cycle derivatives such as fumarate, citrate and succinate were all down-regulated in Ins1LKB1KO islets (NS), Figure 3.10.

The contrasting phenotypes of these Ins1LKB1KO mice, which are the improved glucose tolerance and cell mass whilst cytosolic and mitochondrial function are impaired and the resulting normal *in vitro* insulin secretion indicates that a compensatory mechanism must be present for overall improved GSIS *in vivo*.

3.3.4 *Glutamate release and action may compensate in LKB1 deficient β -cells*

Consistent with the increased expression of the genes *Nptx2*, *Gria3* and *Dlgap2* (Figure 3.13), kainate which binds to and activates ionotropic glutamate receptors [424], caused a more pronounced increase in $[\text{Ca}^{2+}]_{\text{cyt}}$ in Ins1LKB1KO than in WT islets (Figure 3.13). Furthermore, at low glucose concentrations, kainate stimulated insulin secretion in Ins1LKB1KO islets which was not noted in WT islets (Figure 3.14)[427]. In addition, our metabolomics measurements of citrate cycle intermediates found an up-regulation of pyroglutamate, a metabolite of glutamic acid synthesis (NS, data not shown). Here we suggest that LKB1 is required for normal signalling and mitochondrial function within the β -cell and that in mice deleted for LKB1, glutamate production and signalling acts as a compensatory mechanism to maintain normal insulin secretion, as illustrated in Figure 3.16.

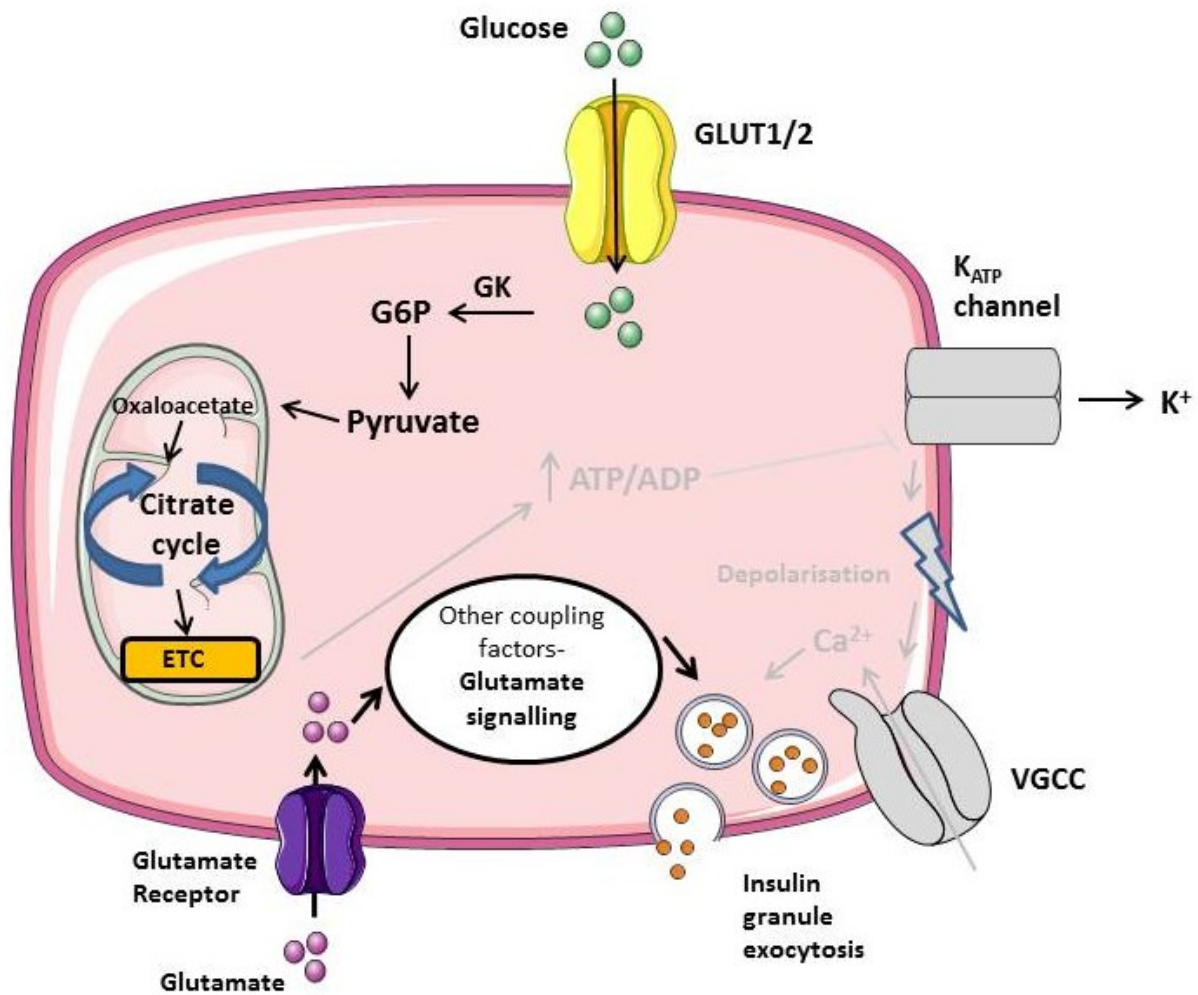


Figure 3.16 - Model showing an alternative signalling system in the β -cell after LKB1 deletion

As discussed in chapter 1, normal GSIS requires increases in glucose concentrations to initiate the transport of glucose into the cell via GLUT2 and is converted to G6P via GK, where glucose undergoes glycolysis. The by-product of glycolysis, pyruvate, enters the mitochondria and ATP is generated via the citrate cycle. Here, K_{ATP} channels close followed by depolarisation of the cell opening of voltage-gated Ca²⁺ channels and the influx of Ca²⁺ into the cytosol, triggering insulin exocytosis. This process is shown in faded grey. However, in β -cells where LKB1 is deleted impairments are apparent in Ca²⁺ and ATP/ADP signalling despite normal insulin secretion. Due to the up-regulation in genes involved in glutamate signalling (Figure 5.13) [427] and insulin secretion in the presence of the glutamate receptor agonist kainite (Figures 5.13 and 14), we propose that glutamate signalling acts as an alternative pathway for GSIS in β -cells deleted for LKB1. The Figure is generated using images from "Servier Medical Art". (<http://www.servier.com/servier-medical-art>).

Similar findings of alternative pathways to maintain normal GSIS in LKB1 deficient mice were found for example, in the Pdx1-Cre LKB1 *floxed* mouse where β -cells lacking LKB1 glucose usage favours citrate and glutamate production [431]. This increase in glutamate enhanced K_{ATP} channel opening and GSIS as well as maintaining higher NADPH and basal mitochondrial membrane potential levels [198]. The authors concluded that LKB1 deletion leads glucose usage away from the citrate cycle and towards other biosynthetic pathways such as amino acid synthesis and lipid generation and that the

enhanced ACC1 production seen in these mice results in the enhanced insulin secretion seen in these β -cells [431].

In addition, metabolic profiling studies conducted in islets of Ins1LKB1KO mice show down-regulation of FA's (Figure 3.9b, c). Lipids are required for normal β -cell function and islets depleted for triglycerides have been found to be irresponsive to glucose both in rats [434, 435] and humans [436]. The link between glucose and lipid metabolism is thought to rely on the glucose-stimulated increase in malonyl-CoA [437] that inhibit carnitine palmitoyltransferase 1 (CPT1) by ACC which blocks FA-CoA transport into the mitochondria [438] resulting in reduced β -oxidation and an increase in cytosolic FA derivatives. Therefore, the up-regulation of ACC1 production found in Pdx1 CreER LKB1 KO mice and down-regulation of FA which may result in ACC inhibition in Ins1LKB1KO suggests a possible link between lipid metabolism and GSIS in LKB1 depleted mice.

3.4 Conclusions

In summary, we found that LKB1 is essential for the maintenance of normal ATP/ADP ratio and mitochondrial function in the pancreatic β -cell. On the other hand, glucose tolerance and β -cell mass is enhanced in mice lacking LKB1 selectively in the β -cell despite normal insulin secretion *in vitro*. Our data, consistent with other findings using different mouse models of β -cell selective LKB1 deletion [430, 431], suggest that LKB1 regulates normal metabolic function and to compensate for these defects when LKB1 is deleted the glutamate signalling pathway becomes amplified in order to maintain normal GSIS upon LKB1 deletion.

Chapter 4

Deletion of LKB1 in α - and L-cells and its effects on glucagon and GLP-1 levels

Abstract

Background and aims: The tumour suppressor LKB1 (STK11) is a serine/threonine kinase that is mutated in the premalignant disorder Peutz Jeghers Syndrome (PJS). PJS is characterised by polyps in the gastrointestinal tract. Proglucagon-expressing enteroendocrine L-cells are involved in the regulation of appetite and glucose homeostasis through the secretion of gut hormones, such as glucagon like peptide (GLP)-1, GLP-2 and peptide YY (PYY). Here, we deleted LKB1 in proglucagon-expressing cells including enteroendocrine L-cells of the gut and pancreatic α -cells to explore the roles of LKB1 in these cells.

Methods: Mice deleted selectively in proglucagon-expressing cells for LKB1 were generated by breeding animals bearing *floxed* alleles for LKB1 with those bearing iGluCre recombinase. Oral and intraperitoneal glucose tolerance tests were carried out in these mice, as were insulin tolerance tests, optical projection tomography (OPT) to measure L- cell mass and immunohistochemistry for α -cell mass. Glucagon secretion was measured both *in vivo* and *in vitro* using radioimmunoassay.

Results: GluLKB1KO mice displayed largely normal glucose homeostasis and circulating GLP-1 levels. However, these mice developed large gastro-duodenal polyps resulting in premature mortality (~120 days) due to blocked food transit. LKB1 deletion in pancreatic α -cells exerted no effect on glucagon secretion either *in vivo* or *in vitro* nor on pancreatic α -cell mass.

Conclusion: The loss of LKB1 within enteroendocrine L-cell progenitor's results in EMT to smooth muscle cells and is sufficient to initiate tumorigenesis. However, the deletion of LKB1 in these cells has no effect on glucose homeostasis, GLP-1 or glucagon synthesis and secretion.

4.1 Introduction

Liver kinase B1 (LKB1), otherwise known as serine/threonine kinase 11 (STK11) (see chapter 1), is a tumour suppressor and an up-stream kinase responsible for the phosphorylation and activation of the 13 members of the AMPK family [204]. These include, Par-1 and MARK2 which are responsible for the regulation of cell polarity [439]. Together with cell growth, metabolism and survival, the control of cell polarity is likely to be an important factor in the actions of LKB1 as a tumour suppressor. On the other hand, activation of AMPK by LKB1 restricts growth factor signalling by stimulating TSC1/2 [440], thus inhibiting mTOR and consequently protein and lipid synthesis [441].

Heterozygous mutations of LKB1 in humans result in the development of the premalignant disorder Peutz Jeghers Syndrome (PJS), characterised by hamatomatous lesions, polyps in the gastrointestinal tract, pigmentation of the lips and an increased risk of developing all cancers [442]. The main cause of mortality in these patients is from obstructions in the abdomen caused by large gastrointestinal polyps which may also result in infarction, intussusception and bleeding. There are currently no approved pharmaceutical treatments for PJS and a more thorough understanding of how LKB1 controls tumour growth is needed for the development of an effective therapy.

Studies in mice lend further support to the control of LKB1 mutations in PJS. Thus, although mice homozygous null for LKB1 (LKB1^{-/-}) are not viable beyond the 10th day of embryogenesis [333, 443], heterozygous null LKB1^{+/-} mice develop polyps similar to those observed in PJS after five months [334, 444].

Enteroendocrine L-cells represent less than 1% of the epithelial cell lining of the intestinal wall of the gut. Despite their small number these cells are essential for normal physiology and energy metabolism [445, 446]. Through the action of prohormone convertase (PC) 1/3 on proglucagon, L-cells generate and secrete the gut hormones glucagon-like peptide-1 (GLP-1), GLP-2 and peptide YY (PYY) [351]. GLP-1 is an incretin hormone that stimulates the release of insulin from the pancreas, and thus is of considerable therapeutic interest in the treatment of Type 2 Diabetes [447].

LKB1 has been found to play a role in insulin secretion, polarity and the control of β -cell mass [318-320] as well as in glucagon secretion [284]. However, the role of LKB1 in enteroendocrine cells has not yet been explored. To determine whether LKB1 may be involved in the growth and function of proglucagon expressing cells and might contribute to the formation of polyps in PJS, we have crossed mice expressing *floxed* alleles for LKB1 to animals carrying Cre recombinase cDNA under the proglucagon promoter (iGlu Cre) [403]. This has allowed us to determine the consequences of

deleting LKB1 from enteroendocrine L-cells, pancreatic α -cells as well as GLP-1 expressing neurones in the brainstem.

4.2 Results

4.2.1 *Deletion of LKB1 from preproglucagon-expressing cells causes dramatic tumour growth in the duodenum*

WT, GluLKB1het and GluLKB1KO mice were generated by crossing mice in which exons 3-6 of the LKB1 gene were flanked with *LoxP* sites [334] to iGluCre mice. These mice were crossed with mice bearing a tandem dimer red fluorescent protein (RFP) transgene at the Rosa26 locus distal to a STOP-LoxP-STOP control cassette [403]. Genotyping of these mice show RFP, iGluCre, WT and *floxed* alleles for LKB1 KO and pPCR demonstrates the successful expression of iGluCre in GluLKB1KO islets (Figure 4.1a-b). This led to Glu-Cre expression in glucagon-positive pancreatic α -cells of which ~70% were positive for tdRFP (Figure 4.1d-e).

The body weight of each group was measured weekly (Figure 4.2a) and showed a dramatic reduction in weight of the GluLKB1KO mice *versus* GluLKB1het and WT after approximately 100 days (12-15 weeks). Weight loss was closely followed by the premature death of GluLKB1KO mice (within a week), as illustrated in the Kaplan Meir curve shown in Figure 4.2c. Although premature death occurred occasionally in WT or GluLKB1het mice, 100% of the GluLKB1KO mice had died by day 120 (Log-rank Mantel-Cox test, $P < 0.001$). By day 197, 93.7% of WT, 64.3% of GluLKB1het mice and 0% of GluLKB1KO mice survived. Vertical deflections in the curve represent censored data where the exact date of death is unknown or when mice were euthanised due to ill-health. Both GluLKB1KO and WT mice were dissected at 15 weeks (Figures 4.3a-f) and it was discovered that whilst WT mice appeared normal, the stomach of GluLKB1KO became bloated in appearance (Figure 4.3c and 4.3d). Upon post-mortem examination large polyps were apparent in the duodenum of GluLKB1KO mice (Figure 4.3f). Therefore, it is likely that the weight loss in these mice was caused by bowel obstructions as a result of the polyp growth (Figure 4.3e). No polys were found during the post-mortem examination in WT or GluLKB1het mice (Figure 4.3b) nor were polys observed lower in the gastrointestinal tract (jejunum, ileum, and colon) of GluLKB1KO mice.

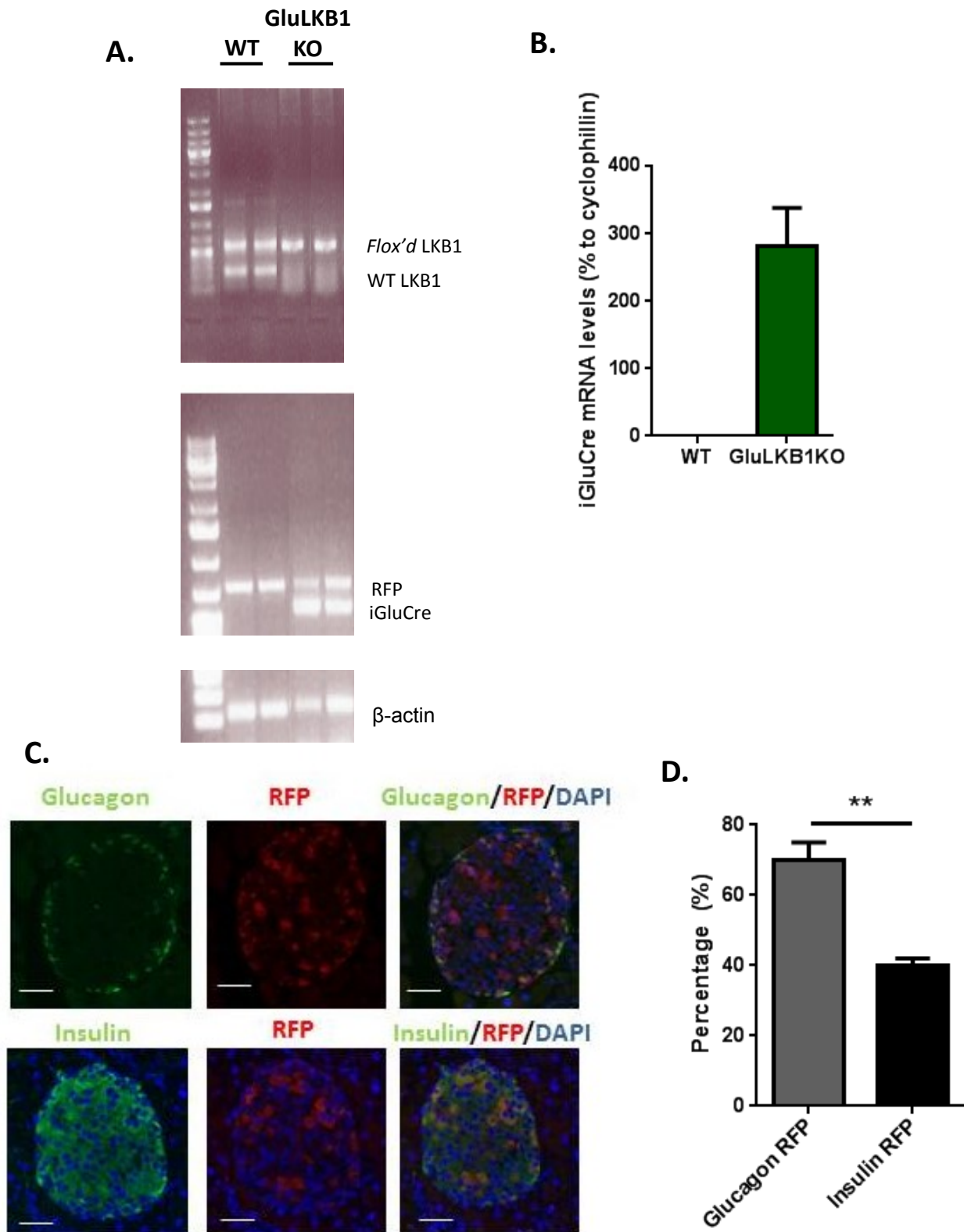


Figure 4.1 - Generation of GluLKB1KO mice use of a Rosa26tdRFP reporter to assess recombination (A) PCR analysis of LKB1, RFP, iGluCre and β -actin expression in mice, (B) GluCre expression, (C) representative immunostaining of pancreatic islets using antibodies against RFP and either glucagon or insulin, nuclei are shown by DAPI (blue) staining. (D) Images of stained islets (6-8) were used to calculate the percentage of cells positive for insulin or glucagon and co-expressing RFP. $N=6$ mice/genotype, ** $P<0.01$ by unpaired Student's t-test. Data are expressed as means $\pm$ SEM.

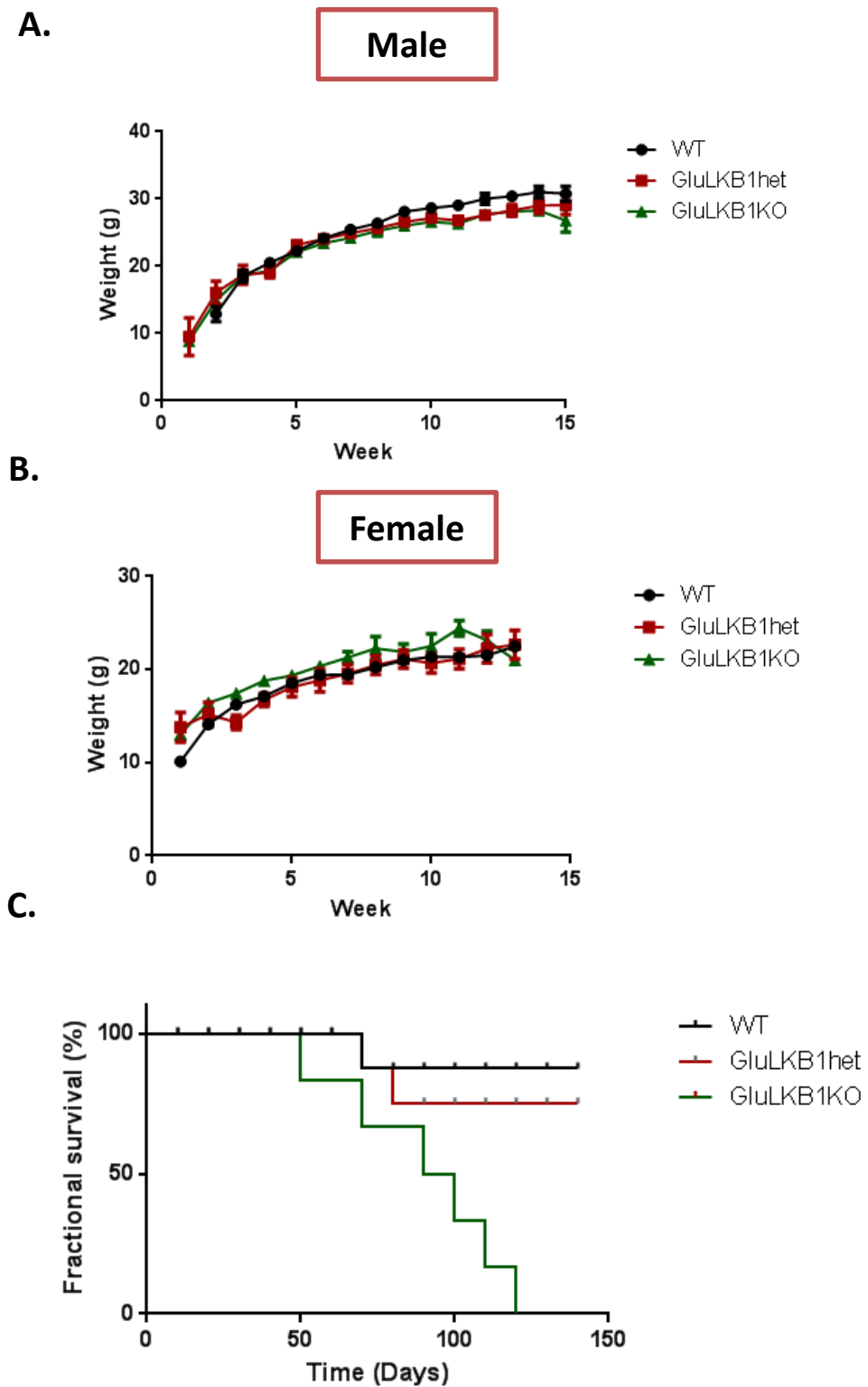


Figure 4.2 - Decreased body weight and life-span of GluLKB1KO mice

Changes in body mass over time in (A) Male and (B) Female WT, GluLKB1het or GluLKB1KO mice were measured weekly between 4 to 15 weeks of age, $n=10-15$. (C) Kaplan-Meier survival curve for WT, $n=16$, GluLKB1het, $n=16$, and GluLKB1KO, $n=21$, mice. GluLKB1KO mice showed premature death from 50-120 days.

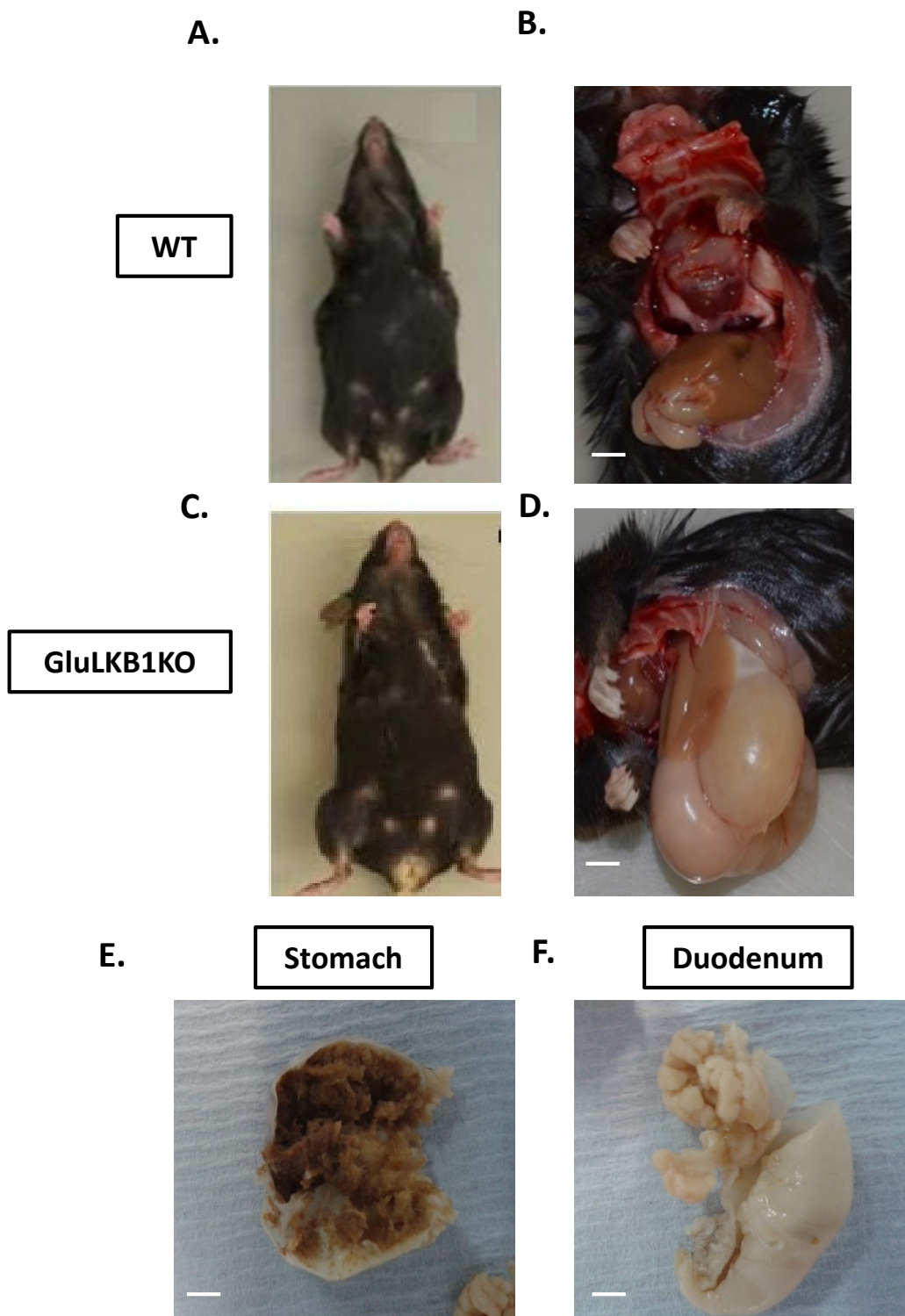


Figure 4.3 - Polyp formation in GluLKB1KO mice

(A) WT mouse with normal phenotype, (B) Abdomen of WT mouse showing normal gastric contents, (C) GluLKB1KO mouse showing bloated appearance, (D) Abdomen of GluLKB1KO mouse showing distended duodenum and stomach due to large duodenum polyp. (E) Stomach of GluLKB1KO mouse which becomes blocked due to polyp formation in the duodenum, (F) Polyp formation within the duodenum of GluLKB1KO mouse. Scale bar: 5mm.

4.2.2 *GluLKB1KO mice have normal glucose tolerance and in vivo GLP-1 secretion*

To investigate the effect of LKB1 deletion from proglucagon-expressing cells on glucose tolerance both oral and intraperitoneal glucose tolerance tests (GTTs) were performed. IPGTTs performed at 10 and 15 weeks (Figure 4.4) failed to reveal any significant change in glucose tolerance between WT, GluLKB1het and GluLKB1KO mice, for both males and females. However, when comparing the area under the curve in 10 week old male GluLKB1KO mice *versus* GluLKB1het mice, glucose tolerance was enhanced ($P < 0.01$, WT: 1235 ± 88.3 mmol/L, het: 1388 ± 51.97 mmol/L, KO: 1276 ± 109.7 mmol/L, Figure 4.4a). On the other hand, though no difference was apparent in glucose tolerance of WT *versus* GluLKB1KO 10 week old female mice, analysis of the area of the curve showed impaired glucose tolerance in GluLKB1KO mice *versus* WT ($P < 0.05$, WT: 972.5 ± 35.36 mmol/L, het: 1009 ± 48.47 mmol/L, KO: 1183 ± 65.45 mmol/L, Figure 4.4b). AUC was unaffected by genotype for either male or female mice at 15 weeks (Figure 4.4c-d).

To determine whether LKB1 deletion affected incretin secretion from L-cells and might thus contribute to improved glucose tolerance, OGTTs were performed (Figure 4.5). As with IPGTT's (Figure 4.4), OGTT's failed to reveal a significant change in glucose tolerance in either male or female WT, GluLKB1het and GluLKB1KO mice at 12-14 weeks of age. AUC were similarly unaffected.

To further examine whether GLP-1 synthesis might be up-regulated in GluLKB1KO mice, OPT was performed using an anti-proglucagon antibody (red) on the duodenum of WT and GluLKB1KO mice [410]. This allowed us to analyse average, total volume, size and total number of proglucagon expressing enteroendocrine L-cells within the duodenum of these mice (Figure 4.6a-e). Relative L-cell mass (WT: $0.51 \pm 0.08\%$, KO: $0.53 \pm 0.17\%$) and L-cell volume (WT: $44.38 \pm 3.34\%$, KO: $46.18 \pm 12.16\%$) was unaffected in GluLKB1KO *versus* WT mice.

To measure GLP-1 secretion *in vivo* plasma was collected from WT, GluLKB1het and GluLKB1KO mice which were either fasted overnight or fed. GLP-1 levels were then measured using 2-site microtitre plate based immunoassay with electrochemical luminescence detection (performed by the Core Biochemical Assay laboratory, Cambridge). Measurement after normal feeding showed GLP-1 levels tended to be higher in GluLKB1KO *versus* WT mice (NS, WT: 22.88 ± 5.87 pg/ml, KO: 33.53 ± 16.79 pg/ml). However, several of the samples had become haemolysed (Figure 4.7a). As expected, GLP-1 levels decrease in mice fasted overnight compared to fed mice as GLP-1 acts as a marker for satiety. In fasted mice GLP-1 levels were found to be similar in GluLKB1KO and WT mice (NS, WT: 10.83 ± 1.21 pg/ml, het: 14.57 ± 0.58 pg/ml and KO: 9.85 ± 1.26 pg/ml, Figure 4.7b).

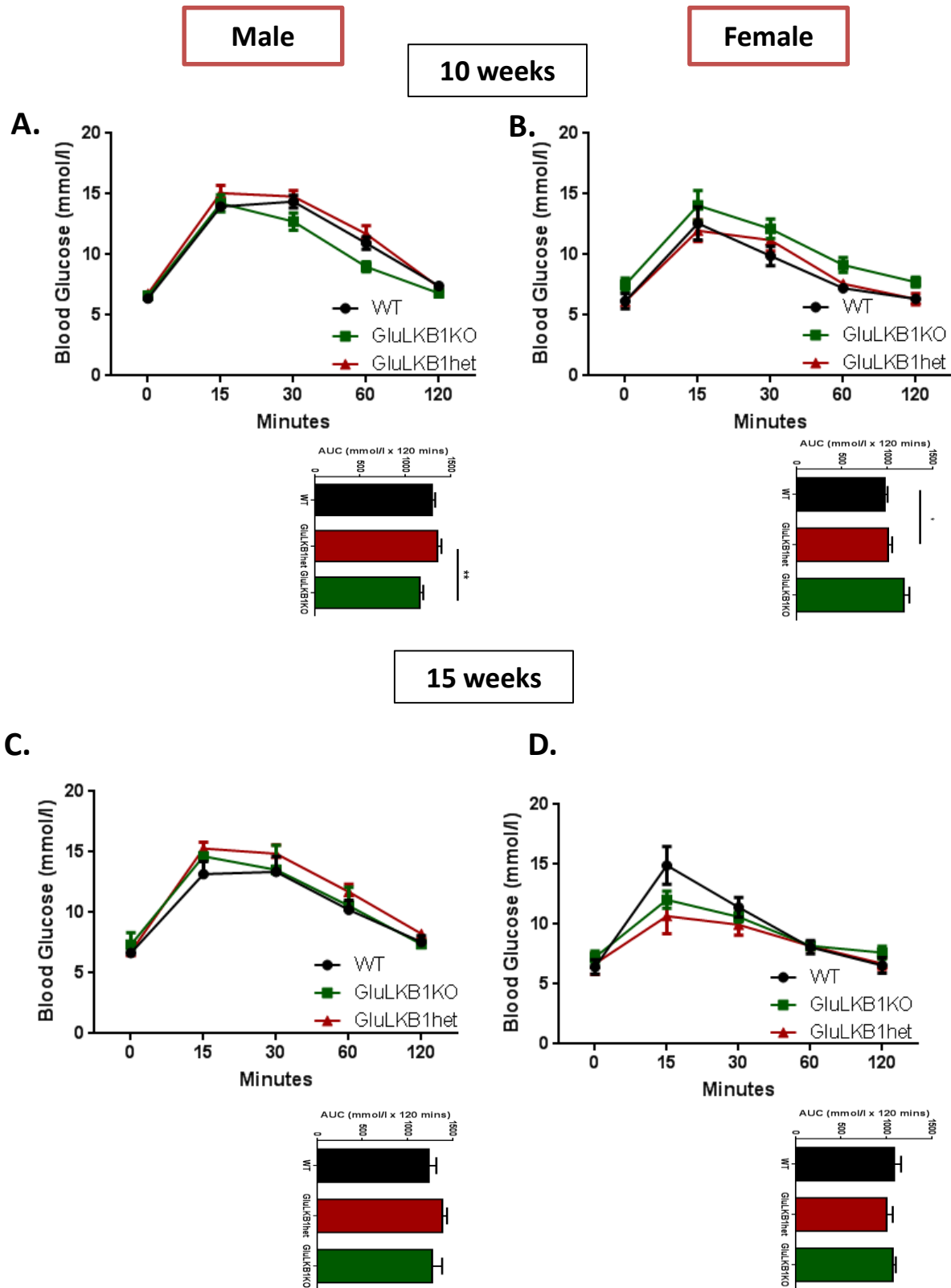


Figure 4.4 - Glucose tolerance is unaffected in GluLKB1KO mice

Intraperitoneal glucose tolerance tests were performed in (A, B) 10 week old male and female mice and (C, D) in 15 week old male and female mice, NS by 2-way ANOVA. AUC is also displayed in bottom right panels, * $P < 0.05$, ** $P < 0.01$, by Student's unpaired t-test. Data are expressed as means $\pm$ SEM; $n = 5-15$ per genotype.

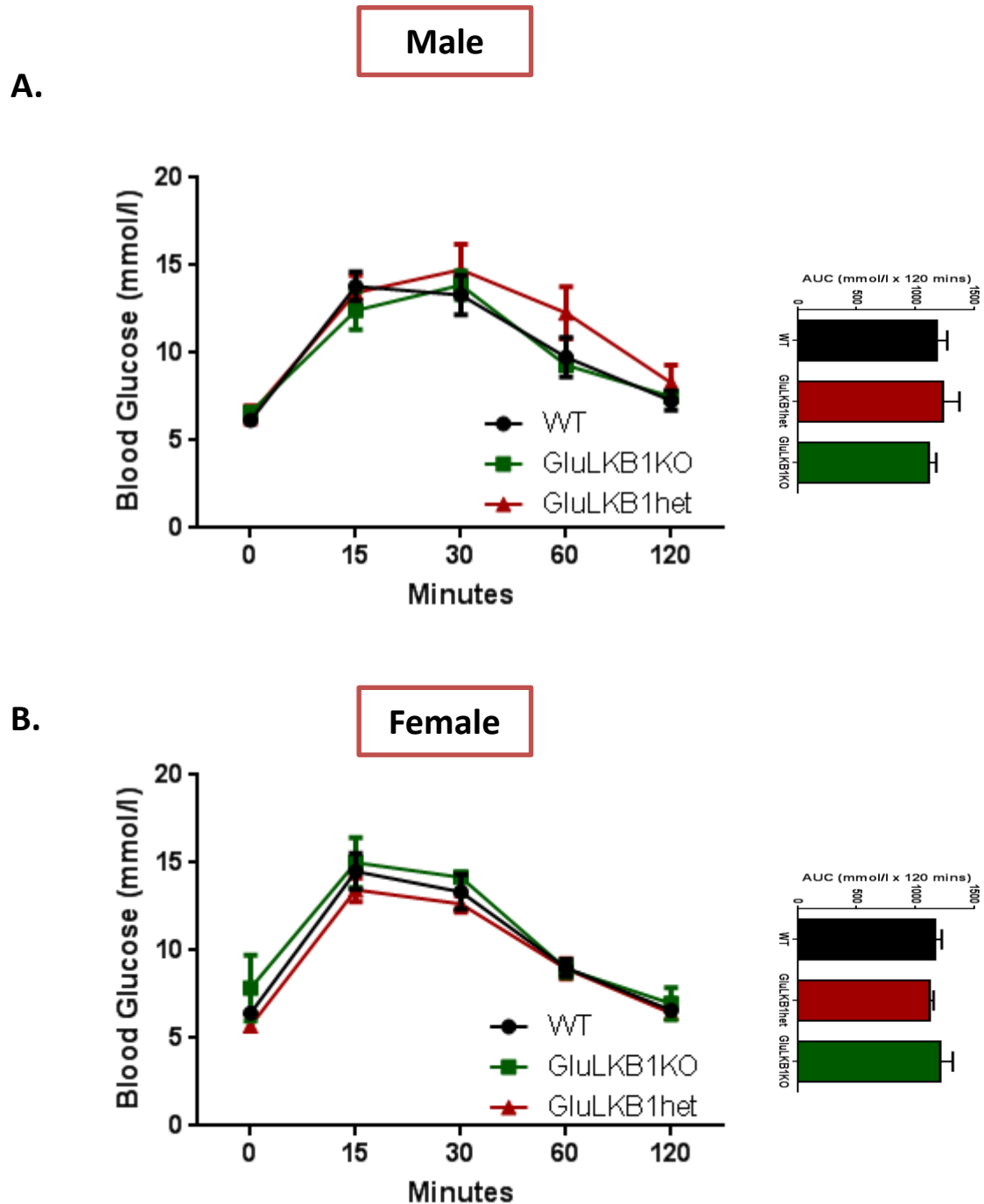


Figure 4.5 - Oral glucose tolerance tests performed in GluLKB1KO mice show no difference in glucose tolerance versus WT littermates

(A) Glucose tolerance of male and, (B) female mice aged 12-14 weeks, NS, by 2-way ANOVA and AUC displayed in right panels, NS by Student's unpaired t-tests. $N=3-9$ per genotype. Data are expressed as means $\pm$ SEM.

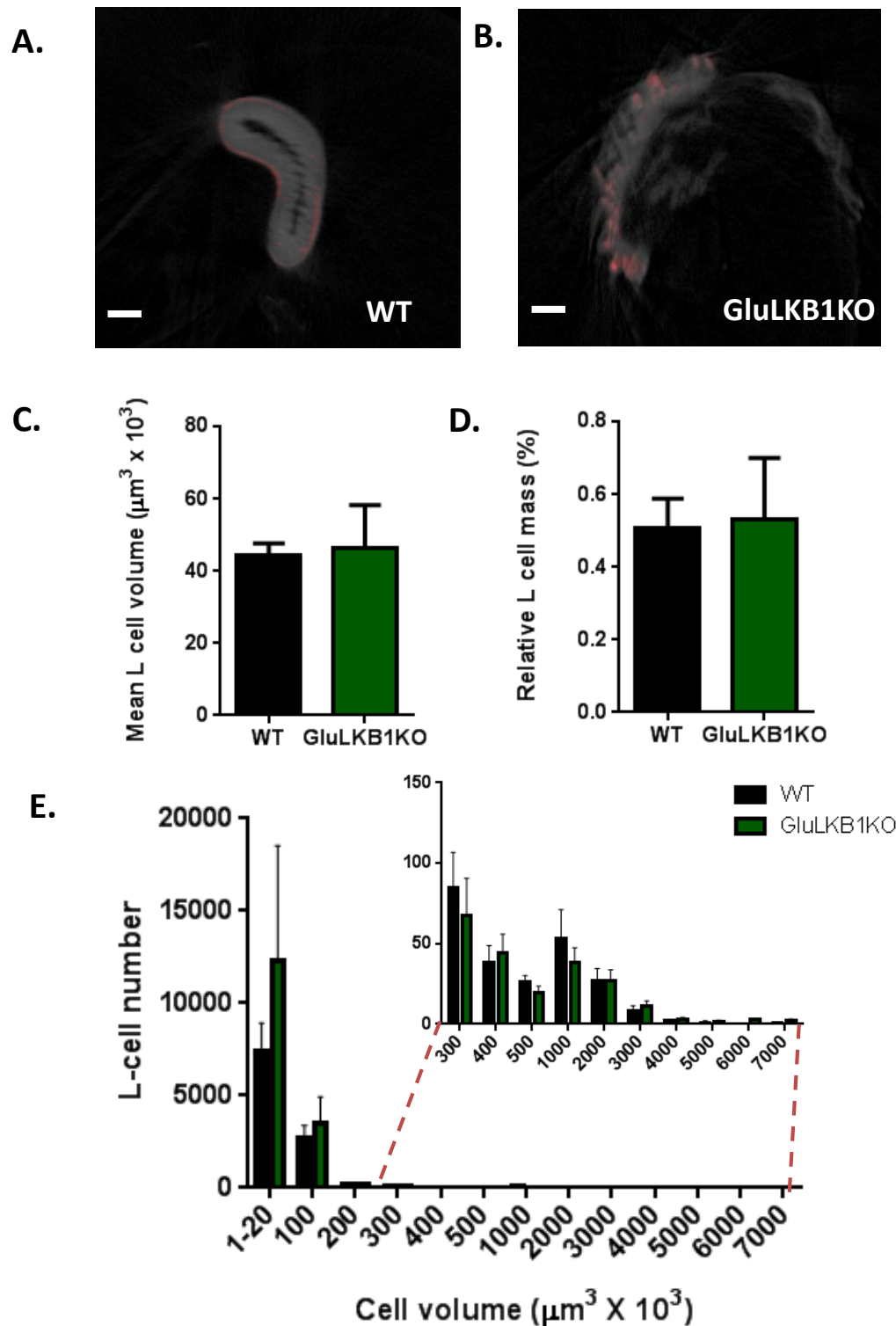


Figure 4.6 - L-cell mass is unaffected in GluLKB1KO mice

(A, B) Representative OPT images of fixed and permeabilised gut tissue. Red staining indicates glucagon-positive structures (likely to be L-cells). The overall shape of the gut was visualised as autofluorescence and is apparent as white/grey shading. Scale bar, 500 μm . (C) The Relative L-cell mass, (D) mean L-cell volume and (E) Distribution of L-cell volume with arrows representing the magnified section was measured, NS, by Student's unpaired t-test and 2-way ANOVA, respectively. Data are from $n=3$ gut tissues per genotype and expressed as means $\pm$ SEM.

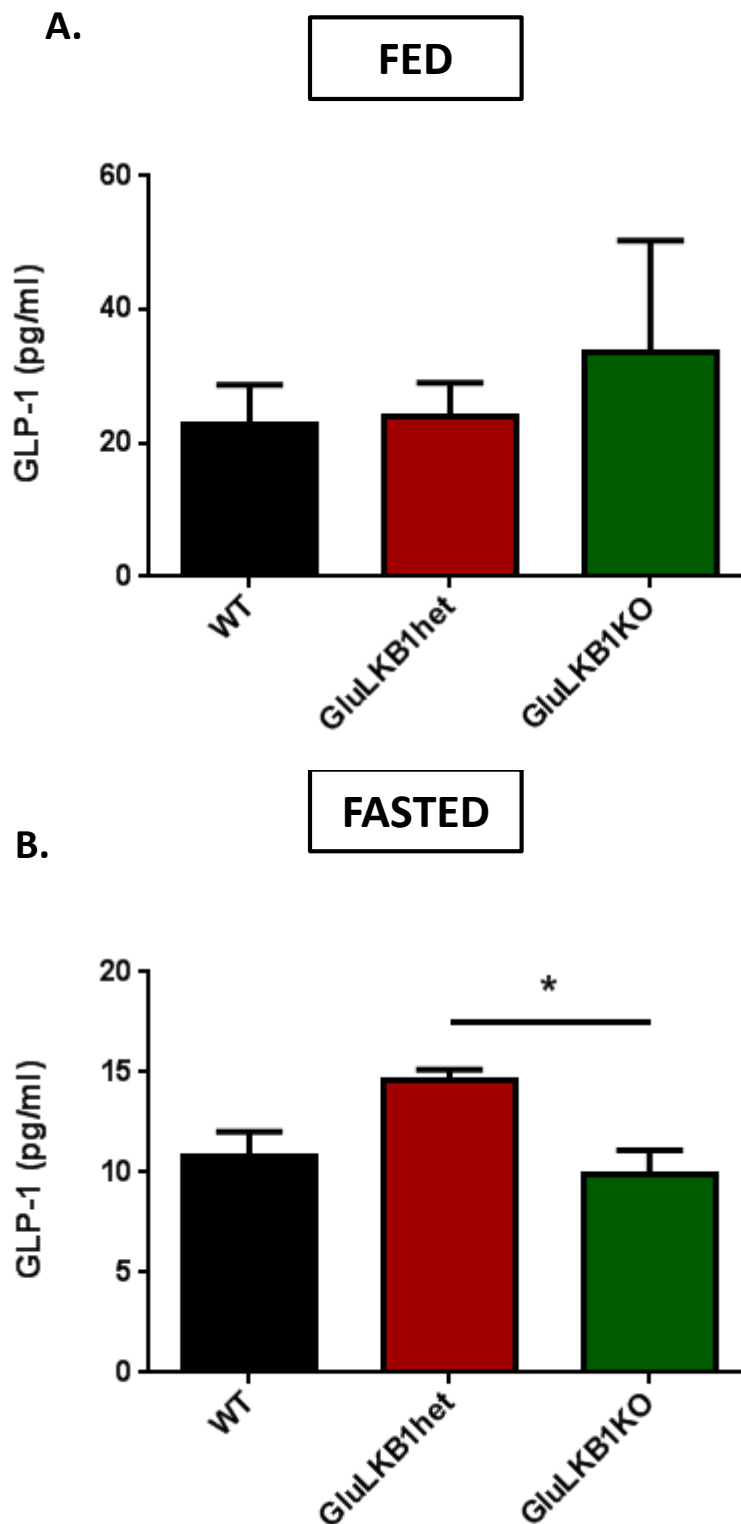


Figure 4.7 - Plasma GLP-1 levels in fed and fasted mice had little effect in GluLKB1KO mice

Plasma GLP-1 levels were measured in WT, GluLKB1het and GluLKB1KO mice with (A) unrestricted access to food and (B) mice fasted for 15 hours and GLP-1 measured by two-site microtitre plate based immunoassay with electrochemical luminescence detection. Data are expressed as means $\pm$ SEM, $n=5-8$ mice/genotype, * $P<0.05$, by Student's unpaired t-test.

4.2.3 Glucagon secretion and α -cell mass is unaffected in GluLKB1KO mice

As LKB1 plays a role in cell growth through inhibition of the mTOR signalling pathway [428], we investigated changes in α -cell mass in GluLKB1KO mice. Wax-embedded pancreatic slices from WT and GluLKB1KO mice were stained with an anti-insulin guinea-pig antibody (green), to identify β -cells, and rabbit anti-glucagon antibody (red), to identify α -cells (Figure 4.8). Through this analysis, the overall α : β cell ratio in islets was found to be increased in GluLKB1KO *versus* WT mice ($P < 0.05$, WT: 0.25 ± 0.026 AU, KO: 0.38 ± 0.04 AU, Figure 4.8d). Neither α - or β - cell mass was altered in GluLKB1KO *versus* WT mice (NS, α -cell WT: $1.01 \pm 0.05\%$, KO: $0.93 \pm 0.07\%$; NS, β -cell WT: $1.82 \pm 0.11\%$, KO: $1.78 \pm 0.14\%$, Figure 4.8b-c).

To determine whether the above increase in the α : β cell ratio (Figure 4.8e) affected overall glucagon secretion from α -cells, insulin tolerance tests (ITT) were performed and plasma collected to assess glucagon levels after insulin injection (Figure 4.9). At 8 and 12 weeks old, insulin sensitivity was unaffected in both male and female GluLKB1KO *versus* WT mice (Figure 4.9a-d). Similarly, *in vivo* glucagon secretion, measured at the 0, 15 and 30 min. *in vivo* glucagon secretion after insulin injection was unaltered between WT and GluLKB1KO mice (Figure 4.9e-f).

Additionally, *in vivo* glucagon secretion was measured in GluLKB1KO and WT mice when fed freely and after overnight fast. Confirming the results obtained by ITT, LKB1 deletion had no effect on *in vivo* glucagon secretion in α -cells (NS, Fed WT: 64.00 ± 3.67 pg/ml, KO: 71.67 ± 3.80 pg/ml; NS, Fasted WT: 28.33 ± 3.33 pg/ml, KO: 23.75 ± 5.54 , Figure 4.10)

To investigate the effects of deleting LKB1 from α -cells on glucagon secretion *in vitro*, islets were isolated from GluLKB1KO and WT mice and incubated in varying concentrations of glucose (0.5, 1, 3, 10 mM) and glucagon secretion measured after 1 hour of incubation (Figure 4.11). Islets isolated from either WT or GluLKB1KO mice and incubated in the lower concentrations of glucose (0.5 and 1 mM) were found to have significantly higher levels of glucagon secretion in comparison to islets incubated in higher glucose concentrations (3 and 10 mM), as expected ($P < 0.01$, 0.5 mM WT: $0.73 \pm 0.20\%$, KO: $0.78 \pm 0.22\%$; 1 mM WT: $0.70 \pm 0.11\%$, KO: $0.75 \pm 0.10\%$; 3 mM WT: 0.40 ± 0.17 , KO: 0.37 ± 0.16 ; 10 mM WT: 0.10 ± 0.02 , KO: $0.13 \pm 0.03\%$). However, as observed *in vivo*, *in vitro* glucagon secretion was identical in islets of GluLKB1KO *versus* WT mice.

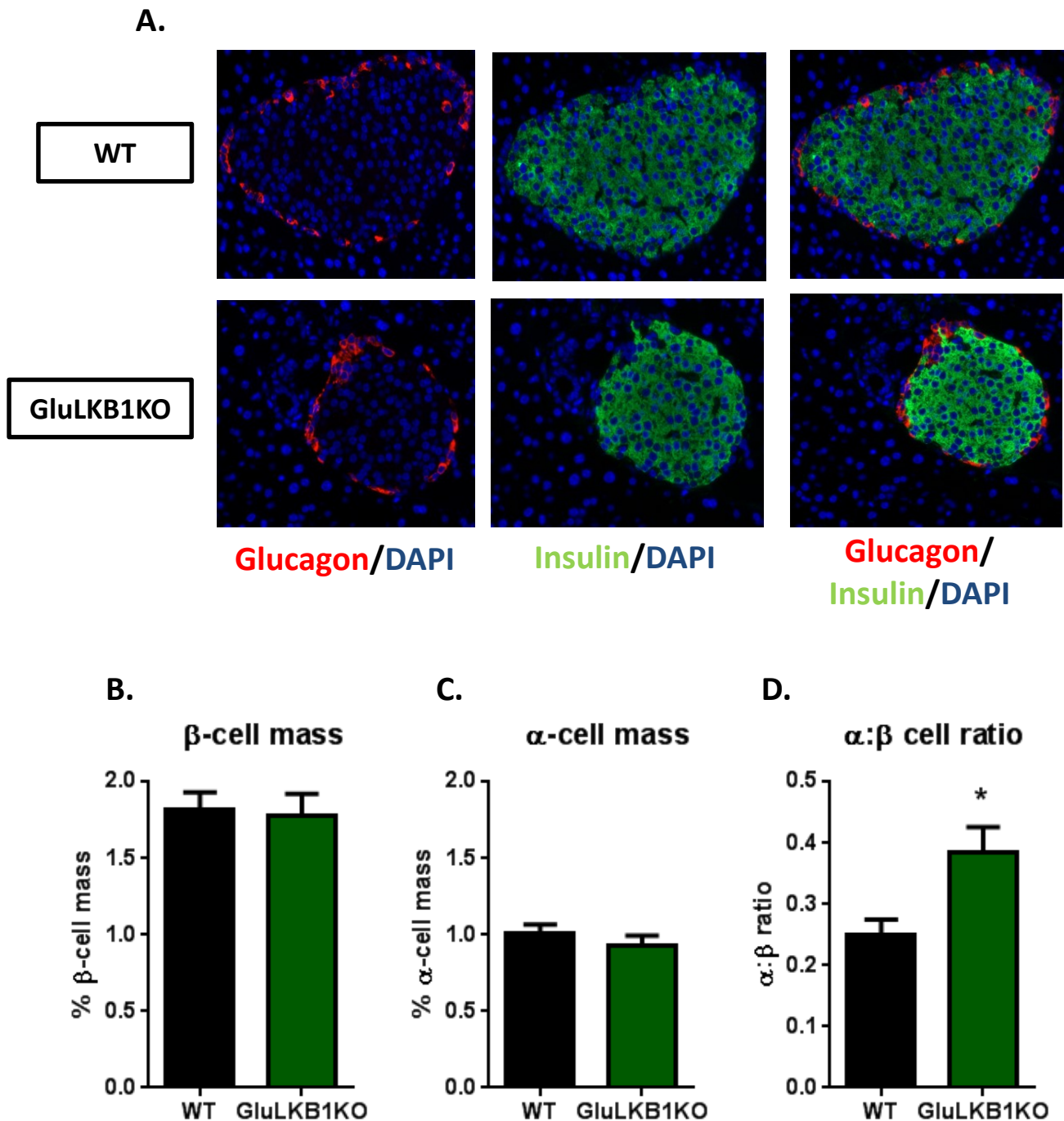


Figure 4.8 - α : β cell ratio increases in GluLKB1KO mice, whilst there is no effect in cell mass

(A) Representative images of wax embedded pancreatic slices of islets stained for insulin, glucagon and DAPI, (B) % β -cell mass and (C) % α -cell mass. (D) α : β cell ratio. Data expressed as means $\pm$ SEM, $n=4$ pancreata per condition, * $P<0.05$, by Student's unpaired t-test.

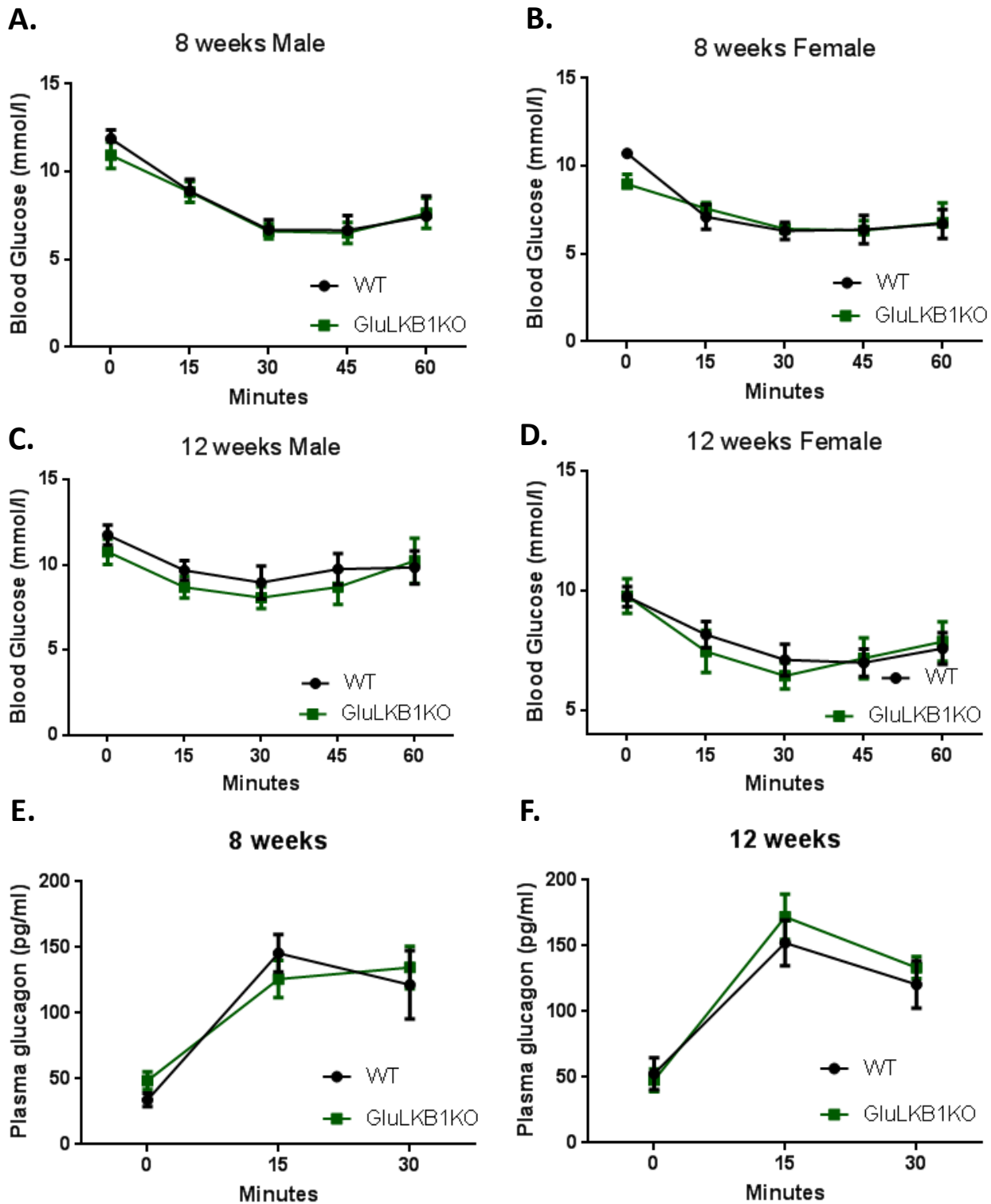


Figure 4.9 - Insulin tolerance and glucagon secretion *in vivo* are unaffected in GluLKB1KO mice

Insulin tolerance tests performed in (A,B) 8 week old male and female mice and (C,D) 12 week old male and female mice ($n=6-9$ mice/genotype). Glucagon secretion was measured after insulin injection after 15 and 30 mins. in (E) 8 week and (F) 12 week old male and female GluLKB1KO and WT mice. NS, by 2-way ANOVA, data are expressed as means $\pm$ SEM, $n=8-11$ mice/genotype.

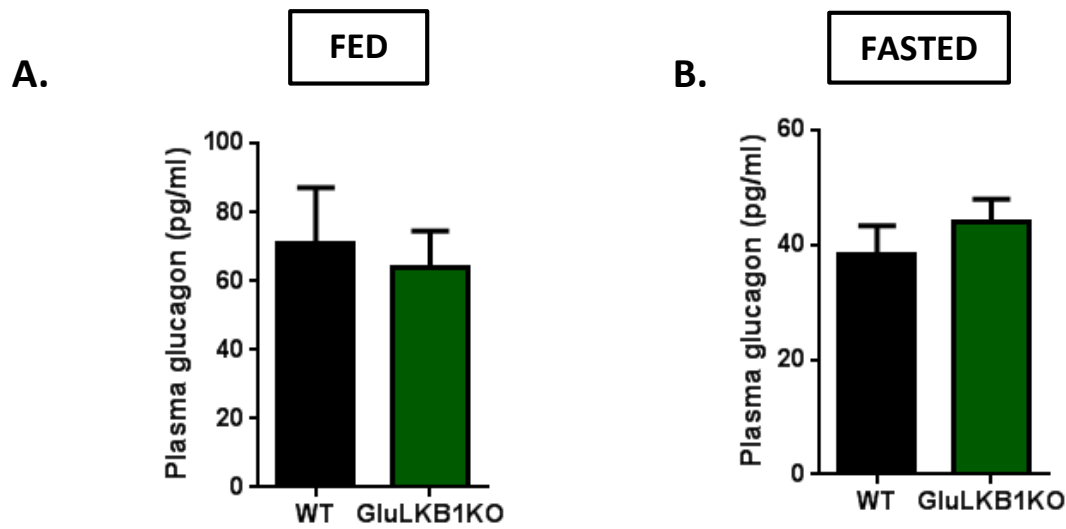


Figure 4.10 - Glucagon levels in plasma of GluLKB1KO mice both fed and fasted overnight are unaffected

Glucagon levels in the plasma of WT and GluLKB1KO mice (A) fed *ad libitum* and (B) fasted overnight (15 hours) measured by RIA. Data are expressed as means $\pm$ SEM, $n=6$ mice/genotype, NS, by Student's unpaired t-test.

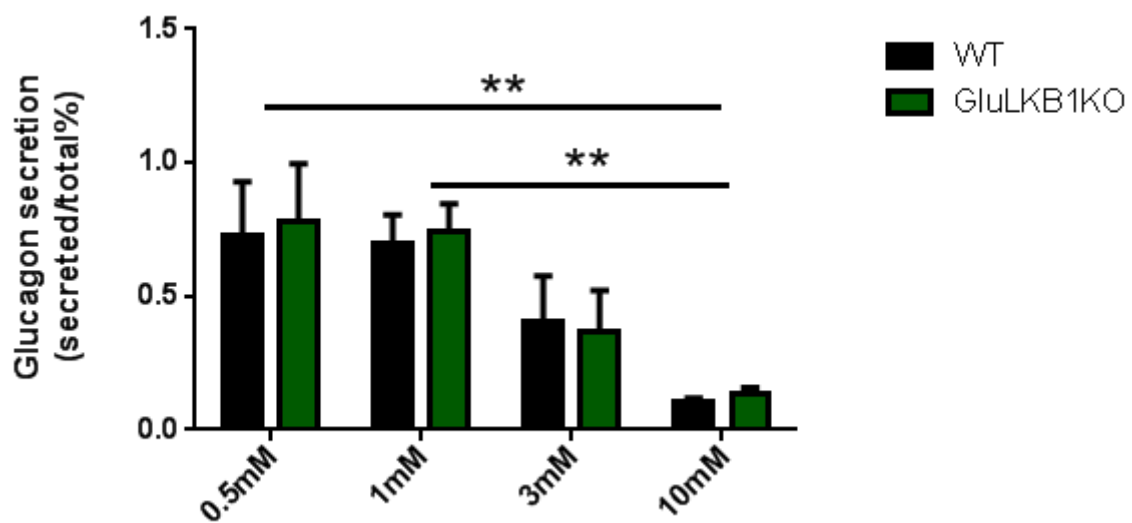


Figure 4.11 - Glucagon secretion is unaffected in GluLKB1KO mice

Glucagon secretion was measured in 10 size-matched islets incubated in 0.5, 1, 3 and 10mM glucose of WT and GluLKB1KO mice. Data are expressed as means $\pm$ SEM, $n=4$ mice/genotype, $**P < 0.01$, by 2-way ANOVA.

4.3 Discussion

The aims of this chapter were to determine whether the deletion of LKB1 from proglucagon-expressing cells may contribute to (1) the development of PJS and/or (2) impact GLP-1 and glucagon secretion, thereby enhancing GSIS. If this was the case then LKB1 in the gut may become a new therapeutic target in the treatment of Type 2 Diabetes.

To address this question we used the proglucagon-specific Cre recombinase, iGluCre, to delete *floxed* alleles of LKB1 so generating GluLKB1KO mice lacking this kinase in L-cells, pancreatic α -cells, as well as other proglucagon-expressing cells in the hind-brain and olfactory bulb (not shown).

4.3.1 *GluCre expression is localised in α - and L-cells*

GluLKB1KO mice and their WT littermates were crossed with mice bearing tdRFP at the Rosa locus (Rosa26tdRFP), thereby, allowing us to determine the degree of deletion of LKB1 in proglucagon-expressing cells. We found approximately 70% recombination of RFP occurred in the α -cells these mice (Figure 4.1b). We note that there may be a potential loss of RFP fluorescence when staining and a difference in recombination efficiency between the Rosa26 and LKB1 *loci* resulting in an over- or under- estimation of the degree of recombination. RFP fluorescence was also seen in a smaller percentage of β -cells, though no phenotypic effect in insulin secretion or β -cell mass was found in these mice. Recombination in enteroendocrine L-cells was also demonstrated when staining for RFP using the same method as Figure 4.1b and shown in [448] and Appendix 6.

4.3.2 *Deletion of LKB1 causes dramatic tumour growth in the duodenum*

Homozygous deletion of LKB1 led to the growth of large polyps at the gastro-duodenal junction (Figure 4.3). These polyps displayed resemblances to the polyps found in human PJS patients as well as to those of mice with heterozygous mutations in LKB1 [444]. However, some differences were noted between these GluLKB1KO polyps and earlier studies of LKB1 KO in the gut. Thus, in GluLKB1KO mice, L-cells account for approximately 1% of cells in the gastrointestinal tract and deletion of LKB1 on these cells resulted in polyps selectively in the duodenum of roughly 3cm in size in mice of 120 days. The size of these polyps was similar to that of polyps found in 6 month old LKB1^{+/-} mice [449] and larger than those found in both LKB1^{+/-} and LKB1^{-/-} mice selectively deleted for LKB1 in smooth muscle cells (~0.6mm)[347]. Additionally, LKB1 deletion in intestinal epithelial cells in previous models did not result in polyp growth [450], but inhibited differentiation of secretory

goblet cells resulting in an immature and less differentiated phenotype, indicating that LKB1 influences epithelial-mesenchymal transition (EMT) in epithelial cells. Polyps derived from GluLKB1KO mice appeared to be larger and were present at an earlier age than apparent in previous models [334, 444]. Out of sixteen GluLKB1het mice, only one developed polyps and mortality appeared to be enhanced in these mice in comparison to their WT littermates. It is possible that such large polyps grow in such an area of low cell population because the deletion of LKB1 affects polarity of these cells resulting in hyperproliferation in this area of the gastrointestinal tract. However, RFP tracing in the gastrointestinal tract of these mice provides no such evidence [448].

As described by Zac-Varghese *et al*, essentially no proglucagon staining or immunoreactive glucagon was found in the GluLKB1KO polyps [448]. We therefore suspect that immature LKB1^{-/-} L-cells undergo EMT and then go on to develop tumours. As explored in parallel studies [448], mice expressing GFP under the glucagon promoter allowed us to show that a small percentage of pro-glucagon-expressing cells express the EMT marker vimentin and so presumably undergo EMT by the 10th day of age. Lineage-tracing studies in GluLKB1KO mice also revealed that there was a greater number of cells in the duodenum with mesenchymal characteristics. Therefore, it is possible that LKB1 plays a role in the prevention of proliferation of cells in this cellular compartment. It seems most likely that polyps develop from this mutated population of smooth muscle cells than L-cells.

4.3.3 *GluLKB1KO display normal glucose tolerance and GLP-1 levels*

We were unable to detect GLP-1 in polyps of the KO mice [448], and levels of GLP-1 in the blood when given normal access to food and after overnight fasting was similar between GluLKB1KO and WT animals. GLP-1 was also measured by staining for proglucagon in the gut and we estimated L-cell mass by OPT. However, no difference was observed between KO and WT mice. Had GLP-1 secretion differed in GluLKB1KO mice *versus* WT we may have expected alterations in glucose tolerance [451]. However, when glucose tolerance tests were performed via both intraperitoneal and oral glucose administration, we found no differences between GluLKB1KO mice and their WT littermates (Figure 4.5 and 4.6). Although this may suggest that LKB1 expression in proglucagon-expressing cells plays little role in the control of incretin secretion and glucose homeostasis, this conclusion must be tentative given the tendency of LKB1 null L-cell progenitors to undergo EMT. In contrast, when LKB1 was deleted specifically in pancreatic β -cells, as discussed in chapter 3, glucose tolerance and insulin secretion were enhanced [318, 452]. In models of LKB1 deletion in α -cells (but not L-cells) using mice crossed with PPGCre recombinase, glucose tolerance was found to improve [453, 454].

Supporting our data that GLP-1 secretion is unchanged in GluLKB1KO mice, GLP-1 mRNA and protein levels were also found to be unaffected in the intestines of these mice [448]. Similarly, in polyps of GluLKB1KO mice GLP-1 protein expression was similar to that of WT duodenum [448].

4.3.4 *GluLKB1KO mice display normal glucagon secretion*

By deleting LKB1 in α -cells, we aimed to explore the effect that LKB1 has on glucagon production/secretion in mice. Modifying glucagon levels may be used as a potential therapeutic approach for diabetes, a concept already introduced and explored in studies in which mice deleted for the glucagon receptors resisted β -cell damage during Streptozocin induced diabetes [168, 455]. In contrast, in studies using the diphtheria toxin to reduced α -cell mass (by <90%), glucose homeostasis was only slightly altered, indicating that α -cells can be flexible in different conditions [456]. However, in our case, glucagon secretion measurements both *in vivo* and *in vitro* revealed that LKB1 deletion in proglucagon secreting cells has little effect on glucagon or glucose homeostasis [430].

As discussed previously, LKB1 plays a role in cell growth via inhibition of mTOR. Models of LKB1 deletion in the β -cell reveal enhancement of β -cell mass due to the up-regulation of this signalling pathway [318-320]. On the other hand, we find that LKB1 deletion in α -cells, alongside LKB1 *floxed* mice crossed with the preproglucagon (PPG) promoter [453], has little effect on the overall α -cell number or size (Figure 4.8). Thus, re-stimulation of mTOR activity by LKB1 does not appear to influence α -cell proliferation.

4.4 *Conclusions*

The role of LKB1 in enteroendocrine cells has not been explored until now. We deleted LKB1 in proglucagon-expressing cells (including enteroendocrine L-cells and pancreatic α -cells) by crossing LKB1 *floxed* mice with the iGlu Cre recombinase and explored the effects on glucose tolerance and GLP-1 and glucagon secretion. We found that LKB1 deletion has little effect on GLP-1 or glucagon secretion but results in large gastro-duodenal polyp growth similar to polyps found in Peutz Jeghers Syndrome.

Chapter 5

Deletion of AMPK α 1 and α 2 in proglucagon-expressing cells up-regulates GLP-1 levels and improves glucose homeostasis

Abstract

Background and aims: Enteroendocrine L-cells synthesise and release the gut hormone glucagon-like peptide-1 (GLP-1) in response to food transit. Deletion of the tumour suppressor kinase LKB1 from proglucagon-expressing cells leads to the generation of intestinal polyps but no change in circulating GLP-1 levels. Here, we explore the role of the downstream kinase AMP-activated protein kinase (AMPK) in these cells.

Methods: Loss of AMPK from proglucagon-expressing cells was achieved using a preproglucagon promoter-driven *Cre* (iGluCre) to catalyse recombination of *floxed* alleles of AMPK α 1 and α 2. Oral and intraperitoneal glucose tolerance tests were performed and L- and α -cell mass measured by immunocytochemistry. GLP-1 and glucagon secretion were measured by electrochemical-based luminescence detection or radioimmunoassay.

Results: Recombination with iGluCre led to efficient deletion of AMPK from enteroendocrine L- and pancreatic α -cells. In contrast to GluLKB1KO mice, mice deleted for AMPK displayed an increase (WT: $0.050 \pm 0.006\%$, KO: $0.090 \pm 0.016\%$, $p < 0.01$) in L-cell mass and elevated plasma fasting (WT: $5.62 \pm 0.80\text{pg/ml}$, KO: $14.5 \pm 1.87\text{pg/ml}$, $p < 0.01$) and fed (WT: $15.7 \pm 1.48\text{pg/ml}$, KO: $22.0 \pm 6.62\text{pg/ml}$, $p < 0.01$) GLP-1 levels. Oral, but not intraperitoneal, glucose tolerance was significantly improved by AMPK deletion, whilst insulin and glucagon levels were unchanged despite an increase in α - to β -cell ratio (WT: $0.23 \pm 0.02\text{AU}$, KO: $0.33 \pm 0.03\text{AU}$, $p < 0.01$).

Conclusions: AMPK restricts L-cell growth and GLP-1 secretion to suppress glucose tolerance. Targeted inhibition of AMPK in L-cells may thus provide a new therapeutic strategy in some forms of Type 2 Diabetes.

5.1 Introduction

As discussed in previous chapters, enteroendocrine L-cells make up less than 1% of the epithelial cells lining of the intestinal wall, but are vital for normal physiology and energy metabolism [445, 446]. L-cells are thus responsible for the synthesis and secretion of the gut hormones glucagon-like peptide-1 (GLP-1), GLP-2, peptide YY (PYY) and oxyntomodulin via the action of prohormone convertases (PC) 1/3 on proglucagon [351]. GLP-1 receptors (GLP-1R) are present on pancreatic β -cell and agonism at these receptors by L-cell-derived hormones or by stabilised analogues such as liraglutide [457] is of considerable therapeutic interest in the treatment of Type 2 Diabetes. Thus, in response to food transit, secreted GLP-1 binding to GLP-1R activates pancreatic β -cell cAMP synthesis and downstream signalling by Protein kinase A (PKA) and Exchange Protein Activated by cAMP-2 (EPAC2), activating insulin secretion (Figure 1.11) [359, 458]. Although the mechanisms involved are debated [459], enhanced ATP synthesis [460], closure of ATP-sensitive K^+ (K_{ATP}) channels and Ca^{2+} influx appear to play a role, particularly in human β -cells [461]. Thus, GLP-1R null mice mouse models display impaired glucose-stimulated insulin secretion (GSIS) in common with patients affected by Type 2 Diabetes [357, 462]. Importantly these actions of incretin hormones complement the effects of GLP-1 and its congeners to control appetite, energy expenditure and body weight. These pleiotropic effects combine to elicit marked improvements in glucose tolerance and in the levels of glycated haemoglobin 1c (HbA1c) [463], a predictor of subsequent disease complications [170, 395, 398]. Whether the effects of GLP-1 are chiefly achieved through the effects of the circulating hormone [464], or reflect the actions of released GLP-1 to act locally in the gut to trigger an autocrine reflex act which ultimately controls blood glucose [465, 466], is contested.

AMP-activated protein kinase (AMPK) is a highly conserved fuel-sensitive serine/threonine protein kinase implicated in the regulation of blood glucose levels [317] by acting as a cellular nutrient sensor [467]. AMPK exists as a heterotrimeric complex comprising a catalytic α (α 1 and α 2) subunit, a scaffold β (β 1 and β 2) and a regulatory γ (γ 1, γ 2 or γ 3) subunit [209, 210]. The AMPK complex is extremely sensitive to intracellular ATP/AMP [206] and ATP/ADP ratios [220]. It has been proposed that stimulation of AMPK in the liver and muscle respectively via the glucose-lowering agents metformin and thiazolidinedione may contribute to the improvements in overall insulin sensitivity [296] brought about by these agents. The activity of AMPK is chiefly controlled by the activity of upstream kinases, notably LKB1 (STK11) and Ca^{2+} calmodulin kinase II (CamKII) [468] which phosphorylate threonine 172 in the key catalytic loop [296].

Glucagon is the main anti-hypoglycaemic hormone available in all mammals [469]. Stimulation of glucagon secretion from pancreatic α -cells occur after blood glucose levels drop and impairment of glucagon release is observed in patients suffering from Type 1 diabetes as well as in long-standing Type 2 Diabetes [470]. However, earlier in disease progression, an increase in circulating glucagon levels is usually present in patients suffering from Type 2 Diabetes, contributing to increased blood glucose levels [163].

At the cellular level, the fall of blood glucose will stimulate depolarisation of the α -cell [69, 137] and the opening L- (and to the lesser extent N-) type voltage-dependent Ca^{2+} channels [128]. This will result in Ca^{2+} entry into the cell and the stimulation of glucagon exocytosis. However, the exact mechanisms through which glucagon secretion is controlled are still debated [471] and likely to involve both cell-autonomous effects of glucose lowering on the α -cell as well as changes in neural inputs and in other islet hormones, notably somatostatin [150].

Previous studies [272, 472] have explored the effects of deleting the catalytic (α 1 and α 2) subunits of AMPK either individually or together in the β -cell in mice using the rat insulin promoter-2 (RIP2) Cre [273]. Studies in islets isolated from α 1/ α 2 null mice indicated that AMPK acts as a negative regulator of insulin secretion at the level of individual cells, consistent with findings in β -cell lines [214, 228]. However, these mice became glucose intolerant and insulin secretion was impaired *in vivo*, demonstrating a requirement for AMPK for normal insulin release *in vivo*. Whilst the reasons for this discrepancy are not fully resolved, studies with the more β -cell selective Ins1Cre [427] suggested both confounding effects of AMPK deletion in key brain nuclei may be involved as well as changes in the expression of key β -cell genes involved in responsiveness to incretins, and the maintenance of normal β -cell identity.

More recently, the role of AMPK in the control of glucagon secretion has also been investigated. Using clonal α TC1-9 cells and pancreatic islets, it was found that forced over-expression of AMPK was able to activate glucagon release, whilst a dominant-negative form of the enzyme blocked release at low glucose concentrations [284]. The effect of AMPK (α 1, α 2 or both) deletion from the α -cell *in vivo* in mice has also been explored recently using a proglucagon deleter strain (PPGCre) [454]. Deletion of AMPK α 1 from these cells resulted in improvement of glucagon release whereas the effects of AMPK α 2 deletion were minimal [453].

As discussed in chapter 4, LKB1, in addition to phosphorylating AMPK, is a tumour suppressor deleted in PJS. In chapter 4, we discovered that deletion of LKB1 from proglucagon-expressing cells, achieved by crossing animals bearing a glucagon promoter-driven Cre recombinase (iGluCre) to mice

with *floxed* LKB1 alleles, leads to the development of large gastro-duodenal polyps, reminiscent of those seen in PJS [473]. Lineage tracing revealed that LKB1 ablation caused an epithelial-to-mesenchymal transition (EMT) in LKB1 null proglucagon-expressing cells, and consequently the formation of a population of smooth muscle-like cells which expanded to form polyps.

This chapter was therefore designed with two goals in mind. Firstly, to determine whether the suppressive effect of LKB1 on EMT in L-cells or their progenitors is mediated by AMPK or via other AMPK-regulated kinases [204], and secondly to explore the role of AMPK in controlling GLP-1 release. We addressed these questions by breeding iGluCre mice to animals bearing floxed AMPK α 1 and AMPK α 2 alleles to generate mice lacking AMPK activity in the majority of proglucagon-expressing cells in the body (GluAMPKKO). Whilst polyp development was not observed in these mice, AMPK α 1/ α 2 deletion led to improved glucose tolerance when glucose was administered using oral gavage, but not through intraperitoneal injection. We show that the improvement in glucose tolerance reflects an increase in the number of L-cells, and up-regulated circulating GLP-1 levels. Thus, AMPK plays a role in the control of GLP-1 secretion from enteroendocrine cells of the gut, acting to improve glucose tolerance.

5.2 Results

5.2.1 *Deletion of AMPK α 1 α 2 from preproglucagon-expressing cells does not lead to polyposis*

GluAMPKKO mice were checked regularly for any sign of tumour development, and body mass was measured weekly (Figure 5.1d-e). Weights were measured weekly until 20 weeks of age in male and 17 weeks in female mice which is when mice were culled for isolation of islets or the harvesting of tissues. Weights in both male and female GluAMPKKO *versus* WT mice were unaltered. GluAMPKKO mice also appeared healthy and not bloated in appearance (data not shown), as was previously observed in GluLKB1KO mice (see chapter 4). When the intestine was explored upon post-mortem no polyp growth was discovered in the null mice (Figure 5.1a-c).

To determine the level that AMPK was knocked-down in proglucagon secreting cells, pancreatic slices were stained for phosphorylated (P) AMPK α 1 and α 2 (red) and proglucagon (green) and the % of α -cells co-stained with both AMPK and pro-glucagon calculated (Figure 5.2a-b). Overall there appeared to be an approximately 2-fold decrease in P-AMPK α 1 α 2 expression in α -cells ($P < 0.01$, WT: $59.6 \pm 5.57\%$, KO: $35.1 \pm 4.04\%$). AMPK expression was also quantified in proglucagon secreting L-cells in the ileum (Figure 5.3a-b), as with pancreatic staining there appears to be a 2-fold decrease in AMPK expression in GluAMPKKO *versus* WT ileum sections ($P < 0.001$, WT: $59.33 \pm 2.95\%$, KO: $35.33 \pm 4.75\%$). To determine whether the iGlu Cre recombinase deletes in β -cells, pancreatic slices of WT and GluAMPKKO mice was co-stained for insulin (green) and AMPK α 1 α 2 (red) and the % of co-staining quantified. We found that expression of AMPK is similar in GluAMPKKO *versus* WT β -cells (NS, WT: $83.45 \pm 5.13\%$, KO: $76.76 \pm 2.07\%$, Figure 5.4a-b). Therefore, we are confident that any changes in insulin expression/secretion in these mice are not due to deletion of AMPK in the β -cell.

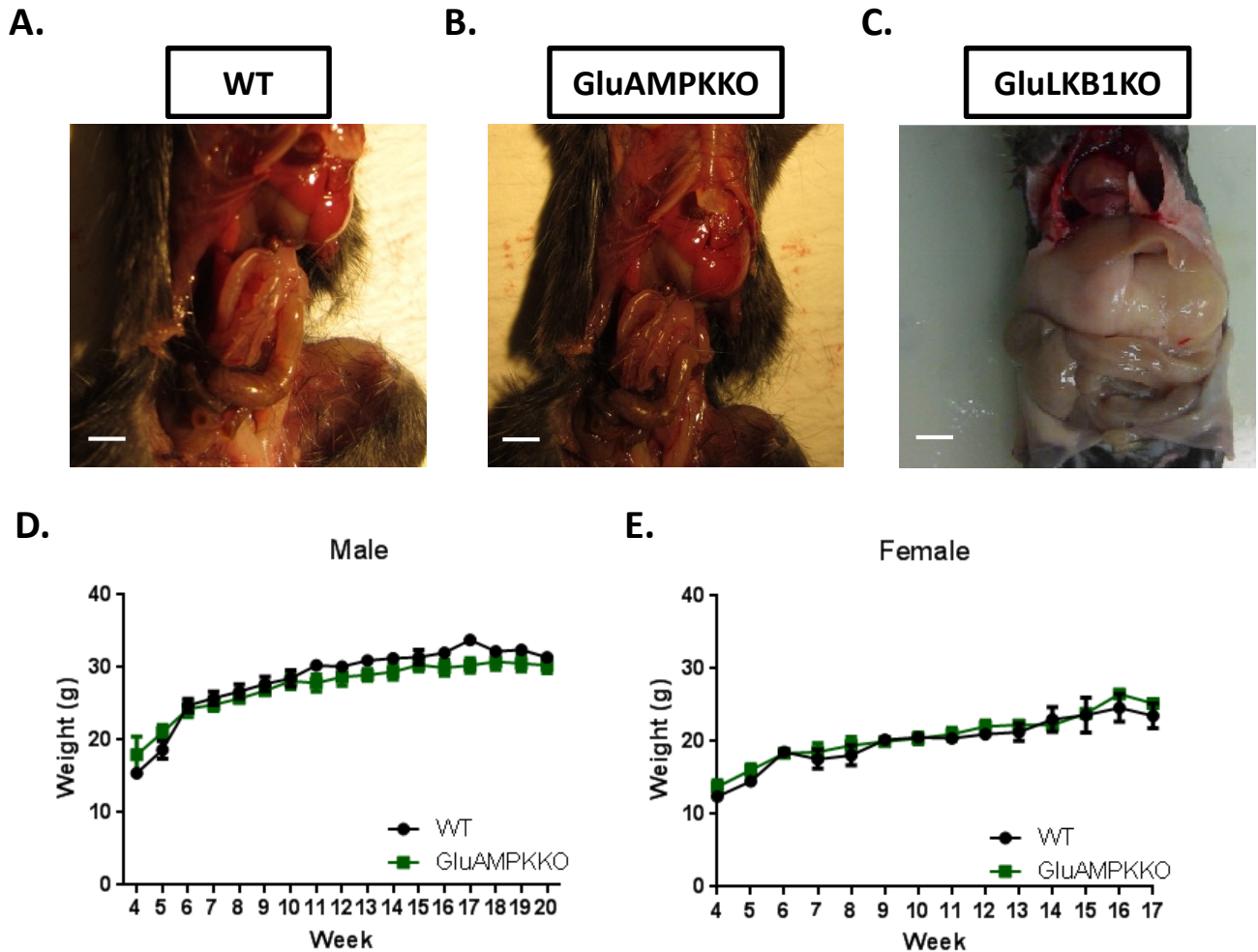


Figure 5.1 - GluAMPKKO mice did not display any weight loss or polyp development as found in GluLKB1KO mice

Photographs of the lower abdomen of (A) WT, (B) GluAMPKKO and (C) GluLKB1KO (chapter 4) mice at 15-20 weeks of age. Unlike GluLKB1KO mice, GluAMPKKO mice did not develop polyps in the duodenum that may have resulted in premature death of the mouse. Changes in body mass over time in (A) Male (4-20 weeks) and (B) Female GluAMPKKO and WT mice (4-17 weeks). $N=9-11$ mice/genotype. Scale bar: 5mm.

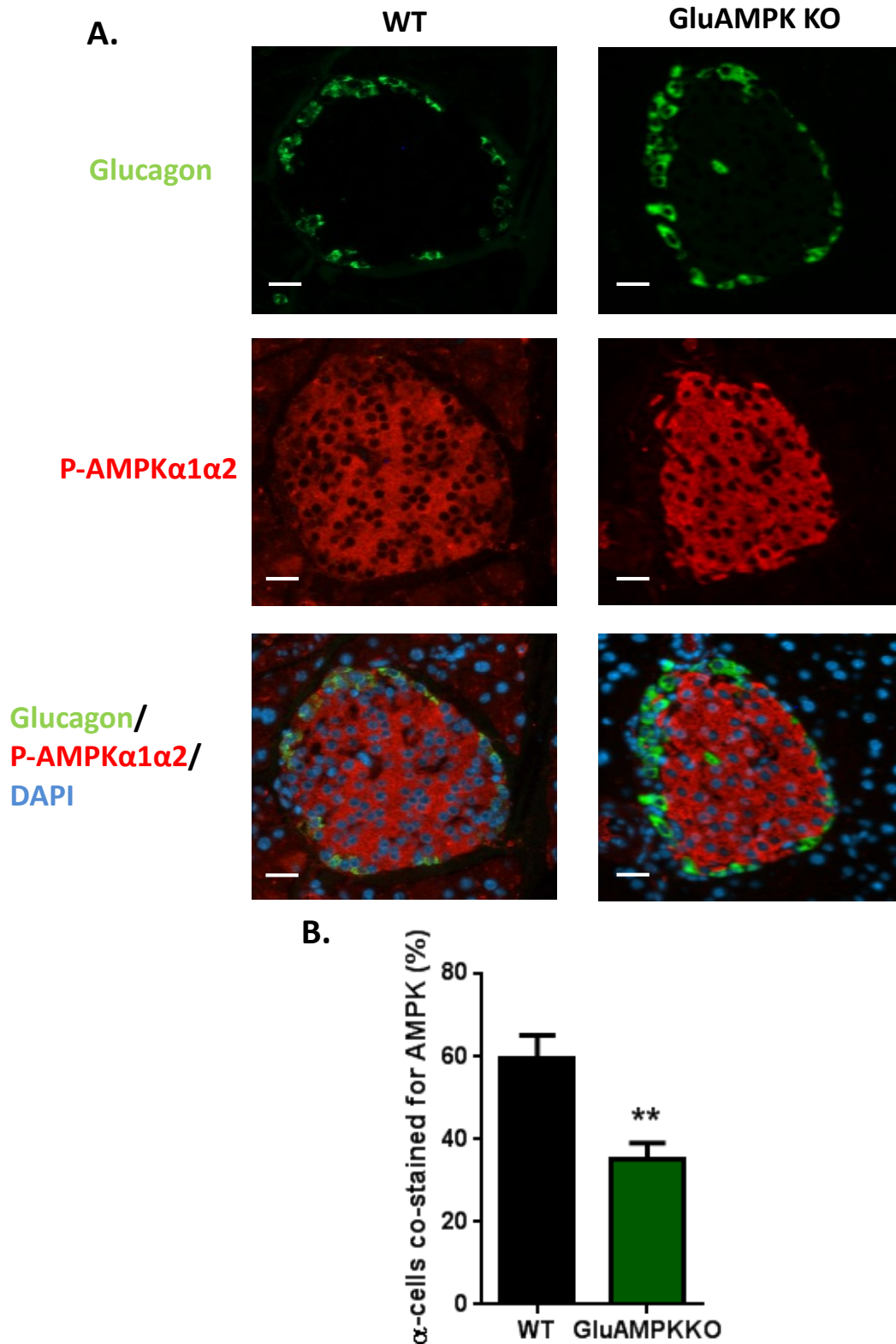


Figure 5.2 - AMPK expression is down-regulated in ~30% α -cells

(A) Immunofluorescent staining of pancreatic sections using anti-rabbit P-AMPK α 1 α 2 (1:100; red) and anti-goat glucagon (1:100; green) antibodies of GluAMPK KO and WT mice, (B) % of cells co-stained for AMPK and glucagon was calculated to measure % AMPK knock-down in α -cells. $N=3$ mice/genotype, ** $P<0.01$ by unpaired Student's t-test, data are expressed as means $\pm$ SEM. Scale bar: 50 μ m.

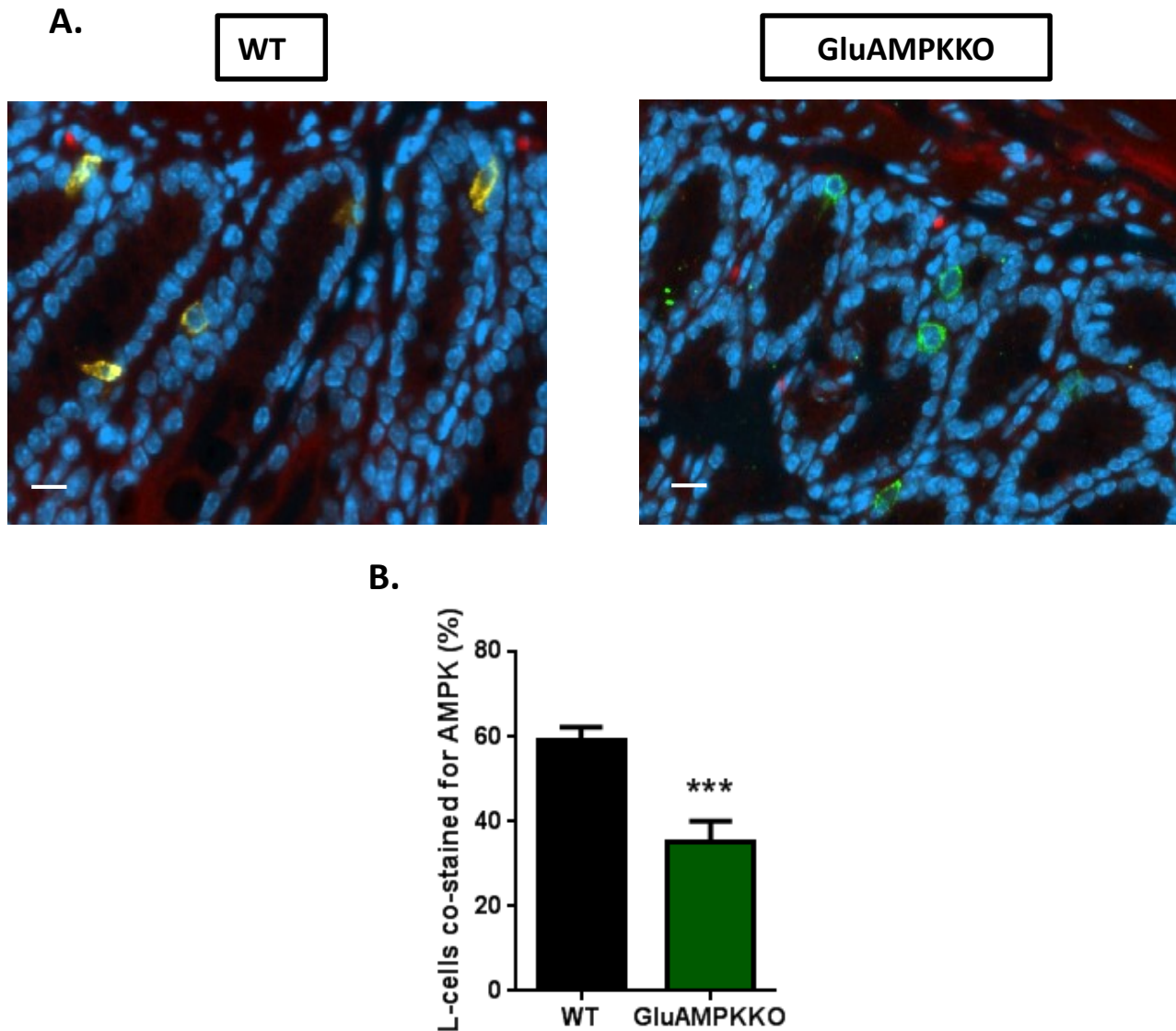
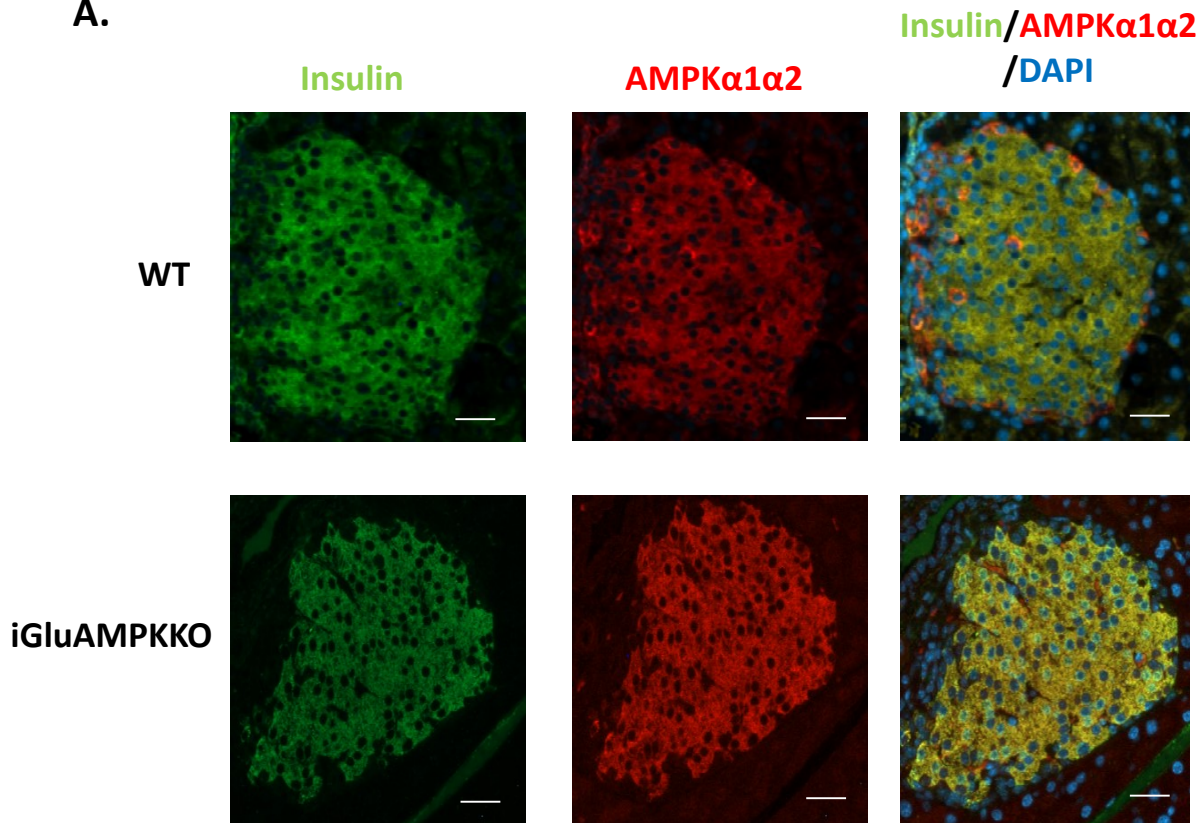


Figure 5.3 - AMPK expression in enteroendocrine L-cells is down-regulated in GluAMPKKO versus WT mice
(A) Immunohistochemical staining of ileum sections using P-AMPK α 1 α 2 (red) and proglucagon (green) antibodies show co-staining between proglucagon and AMPK, (B) % of L-cells expressing AMPK in GluAMPKKO mice and their WT littermates. **P<0.001 by unpaired Student's t-test. Data are expressed as means $\pm$ SEM, n= 3 mice/genotype. Scale bar: 50 μ m.

A.



B.

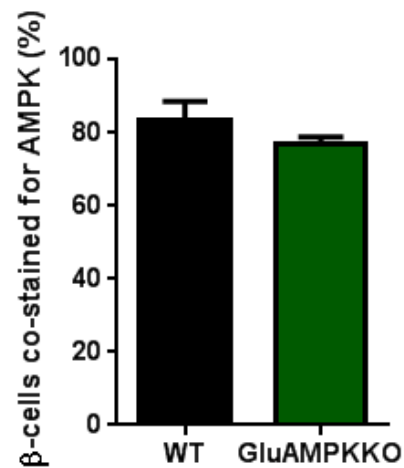


Figure 5.4 - iGlu Cre driven recombination does not cause deletion of AMPK in pancreatic β -cells

(A) Immunofluorescent staining of pancreatic slices co-stained for insulin (green) and AMPK (red) and (B) % of co-staining quantified to analyse the % of AMPK knocked-out in WT and GluAMPKKO β -cells. $N=3$ mice/genotype, NS, by Student's unpaired t-test, data are expressed as means $\pm$ SEM. Scale bars: 50 μ m.

5.2.2 *Deletion of the AMPK α -subunits improves glucose tolerance after oral gavage but not after intraperitoneal injection*

Glycaemia was unchanged between male and female AMPK α 1 α 2KO mice *versus* their WT littermates with all animals given free access to food (WT: 10.03 $\pm$ 0.45 mmol/L and KO: 9.7 $\pm$ 0.63 mmol/L, Figure 5.5a-b).

The iGluCre promoter deletes selectively in proglucagon-expressing cells for example, enteroendocrine L-cells [403]. Therefore, OGTT was performed to assess any changes in glucose homeostasis in these mice in comparison to their WT littermates due to changes in the release of glucose lowering, gut secreting hormones such as GLP-1. During OGTT, basal glycaemia was found to be unchanged in 10 week old GluAMPKKO *versus* WT mice in mice fasted overnight but significantly improved 30 mins. after glucose administration ($P < 0.01$, WT: 17.3 $\pm$ 1.4.8 mmol/L and KO: 12.5 $\pm$ 0.70 mmol/L, Figure 5.6a). Glucose tolerance was enhanced further in 15 week old male mice in GluAMPKKO *versus* WT mice (15 mins.; WT: 17.2 $\pm$ 0.65 mmol/L and KO: 13.8 $\pm$ 0.65 mmol/L ($P < 0.05$), 30 mins.; WT: 18.3 $\pm$ 0.97 mmol/L and KO: 13.9 $\pm$ 0.97mmol/L ($P < 0.01$), and 45 mins.; WT: 15.9 $\pm$ 1.02mmol/L and KO: 12.4 $\pm$ 0.69 mmol/L ($P < 0.01$), Figure 5.6c). In comparison, glucose tolerance was unaltered in female GluAMPKKO *versus* WT mice at both 10 and 15 weeks of age when OGTTs were performed (Figure 5.6b, d).

In addition to OGTT, IPGTTs were performed on these mice at 20 weeks of age to examine if these improvements in glucose tolerance in these mice can also be seen after intraperitoneal injection of glucose. We found that, unlike oral administration of glucose, intraperitoneal injected glucose does not show alterations in glucose tolerance in GluAMPKKO *versus* WT (Figure 5.7a-b).

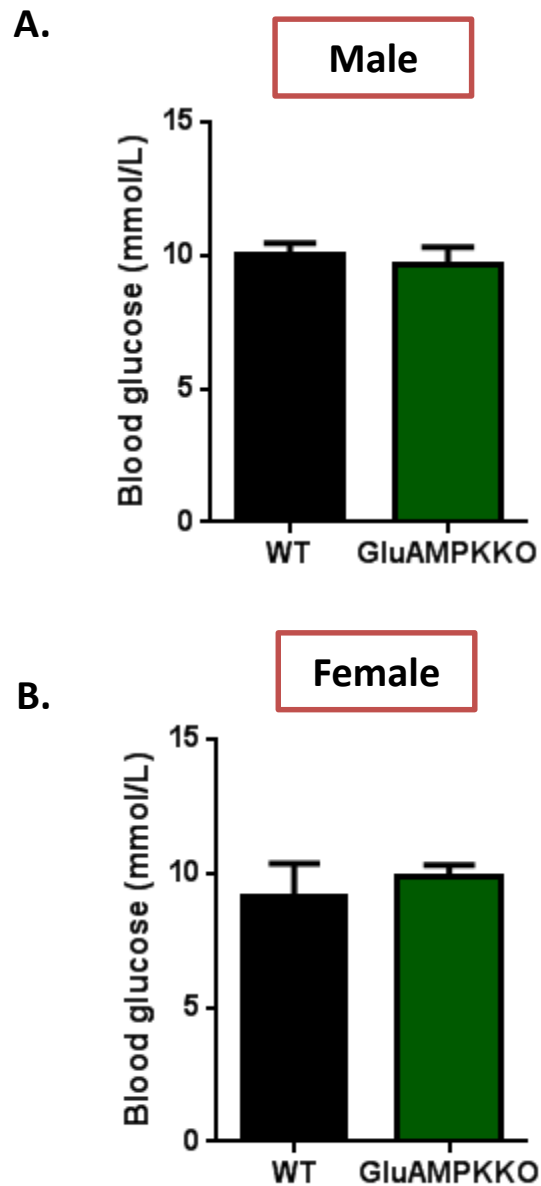


Figure 5.5 - Fed glycaemia is unchanged in GluAMPKKO versus WT mice

Glycaemia was measured in (A) male and (B) female GluAMPKKO and WT mice when fed *ad libitum* and was found to be the same between these mice. NS, by Student's unpaired t-test, data are expressed as means $\pm$ SEM, $n=6-9$ mice/genotype/sex.

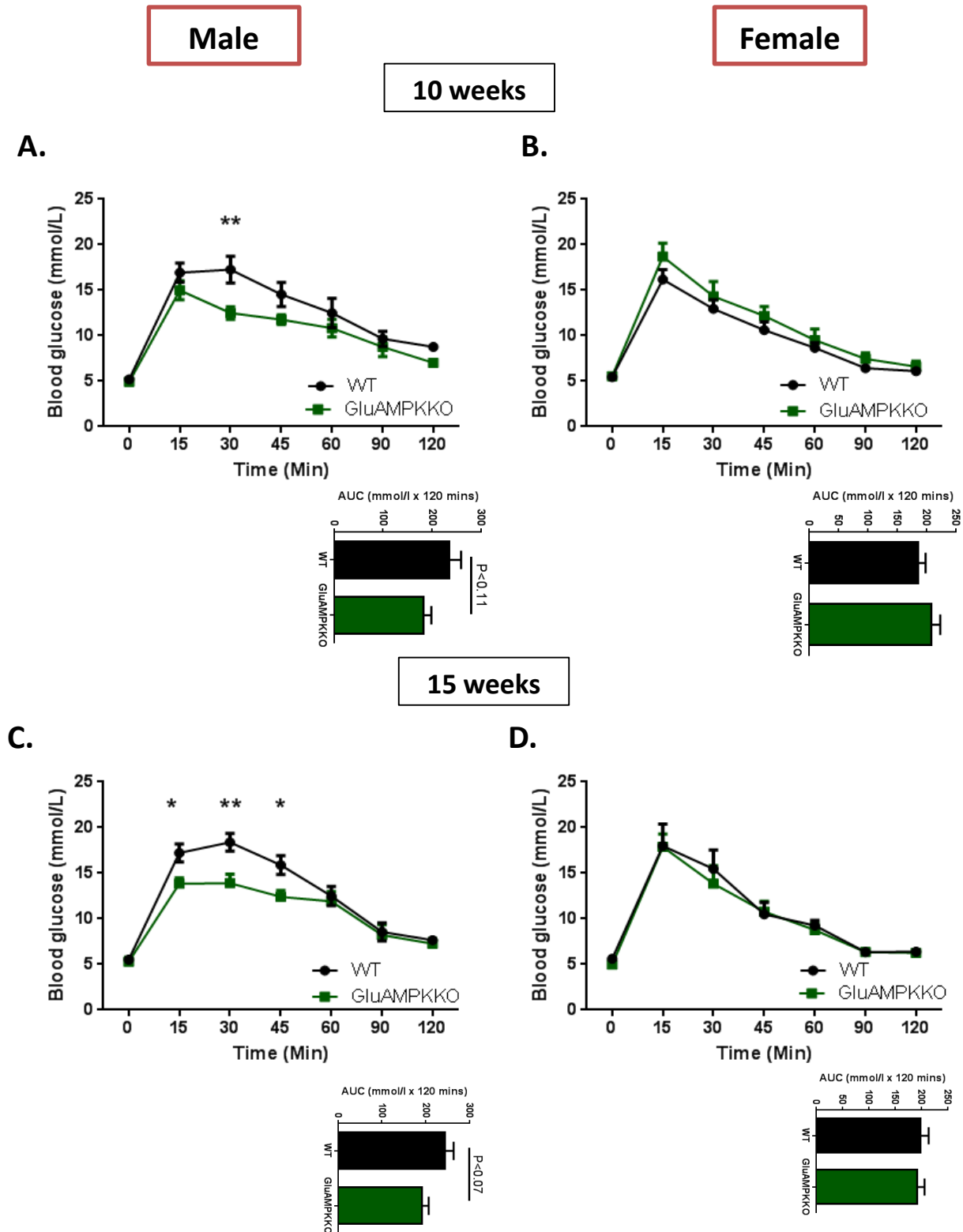


Figure 5.6 - Glucose tolerance is improved in GluAMPKKO mice after oral administration of glucose

Glucose was administered using oral gavage (1 g/kg) after mice were fasted overnight and blood glucose levels measured at 0, 15, 30, 45, 60, 90 and 120 mins. after glucose administration in (A-B) 10 week old male and female mice and (C-D) 15 week old male and female mice, AUC is also displayed in bottom right panels. $N=5-9$ mice/genotype, * $P<0.05$, ** $P<0.01$ by 2-way ANOVA and Student's unpaired t-test. Data are expressed as means $\pm$ SEM.

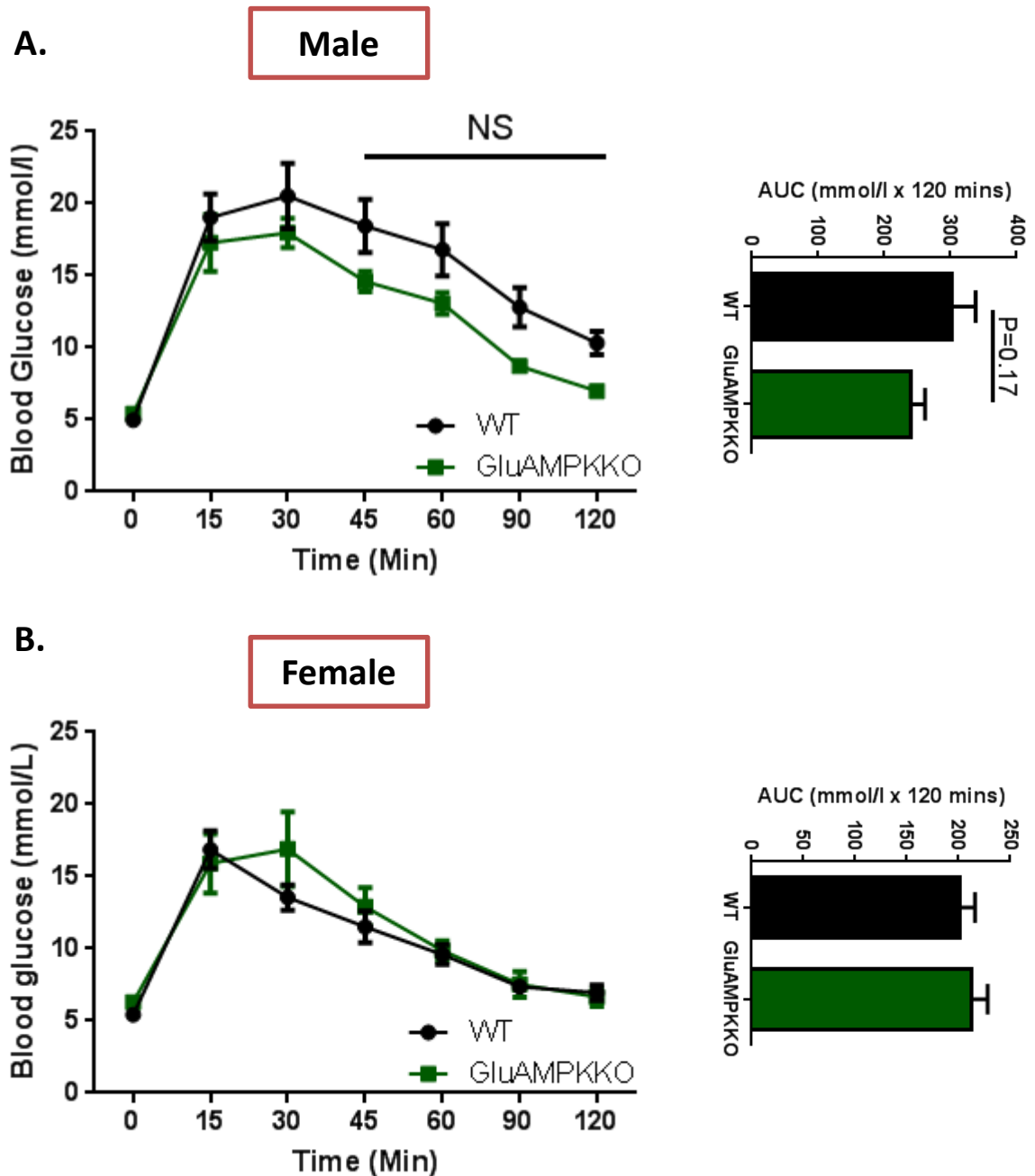


Figure 5.7 - Glucose tolerance is unaffected in GluAMPKKO mice after intraperitoneal administration of glucose

IPGTT's were performed on 20 week old (A) male and (B) female mice, AUC displayed on the right. $N=5-9$ mice/genotype, NS, by 2-way ANOVA and Student's unpaired t-test, data are expressed as means $\pm$ SEM.

5.2.3 *AMPK α 1 α 2 deletion in proglucagon expressing cells has no effect on insulin tolerance or circulating insulin levels*

To determine whether deletion of the AMPK α -subunits in proglucagon-expressing cells has an effect on insulin tolerance, insulin tolerance tests (ITTs) were performed (Figure 5.8). ITTs were carried out on 10 and 15 week old male and female mice. These tests showed no changes in insulin tolerance in GluAMPKKO *versus* WT mice apart from at the 60 min. time point in 10 week old male mice (Figure 5.8a). Here, blood glucose was significantly higher in GluAMPKKO *versus* WT mice ($P < 0.01$, WT: 7.12 ± 0.36 mmol/L and KO: 10.62 ± 1.52 mmol/L).

Insulin levels were measured *in vivo* by looking at insulin levels in the plasma of mice given free access to food, or after an overnight fast (Figure 5.9a-b). These measurements showed no changes in insulin levels between GluAMPKKO *versus* WT mice (NS, Fed; WT: 2.43 ± 0.18 ng/ml and KO: 2.07 ± 0.39 ng/ml, Fasted; WT: 1.21 ± 0.46 ng/ml and KO: 1.67 ± 0.06 ng/ml). Plasma insulin levels were also measured 0, 15 and 30 mins. after glucose injection, and again, no alterations were found in insulin levels between GluAMPKKO mice and their WT littermates (NS, 0 mins. WT: 0.09 ± 0.006 ng/ml, KO: 0.10 ± 0.02 ng/ml; 15 mins. WT: 0.18 ± 0.07 ng/ml, KO: 0.19 ± 0.02 ng/ml; 30 mins. WT: 0.22 ± 0.08 ng/ml, KO: 0.25 ± 0.07 ng/ml, Figure 5.10).

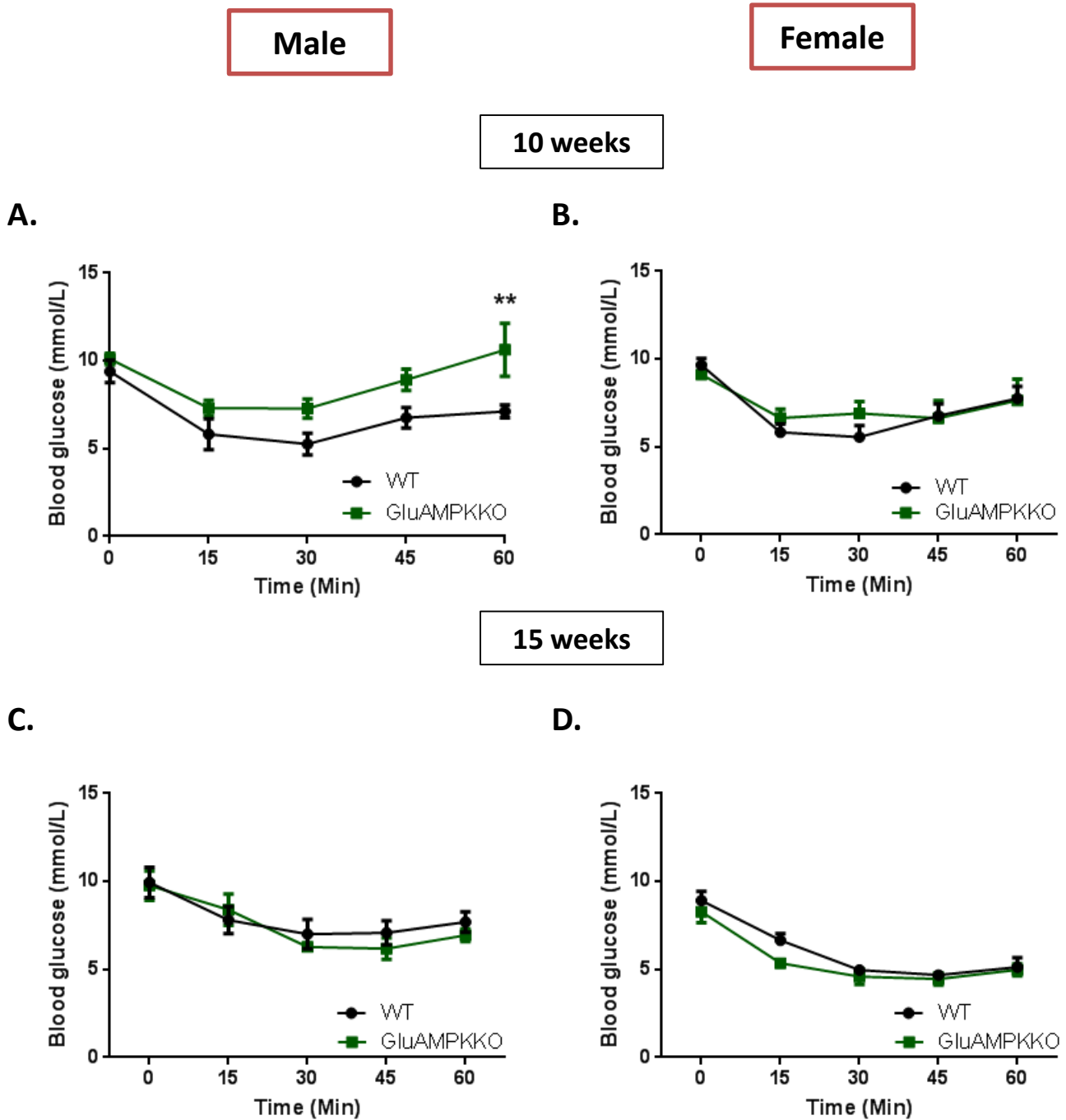


Figure 5.8 - AMPK α 1 and α 2 deletion has no effect on glycaemia after insulin injection

ITT's were performed on (A-B) 10 week old male and female and (C-D) 15 week old male and female GluAMPKKO and WT mice after a 5 hour fast and injected with 0.75U/kg insulin, blood glucose was measured 0, 15, 30, 45 and 60 mins. after insulin injection. N= 5-9 mice/sex/genotype, **P<0.01 by 2-way ANOVA data are expressed as means $\pm$ SEM.

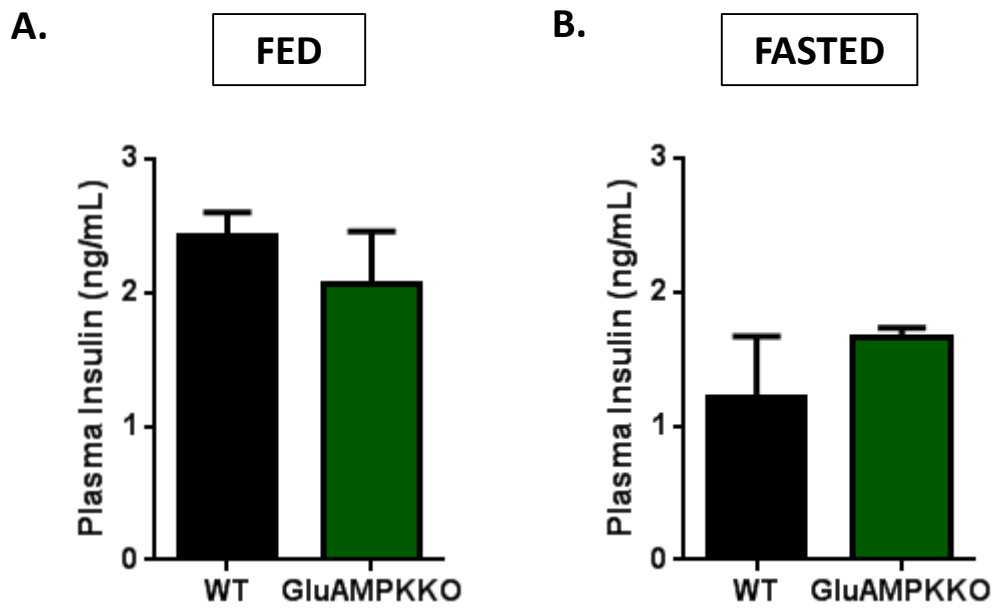


Figure 5.9 - Plasma insulin levels are unaffected in GluAMPKKO mice when fed *ad libitum* or fasted overnight

Plasma insulin levels were measured in GluAMPKKO and WT mice when (A) fed *ad libitum* and (B) fasted overnight. By Student's unpaired t-test, $N=6$ mice/genotype, data are expressed as means $\pm$ SEM.

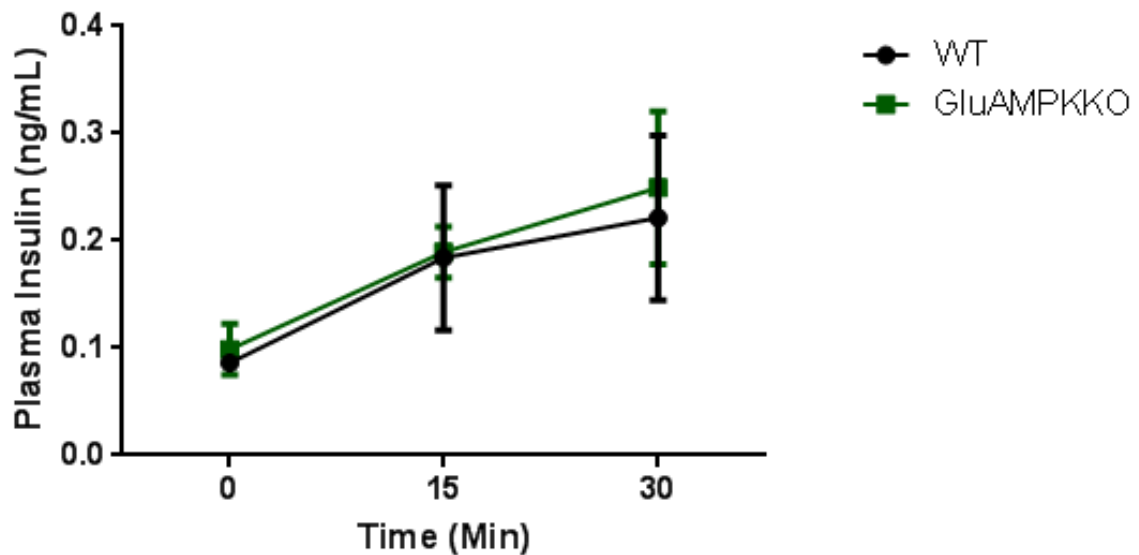


Figure 5.10 - The iGlu Cre transgene has no effect on β -cells function

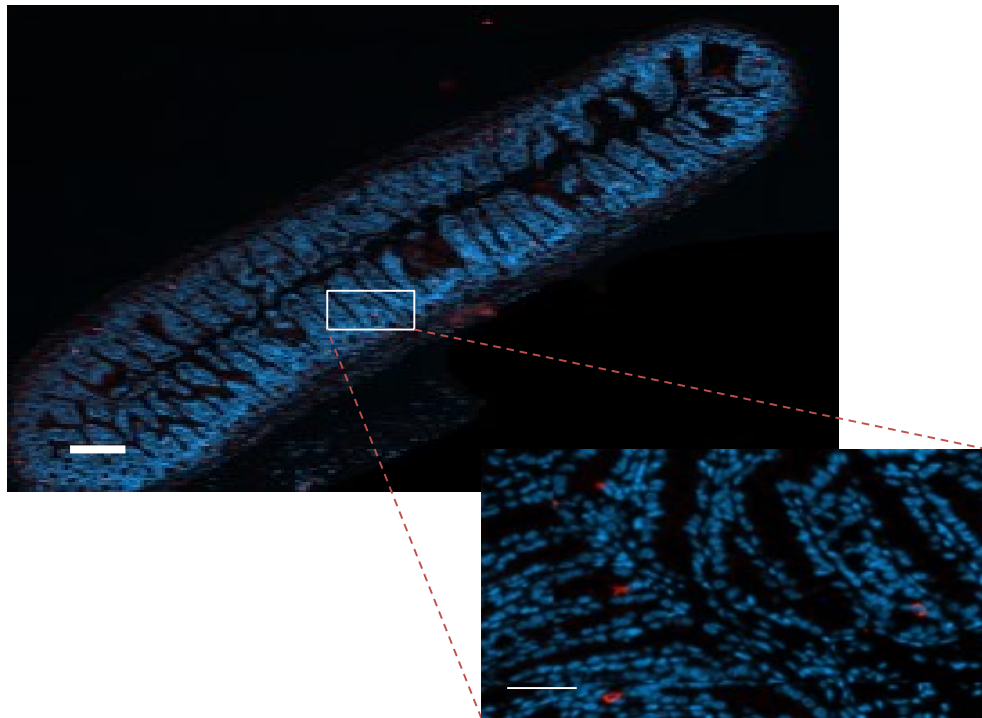
Plasma insulin levels were measured at 0, 15 and 30 mins. after glucose injection in WT *versus* GluAMPKKO mice. $N=3$ mice/genotype, NS, by 2-way ANOVA, data are expressed as means $\pm$ SEM.

5.2.4 *AMPK α 1 and α 2 deletion in proglucagon-expressing cells results in increased L-cell mass and circulating GLP-1 levels.*

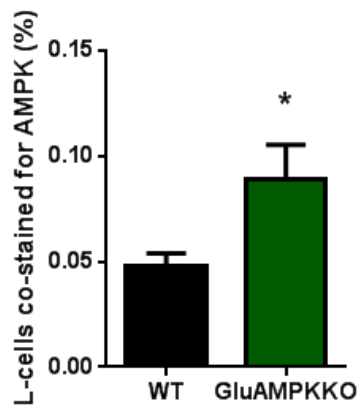
The improvement in oral (Figure 5a, c) but not intraperitoneal (Fig. 6a) glucose tolerance is suggestive of an improved incretin response in GluAMPKKO *versus* WT mice. To explore this possibility, L-cell mass and circulating GLP-1 levels were measured. Ileum sections of GluAMPKKO mice and their WT littermates were stained with a proglucagon staining antibody which allowed us to measure the percentage of proglucagon-expressing cells (Figure 5.11a-b). We found that L-cell mass was increased in GluAMPKKO *versus* WT mice ($P < 0.05$, WT: $0.050 \pm 0.006\%$ and KO: $0.090 \pm 0.016\%$, Figure 5.11b) and cell number increased though not significantly ($P = 0.06$, WT: $0.0092 \pm 0.002\mu\text{M}^{-2}$, KO: $0.015 \pm 0.002\mu\text{M}^{-2}$, Figure 5.11C). Epithelial cell number was unaltered in GluAMPKKO *versus* WT mice (NS, WT: 118.9 ± 4.33 KO: 121.2 ± 4.93).

Circulating GLP-1 secretion was measured by collecting plasma of GluAMPKKO mice and WT mice either when given free access to food or after overnight fast (Figure 5.12a-b). We found that plasma GLP-1 levels in GluAMPKKO mice both when fed and fasted were higher than in WT mice ($P < 0.01$, Fed; WT: $15.69 \pm 1.48\text{pg/ml}$ and KO: $21.99 \pm 6.52\text{pg/ml}$, Fasted; WT: $5.62 \pm 0.80\text{pg/ml}$ and KO: $14.46 \pm 1.87\text{pg/ml}$).

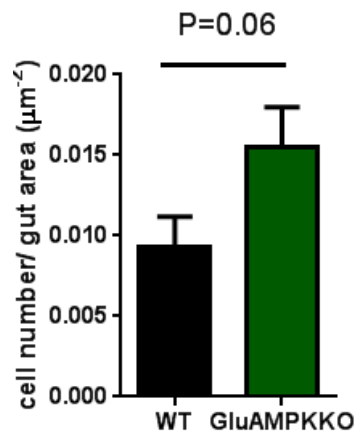
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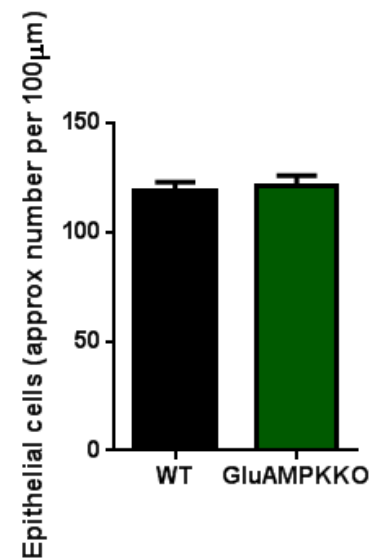


Figure 5.11 - L-cell mass is increased in GluAMPKKO mice

(A) Immunohistochemical analysis of an ileum section from GluAMPKKO *versus* WT male mouse; red staining represents proglucagon (GLP-1) staining in an enteroendocrine cell, dotted lines represent magnified section. (B) Percentage of proglucagon staining cells in the ileum. (C) L-cell count/area of gut, $P=0.06$ (D) Approximate endothelial cell count for the whole gut section. $N=3$ mice/genotype (male), * $P<0.5$ by unpaired Student's t-test. Data are expressed as means $\pm$ SEM.

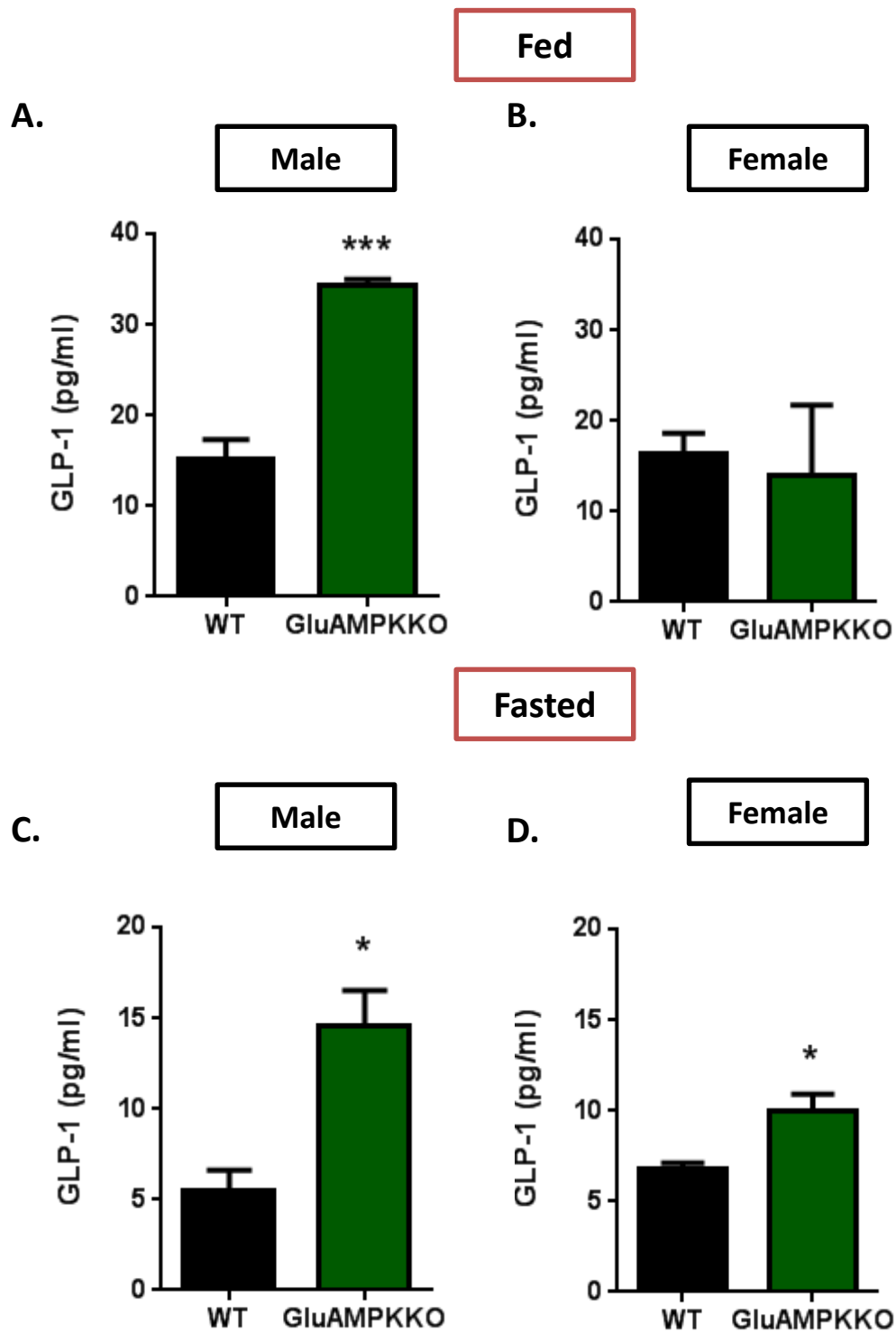


Figure 5.12 - GLP-1 secretion *in vivo* is enhanced in GluAMPKKO mice

GLP-1 levels were measured in the plasma of (A) fed and (B) fasted GluAMPKKO and WT mice, $n=6$ mice/genotype. ** $P<0.01$ by unpaired Student's t -test. Data are expressed as means $\pm$ SEM.

5.2.5 AMPK α 1 and α 2 deletion in proglucagon-expressing cells has no effect on α -cell mass or glucagon secretion

To investigate whether AMPK plays a role in pancreatic α -cell growth or survival, pancreatic sections from male mice were co-stained with insulin and glucagon in order to measure pancreatic α - and β - cell mass as well as the α : β cell ratio (Figure 5.13a-d). Though the α : β cell ratio was found to be increased in GluAMPKKO mice in comparison to WT littermates ($P < 0.05$ WT: 0.23 ± 0.02 AU, KO: 0.33 ± 0.03 AU, Figure 5.13d), no changes were found in overall α - or β -cell mass (NS α -cell WT: $0.15 \pm 0.02\%$, KO: $0.17 \pm 0.03\%$; β -cell WT: $0.82 \pm 0.10\%$, KO: $0.89 \pm 0.17\%$, Figure 5.13b-c).

We determined whether deleting the AMPK α -subunits in pancreatic α -cells might affect glucagon levels in the blood by measuring plasma glucagon levels of GluAMPKKO and WT mice fed *ad libitum* or fasted overnight. This analysis revealed little difference in glucagon levels between the two mouse strains (NS, Fed WT: 64.0 ± 3.67 pg/ml, KO: 71.7 ± 3.80 pg/ml; Fasted WT: 28.3 ± 3.33 pg/ml, KO: 23.7 ± 5.54 pg/ml, Figure 5.14a-b). To further examine whether AMPK α 1 α 2 deletion has an effect on pancreatic α -cells *in vitro*, glucagon secretion was investigated in isolated islets incubated in 0.5, 3 or 10 mmol/L glucose (Figure 5.15). In both GluAMPKKO and WT mice, islets incubated in lower concentrations of glucose (0.5, 3 mM) showed significantly higher levels of glucagon secretion compared to higher glucose concentrations (10 mM, $P < 0.0001$). However, deletion exerted no apparent effect on regulated glucagon secretion (0.5 mM WT: $0.97 \pm 0.13\%$, KO: 1.00 ± 0.22 ; 3 mM WT: $0.40 \pm 0.17\%$, KO: $0.37 \pm 0.16\%$; 10 mM WT: $0.13 \pm 0.03\%$, KO: $0.10 \pm 0.02\%$, Figure 5.15).

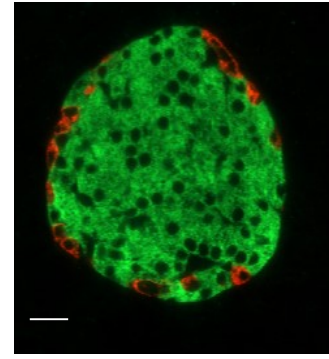
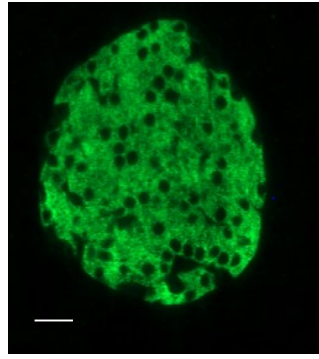
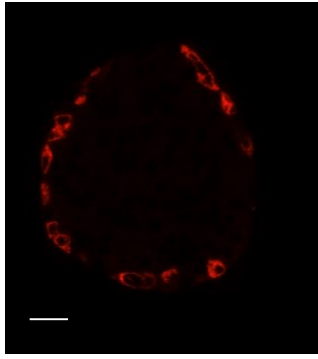
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Glucagon

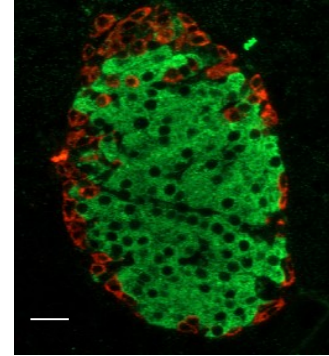
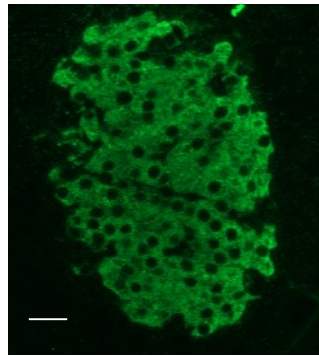
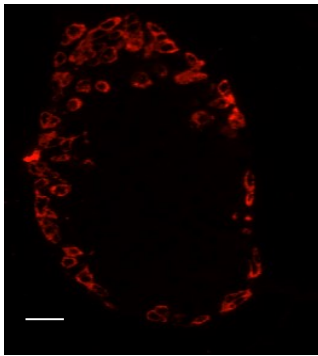
Insulin

Glucagon/Insulin

WT

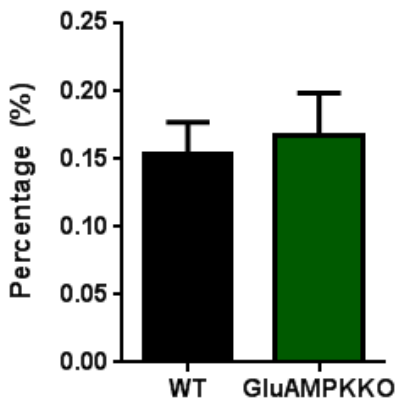


GluAMPKKO



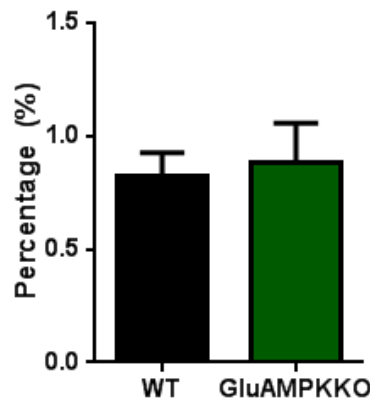
B.

% α -cell Mass



C.

% β -cell Mass



D.

α : β cell Ratio

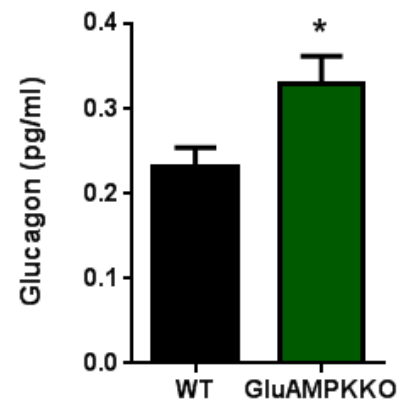


Figure 5.13 - α : β cell ratio increases in GluAMPKKO mice versus WT, whilst there is no effect on overall islet cell mass

(A) Representative images of wax-embedded pancreatic slices of islets stained for insulin, glucagon and DAPI, (B) % α -cell mass of pancreatic sections, (C) % β -cell mass of pancreatic sections and (D) the α : β cell ratio of the pancreas. Data are expressed as means $\pm$ SEM, $n=3$ pancreata per condition, * $P<0.05$ by unpaired Student's t -test. Scale bar: 50 μ m.

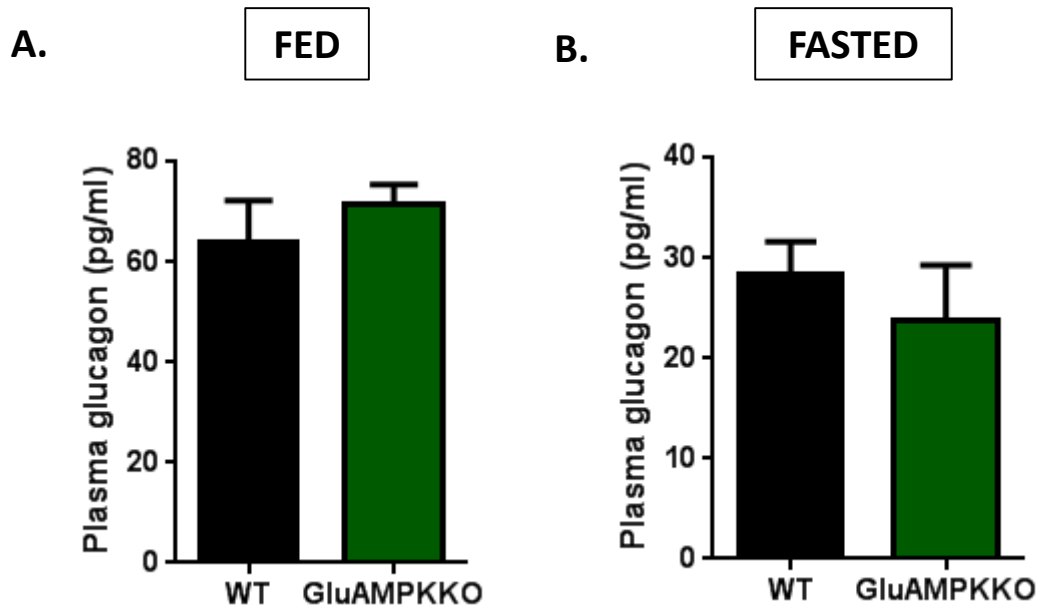


Figure 5.14 - Glucagon levels in the plasma is unaffected in GluAMPKKO mice *in vivo*

Glucagon levels in the plasma of GluAMPKKO and WT mice was measured in (A) fed and (B) overnight fasted mice. Data are expressed as means $\pm$ SEM, $n=6$ mice/genotype, NS, by Student's unpaired t-test.

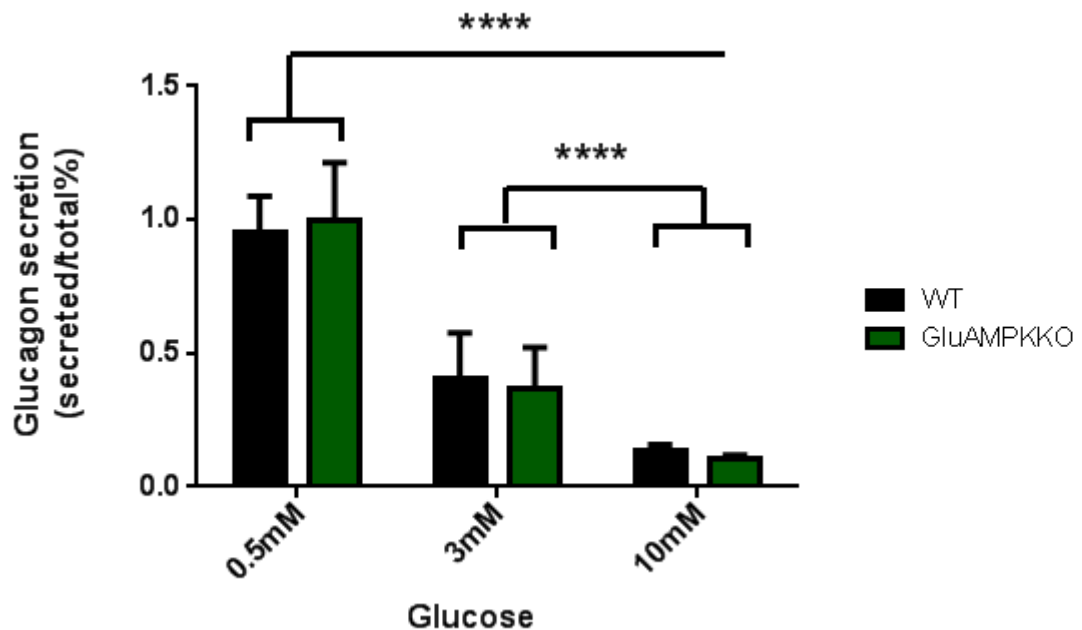


Figure 5.15 - Glucagon secretion *in vitro* is unaffected in GluAMPKKO mice

12 size-matched islets of GluAMPKKO and WT mice were picked and incubated in 0.5mM, 3mM or 10mM glucose and levels of glucagon secretion and total glucagon measured using radioimmunoassay. **** $P < 0.0001$ by 2-way ANOVA, $n=6$ mice/genotype. Data are expressed as means $\pm$ SEM.

5.3 *Discussion*

The overall aims of this study were (1) to determine whether deletion of the AMPK α -subunits in enteroendocrine L-cells results in polyp formation as observed after LKB1 deletion in chapter 4 and (2) whether deleting AMPK α 1 α 2 specifically in proglucagon-expressing cells improves GLP-1 production and secretion. To do this we used the proglucagon specific Cre recombinase (iGlu Cre) crossed with mice bearing *floxed* alleles for AMPK α 1 α 2 to generate GluAMPKKO mice and their WT littermates.

5.3.1 *Deletion of AMPK α 1 and α 2 does not stimulate polyposis*

The first aim of this study was to determine whether deleting AMPK α 1 and α 2 subunits in proglucagon-expressing cells would phenocopy the effect of deleting LKB1 in these cells using the same iGluCre transgene [448] and produce large gastro-duodenal PJS like-polyps (chapter 4). In marked contrast to our earlier findings (chapter 4) [448], GluAMPKKO mice showed no signs of tumour development or premature death (Figure 5.1). This suggests that members of the AMPK-related kinase (AMPK-RK) family [204], rather than canonical AMPK α 1 or α 2-containing complexes, are responsible for the suppression of polyposis from proglucagon-expressing cells [474] in the presence of LKB1. Possible candidates include Par1b/MARK2 [324], which controls cell polarity and is implicated in β -cell hyperplasia after LKB1 deletion [318, 319], as well as ARK5/SNARK [475, 476].

5.3.2 *Deletion of AMPK α 1 and α 2 in L-cells of the gut improves GLP-1 secretion resulting in these mice to become more glucose tolerant*

GLP-1 plays an important role in GSIS by binding to GLP-1R on the pancreatic β -cell, enhancing adenylate cyclase activity and the downstream signalling pathways stimulating PKA and EPAC2, resulting in K_{ATP} channel closure and depolarisation which initiates insulin secretion [359, 458]. GLP-1R KO models have been found to have impaired GSIS and GLP-1R is found to be miss-regulated in patients with Type 2 Diabetes [357, 462].

AMPK plays a role in insulin secretion as found in mice with AMPK α 1 and - α 2 selectively deleted in pancreatic β -cells [472]. AMPK α 1 α 2 KO mice became hyperglycaemic and presented decreased levels of insulin secretion *in vivo*. In contrast, AMPK α 1 and - α 2 deletion in enteroendocrine L-cells improved glucose tolerance in male KO mice during OGTT (Figure 5.5). Interestingly, glucose tolerance during IPGTT did not seem to be affected in these KO mice (Figure 5.6), although it should be noted that the latter tests were performed in the same animals at a slightly later stage (at 20

versus 15 weeks of age). These findings suggest that the incretin response is selectively enhanced in the GluAMPKKO model. Providing direct evidence for this view GLP-1 release into the plasma was significantly increased in GluAMPKKO mice in both fed and fasted mice (Figure 5.12). Surprisingly, however, this was not associated with any changes in detectable plasma insulin in fed or fasted states (Figure 5.9), nor in the increase in plasma insulin during IPGTT (Figure 5.7). These observations may suggest that the actions of GLP-1 on glucose tolerance may chiefly reflect local effects on neurons close to the site of release which then initiates an autocrine loop to regulate the production of hepatic glucose [477]. Of note, the latter study showed that metformin action in the gut acutely improved hepatic production of glucose in an AMPK-dependent manner. Whilst this did not modulate AMPK specifically within enterendocrine cells, the apparent contrast between the present study and our earlier findings [477] may thus suggest that metformin acts chiefly in the intestine on the a non-L-cell enteroendocrine population, largely localised in the duodenum, or that there are significant differences between the acute and chronic activation of AMPK on a “gut-brain-liver axis”.

5.3.3 *AMPK α 1 and α 2 deletion from pancreatic α -cells does not alter α -cell mass or glucagon secretion but increases L-cell mass*

In the present study no changes in α - or β -cell mass were observed in GluAMPKKO mice, consistent with previous findings using the shorter glucagon promoter-*Cre* to drive deletion in these cells [453]. AMPK is a regulator of the mTOR complex (as mentioned in previous chapters), that goes on to phosphorylate the mTORC1 components Raptor and TSC2 [428] which are involved in the regulation of cell size [478] and proliferation. Interestingly, a clear increase in total L-cell mass, expressed as a fraction of intestinal surface, was apparent (Figure 4a,b) alongside a strong tendency towards an increase, of similar magnitude, in the number of L-cells per unit area. Although further investigations are required to explore the mechanisms involved, these might conceivably include the activation of mTORC1 as a result of AMPK deletion. Additionally, Carbohydrate Response Element Binding Protein (ChREBP), a transcriptional regulator of proglucagon gene expression and carbohydrate metabolism in L-cells [479], is also inhibited by AMPK [480], and may further increase GLP-1 synthesis and secretion.

Consistent with previous findings [453] in which both AMPK α 1 and α 2 were deleted in the α -cell using a shorter (1.6 kB) preproglucagon promoter [454] to drive *Cre*-expression, no evidence was obtained for changes in circulating glucagon levels after deletion of AMPK activity. Of note, the longer proglucagon promoter used in our study deletes additionally in enteroendocrine L-cells as well as other proglucagon-expressing cells throughout the body (e.g. in the brain stem and olfactory

bulb) [481]. Thus, we show that deleting both AMPK α -subunits in pancreatic α -cells has little effect on glucagon release in response to low glucose levels *in vitro* or *in vivo*. Likewise, in GluAMPKKO mice, glucagon secretion was not enhanced in comparison to WT littermates during hypoglycaemia induced by insulin injection (Figure 5.8). It should be noted, however, that when AMPK α 1, but not α 2, was deleted selectively in the α -cell with the shorter proglucagon promoter-driven Cre, impaired glucagon secretion was observed both *in vivo* during hypoglycemic clamp and *in vitro* [482]. Furthermore, AMPK activation was previously found to regulate glucagon secretion *in vitro* in a study using clonal α TC1-9 cells [284]. In the latter study, AMPK expression was stimulated using pharmacological agents such as metformin, and resulted in enhanced glucagon secretion at both high and low glucose concentrations. One possible explanation for the apparent discrepancy between the present and earlier studies is that complete inactivation of AMPK in the α -cell *in vivo* leads to robust compensatory changes that reset glucagon release back to wild-type levels as also observed in our previous study [482] after deletion of both AMPK α 1 and α 2 in the α -cell using the shorter Cre strain developed in [454].

5.4 Conclusions

Deletion of AMPK α 1 α 2 in proglucagon secreting cells including pancreatic α -cells and enteroendocrine L-cells does not lead to polyposis seen after deletion of LKB1 in the same cell population [448], implicating non canonical members of the AMPK-RK family in EMT and polyposis. On the other hand, glucose tolerance after oral administration of glucose is improved, L-cell number increased and GLP-1 secretion enhanced. Our results suggest that AMPK limits expansion of the L-cell population and GLP-1 release, processes which may provide a new therapeutic avenue for the treatment of Type 2 Diabetes.

Chapter 6

Final Discussion

6.1 Investigating the relevance of AMPK and LKB1 in glucose homeostasis using tissue-specific knock-out mice

The overall aim of this thesis has been to study the roles of the protein kinase LKB1 and one of its key down-stream kinases AMPK in regulated hormone secretion and the control of glucose homeostasis. Our main approach has been in the breeding and generation of mice deleted for either LKB1 or AMPK specifically in selected cell types. The role of LKB1 in the pancreatic β -cell has been previously studied in KO mice using relatively unrelated Cre recombination lines [318-320]. These earlier studies found that inhibiting LKB1 activity selectively in β -cells facilitates their expansion through hypertrophy and hyperplasia and improves glucose tolerance *in vivo* [318-320]. In chapter 3 we use a novel strain of mice expressing a much more selective and efficient Cre recombinase (Ins1 Cre), known to delete LKB1 *floxed* alleles in approximately 94% of β -cells without off-target effects in other tissues [400].

AMPK is thought to be an important target for the treatment of diabetes due to the role this kinase plays in the liver and muscle during the glucose-lowering treatment using metformin and thiazolidinedione [296]. AMPK has previously been studied in the pancreatic β -cell using tissue specific RIP-2 Cre deletion of both α -subunits of AMPK, as described above for LKB1 [483]. It was discovered through this approach that the regulation of AMPK in the β -cell is important for normal glucose homeostasis.

Information gathered on the roles of AMPK in the pancreatic α -cell is limited. Similarly, the role of both LKB1 and AMPK in the enteroendocrine L-cells and its contribution to the control of GLP-1 secretion and glucose homeostasis has not been investigated previously. In this thesis, we bred mice *floxed* for LKB1 (chapter 4) or AMPK (chapter 5) with the proglucagon deleting iGlu Cre promoter to investigate the impact that these kinases have on L- and α -cells.

6.2 Roles of LKB1 in the pancreatic β -cell

In chapter 3 we build from previous studies exploring the effects of LKB1 deletion in the pancreatic β -cell by using the Ins1 Cre promoter. In these earlier studies, LKB1 was deleted in the β -cell of adult mice using the Pdx1 CreER [318, 319] or the RIP2 Cre transgene [320]. Encouragingly, our new findings using Ins1LKB1KO mice largely mirror these previous observations with glucose tolerance and *in vivo* insulin secretion becoming enhanced in the KO mice [427] as they are in the earlier models.

β -cell mass is ruled by the balance of four known parameters: proliferation, cell size, apoptosis and neogenesis [484]. Using the same methods as in [320], β -cell mass was measured in our mice and similar to the effect LKB1 deletion has in earlier models, β -cell size was increased. Although not tested directly in the current studies, this may be due to up-regulation of mTORC signalling, as discussed in chapter 3 and illustrated in figure 6.1.

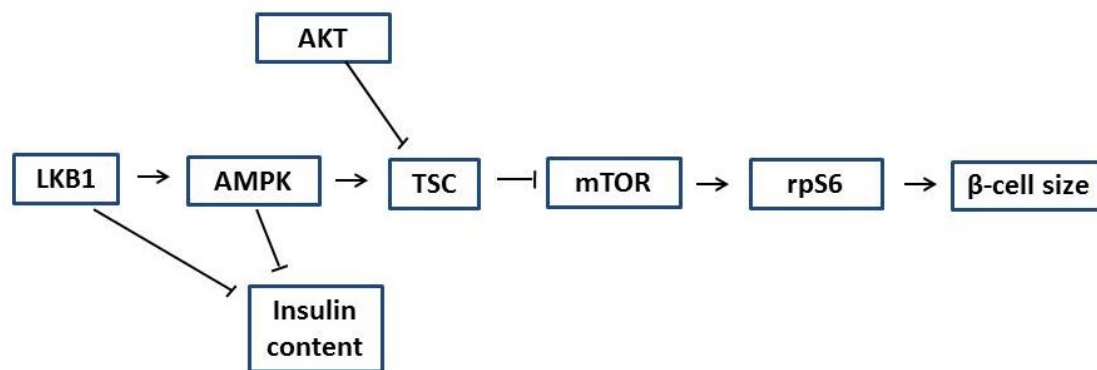


Figure 6.1 - The proposed pathway for the modulation of β -cell size, utilising the LKB1-AMPK-mTOR-rpS6 pathway

In this pathway we show that LKB1 and AMPK are both detrimental to insulin content and β -cell size via the activation and consequent inhibition of the mTOR pathway.

Unexpectedly, in our Ins1LKB1KO mice *in vitro* insulin secretion was found to be unaltered (figure 3.3), despite GSIS being markedly enhanced *in vivo*. Nonetheless, and perhaps arguing against the above model, deletion of both catalytic subunits of AMPK failed to phenocopy the action of LKB1 deletion in this study [427]. This led us to further investigate the insulin secretion pathway components in the KO model to identify the signals responsible for the enhanced *in vivo* insulin secretion and glucose tolerance seen in these mice. Surprisingly, we found that LKB1 deletion in β -

cells causes a dramatic deterioration in glucose-induced cytosolic ATP/ADP and Ca^{2+} signalling as well as mitochondrial membrane potential (figures 5.4-5.8).

Similar findings were obtained in Pdx1 CreER [427], RIP2Cre [320] and the Ins1Cre strain LKB1 deleted mice [430, 431]. In the latter mice, mitochondrial oxidation of glucose and the citrate cycle intermediate succinate was found to be defective in triggering GSIS [430] and ACC activation was enhanced [431]. From our work [427] and others [430, 431] we conclude that the improvement in glucose tolerance in LKB1-deficient mice is not simply a consequence of enhanced β -cell mass or insulin content. In tamoxifen-induced Pdx1 CreER LKB1 deleted strains, insulin content and β -cell mass were not found to be significantly increased to a degree that could explain the increase in insulin secretion [320, 427]. Instead an increase in GSIS in perfused islets suggested that insulin secretion is up-regulated in a cell-autonomous manner.

Given the observed defects in cytosolic Ca^{2+} and ATP/ADP signalling, we investigated mitochondrial and citrate cycle components in our Ins1LKB1KO mice using a metabolomic approach. Here, we found that certain citrate cycle intermediates such as succinate and citrate were present in lower levels in Ins1LKB1KO islets, as were glycerol and fatty acids (Figure 5.9). Mitochondrial membrane potential in β -cells from these mice was also found to be lowered (Figure 5.8). Similarly, β -cells deleted for LKB1 using tamoxifen-induced Pdx1 CreER deletion revealed mitochondrial defects. In the latter KO mice, it was proposed that leucine may be further metabolised increasing the concentration of glutamate which has been found to stimulate insulin secretion [177]. Interestingly, transcriptomic analysis of islets from Ins1LKB1KO mice revealed up-regulation in genes involved in glutamate signalling, as discussed in chapter 3 [427]. Thus, we speculate that intracellular levels of glutamate increase in cells abolished for LKB1, stimulating a more effective GSIS and less ATP/ADP and Ca^{2+} is required to trigger insulin release (as shown in figure 3.15). Supporting this idea stimulation using the glutamate receptor agonist kainite resulted in a more pronounced increase in cytosolic Ca^{2+} ($[\text{Ca}^{2+}]_{\text{cyt}}$) and insulin secretion in Ins1LKB1KO islets in comparison to WT. Consistent with our results, Pdx1 CreER LKB1-deleted mice showed an up-regulation of glutamate levels in LKB1-deficient islets [430]. Taken together, these findings suggest that improved insulin secretion in LKB1 null β -cells is due to amplification of a glutamate induced-signalling pathway. An overview of LKB1 regulation in the β -cell is illustrated in figure 6.2.

6.3 *Impact of LKB1 and AMPK deletion in enteroendocrine L-cells*

Enteroendocrine L-cells make up a small but significant population of cells lining the intestinal walls within the gut (less than 1% of cells) that are essential for normal physiology and metabolism [403]. Through expression of proglucagon and PC-1; L-cells (and K-cells to a lesser extent) are responsible for the synthesis and release of the gut hormones, including GLP-1, GLP-2 and oxytomodulin [485]. These hormones target GLP-1R which are present on the pancreatic β -cell as well as in the CNS [486]. Given the role that the incretin hormone GLP-1 plays in glucose tolerance, appetite and energy expenditure, the GLP-1R is considered an important therapeutic target in the treatment of diabetes, as exemplified by the use of the analogue liraglutide and DPPIV inhibition [457]. In recent years, GLP-1 has been found to play a role in insulin secretion during food transit, initiating cAMP synthesis and downstream signalling of PKA and EPAC2, stimulating insulin secretion [359, 458]. Thus, GLP-1R agonists and inhibitors of GLP-1 now represent a front-line therapy for diabetes [487], and enhanced secretion of these hormones has also been implicated in the beneficial effects of bariatric surgery on diabetes [488]. Up until now, the roles of LKB1 and AMPK in enteroendocrine L-cells have not been explored.

To investigate the role of these kinases in the control of GLP-1 production and secretion, mice were crossed with either LKB1 (chapter 4) or AMPK α 1 and - α 2 (chapter 5) *flxed* mice and mice expressing Cre-recombinase under the control of the proglucagon promoter [403]. This resulted in mice that were deleted for AMPK or LKB1 in proglucagon-secreting cells, notably enteroendocrine L-cells and pancreatic α -cells.

6.3.1 *Deletion of LKB1 in L-cells*

LKB1, in addition to phosphorylating AMPK and its downstream kinases [204], acts as a tumour suppressor and is mutated in PJS, a syndrome characterised by the appearance of hamartomatous polyps in the gut (as discussed in chapter 1). Our findings in GluLKB1KO mice (chapter 4) show that LKB1 expression in regulating L-cells has little or no role in play in L-cell number or GLP-1 production and secretion. Thus, no changes were observed in incretin-induced glucose tolerance (Figure 4.5) or circulating GLP-1 (Figure 4.7) in GluLKB1KO mice.

However, Glu1LKB1KO mice were found to develop large gastro-duodenal polyps reminiscent of those seen in PJS [473] and previously in mice lacking a single LKB1 allele throughout the body [444]. These gastro-duodenal polyps eventually resulted in the death of the mice due to intestinal obstruction [448].

Proglucagon-expressing cells are scattered throughout the gastrointestinal tract, the most concentrated area of these cells being within the colon and rectum [489]. Development of polyps within the gastro-duodenal junction was surprising as proglucagon-secreting cells are known to be sparse in this area. Encouragingly, similar findings were observed in three independent Glu-Cre founder lines [448]. Therefore, it is unlikely that these findings are due to some mis-expression of Cre due to random insertion events [448].

LKB1 deletion in the intestinal epithelium has been shown previously to prevent terminal differentiation of secretory goblet cells. This resulted in an immature and less differentiated cell phenotype, suggesting that LKB1 plays a role in EMT in the intestinal epithelium [450]. During development, L-cells originate from common progenitor cells located at the base of the intestinal crypt, and neurogenin 3 commits the cells to their endocrine fate [490]. Though we are unable to make a definitive assignment of the progenitor cells from which the polyps develop after LKB1 deletion, the rapid turn-over of mature L-cells and their inability to divide [491] make them unlikely candidates as the immediate precursors [448]. However, as discussed in [448] LKB1 deletion occurs before the loss of promoter activity and because relatively little proglucagon staining was observed within polyps we propose that offspring of immature LKB1 deleted cells undergo transition and become transformed to develop tumours. In studies performed in [448], GluLKB1KO mice expressing GFP demonstrate that a small proportion of proglucagon-expressing cells express the EMT marker vimentin. Proglucagon-expressing cells were found to undergo EMT by the 10th day post-partum [448]. Lineage tracing studies in our mice show that there is an increased number of cells within the duodenum with mesenchymal characteristics and localisation [448]. It seems likely that polyps develop from this population of smooth-muscle like cells (rather than mature L-cells).

It is unclear how mutations in LKB1 lead to tumour development. Our study suggests there is an increased susceptibility of proglucagon-expressing precursors to proliferate following deletion of LKB1. Thus, it is possible that this pool of LKB1 null cells provides a supply of precursors for polyp development and contributes to tumour growth [448]. However, the few studies that have looked at the role of endocrine cells towards PJS development are conflicting [335, 492]. Earlier suggestions for tumour development focussed on the inhibition of the mTOR pathway after LKB1 deletion (as mentioned above). In whole body LKB1^{-/+} mice rapamycin was shown to reduce tumour size [493]. However, in human PJS patients there is insufficient evidence to support the use of rapamycin as a preventative treatment [494]. An interesting follow-up to our study would be to investigate whether treatment of GluLKB1KO mice using oral rapamycin treatment will also reduce tumour size. In the context of manipulating LKB1 expression in the intestinal epithelium for the treatment of Type 2

Diabetes, due to the role this kinase plays in the initiation of tumorigenesis its use as a therapy should be approached with great caution.

6.3.2 *Deletion of AMPK α 1 and α 2 in L-cells*

In our second study using the proglucagon iGlu Cre promoter, we crossed mice expressing this promoter with mice *floxed* for both AMPK α -subunits (chapter 5). Cre deleted GluAMPKKO mice show improved glucose tolerance after glucose was administered using oral gavage, but not after interperitoneal injection. Providing a likely explanation for the improvements in glucose tolerance are the improvements in L-cell number and circulating GLP-1 levels that was found in this model (chapter 5). In future experiments, mTORC1 activation might be investigated to further explore the role of this critical kinase [429]. Another potential mediator is Carbohydrate Response Element Binding Protein (ChREBP) a transcriptional factor [495] inhibited by AMPK and which has recently been shown to regulate the output of GLP-1 from L-cells [496].

AMPK was previously found to play a role in insulin secretion. Thus, mice in which AMPK α 1 and - α 2 was deleted selectively in pancreatic β -cells using the RIP2Cre transgene [483] became hyperglycaemic and insulin secretion was impaired. In contrast, glucose tolerance was improved in our GluAMPKKO mice during OGTT, but was not affected during IPGTT, whilst *in vivo* GLP-1 secretion was increased in these mice. Therefore, our findings indicate that the incretin response is impaired by AMPK expression in L-cells.

These observations may reflect an action of GLP-1 on enteric neurones close to its site of release that will then initiate an autocrine loop which regulates hepatic glucose production as recently proposed [477]. However, in the latter study the acute action of metformin in the duodenum reduced hepatic glucose production via an AMPK-dependent mechanism. This contrast between these earlier studies [477] and our findings suggest that metformin may act chiefly on the non-L-cell enteroendocrine population of the gut (localised largely in the duodenum).

Initially, we bred our GluAMPKKO colony of mice to determine whether the suppressive effect of LKB1 on EMT in the enteroendocrine L-cells or their progenitors is mediated via AMPK or by other members of the AMPK-related kinase family [204]. These mice showed no signs of tumour development or premature death, suggesting that members of the AMPK-related kinase family, rather than canonical AMPK α 1 or α 2-containing complexes, are responsible for the suppression of polyposis from proglucagon secreting cells [474]. Possible candidates include Par1b/MARK2 which plays a role in the control of cell polarity and is involved in β -cell hyperplasia during LKB1 deletion

[318-320, 497] as well as NUA1/SNARK [475] and NUA2 [476]. An overview of LKB1 and AMPK regulation in L-cells is illustrated in Figure 6.2.

6.4 Impact of AMPK and LKB1 in pancreatic α -cells

As discussed in previous chapters, glucagon, generated via the action of PC-2 and secreted from pancreatic α -cells, acts as the main anti-hypoglycaemic hormone in mammals [469]. Impaired release of this hormone is observed in patients suffering from Type 1 and long-standing Type 2 Diabetes [498]. As well as deleting in enteroendocrine L-cells the iGlu Cre promoter leads to recombination of proglucagon-expressing cells of the pancreas, namely the α -cell.

6.4.2 Deletion of LKB1 in α -cells

The role of LKB1 in the α -cell and the control of glucagon secretion have not previously been explored *in vivo*. In chapter 4, we show that deletion of LKB1 in the α -cell has no phenotypic effect on *in vivo* or *in vitro* glucagon secretion, α -cell mass or in insulin tolerance. In a previous study, the shorter PPG promoter (1.6kB) [454] was used to drive Cre expression, and crossed with mice *floxed* for LKB1. Unlike our GluLKB1KO mice, blood glucose was found to improve in PPG Cre: LKB1 *floxed* mice after overnight fast and fasting glucagon levels were increased enhanced. As with GluLKB1KO mice, α -cell size and number is unaffected in LKB1 deleted PPG Cre mice, despite up-regulation in glucagon secretion [482]. Unlike LKB1-deficient β -cells [318, 319, 427], attenuation of mTOR signalling by LKB1 appears to be absent from α -cells. Reasons for this are unclear.

6.4.2 Deletion of AMPK in α -cells

As with our GluLKB1KO mice mentioned above, neither α - or β - cell mass were altered in GluAMPKKO mice (chapter 5). Consistent with our results, no changes in cell size or circulating glucagon levels were observed after deletion of AMPK using the PPG Cre promoter [482]. However, it should be noted that in AMPK α 1- (though not α 2-) deficient mice lacking AMPK selectively in α -cells after recombination with the shorter proglucagon promoter-driven Cre [482] glucagon secretion was impaired both *in vivo* during hypoglycaemic clamp and *in vitro*. Thus, we find that deleting AMPK in both α -subunits in pancreatic α -cells has little effect on glucagon secretion *in vitro* or *in vivo*. This is in contrast to the effects of deleting AMPK α 1 alone in these cells [453] and

suggests that the complete ablation of AMPK in pancreatic α -cells *in vivo* leads to robust compensatory changes that reset glucagon back to WT levels. An overview of LKB1 and AMPK regulation in α -cells is illustrated in Figure 6.2.

Of note, activation of AMPK was previously discovered to regulate glucagon secretion *in vitro* in a study that used clonal α TC1-9 cells [284]. Here, expression of AMPK was stimulated via pharmacological agents such as metformin, and this resulted in the enhancement of glucagon secretion at both high and low glucose concentrations.

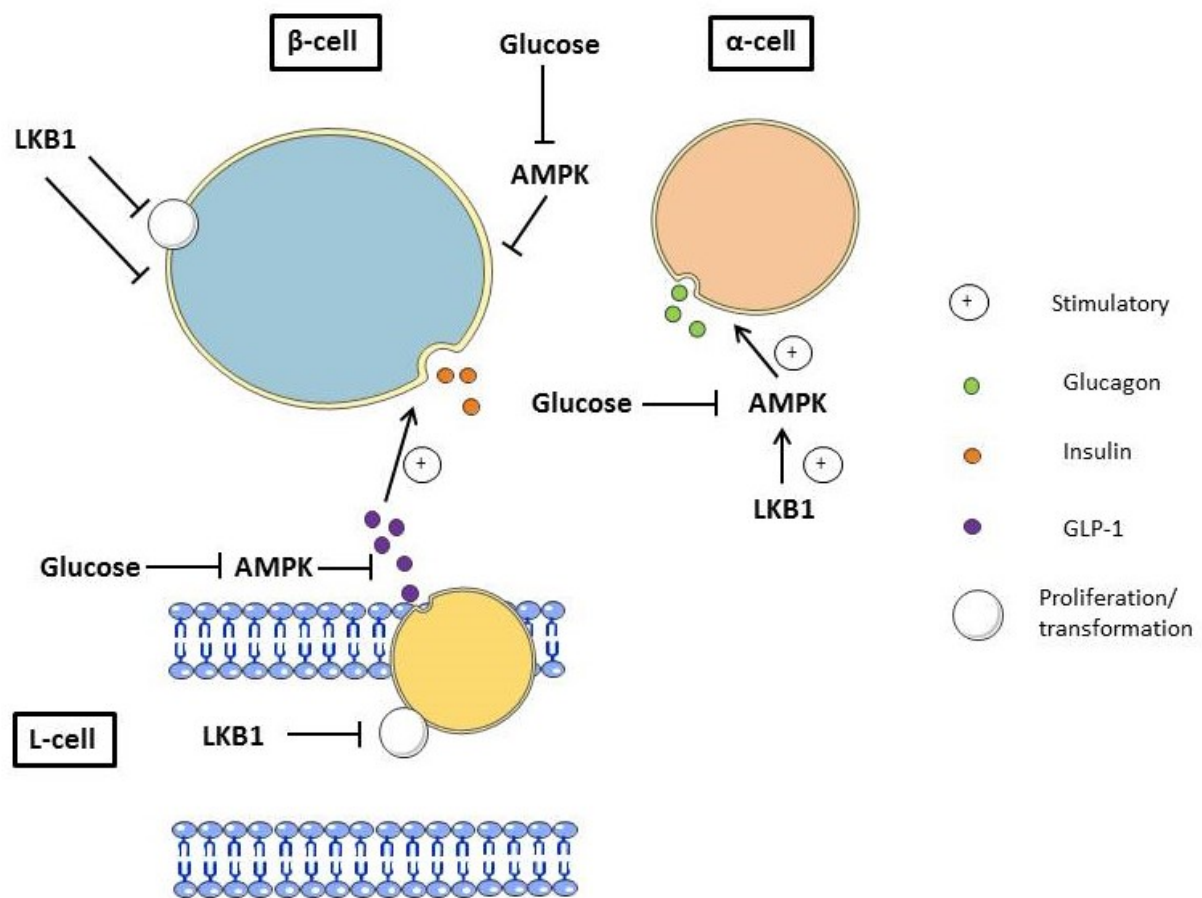


Figure 6.2 – A brief overview of the roles LKB1 and AMPK play in the regulation of the pancreatic β -, α - and enteroendocrine L-cell

GLP-1 secretion from the L-cell plays a role in stimulating normal insulin secretion from the pancreatic β -cell. In the β - and L-cell, activation of LKB1 regulates normal proliferation and plays a role in inhibiting normal insulin secretion. Impairment of LKB1 in these cells results in enhanced cell growth and in the case of L-cells, polyp formation. Glucose stimulation results in inhibition of AMPK and further inhibition of GLP-1 and insulin secretion. Therefore, in AMPK deficient cells, glucose stimulation will enhance GLP-1 secretion, thus, improving glucose homeostasis. In the pancreatic α -cell, glucose stimulation will prevent the secretion of glucagon from the cell.

6.5 *Final conclusion*

This thesis describes the impact of deleting LKB1 or AMPK selectively in the pancreatic α -cell and enteroendocrine L-cell as well as LKB1 selectively in the pancreatic β -cell. Through the generation of these three novel models we show that these kinases play an important role in the regulation of key gluco-regulatory hormones and regulation in glucose homeostasis. Although deletion of LKB1 in L-cells exerted little effect on GSIS, this study provided new information on the origin of PJS as well as the role that LKB1 plays as a tumour suppressor. Moreover, the failure of AMPK deletion in L-cells (chapter 4) to recapitulate the effects of LKB1 deletion in these cells provides compelling evidence that alternative pathways and AMPK-related kinases probably mediate the effects of LKB1 in L-cells. Our findings support the view that LKB1, AMPK and/or their down-stream kinases may provide therapeutic targets in the pancreatic β -cell and enteroendocrine L-cell to treat some forms of diabetes.

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Appendix 1

Antibody List

Primary Antibody

Antibody	Species	Procedure	Dilution	Company
DsRed	anti rabbit	IHC	1:200	Clontech
Glucagon	anti goat	IHC	1:100	Dako
Glucagon	anti mouse	IHC	1:100	Sigma
Glucagon	anti rabbit	IHC/OPT	1:100	Santa Cruz
Insulin	anti guinea-pig	IHC/OPT	1:200	Dako
P-AMPK α 1/ α 2	anti rabbit	IHC	1:100	NEB Cell signalling

Secondary Antibody

Laser	Species	Procedure	Dilution	Company
488	donkey anti mouse	IHC	1:1000	Life technologies
488	donkey anti rabbit	IHC/OPT	1:500	Life technologies
488	goat anti guinea-pig	IHC	1:1000	Invitrogen
488	goat anti mouse	IHC	1:1000	Invitrogen
568	donkey anti goat	IHC	1:1000	Life technologies
568	goat anti guinea-pig	IHC	1:1000	Invitrogen
568	goat anti rabbit	IHC	1:1000	Invitrogen
594	goat anti guinea-pig	IHC/OPT	1:1000	Life technologies

Appendix 2

PCR primers (for genotyping) and T_m

Gene	Primer sequence	T_m
<i>AMPKα1</i>	Fwd: TATTGCTGCCATTAGGCTAC	53
	Rev: GACCTGACAGAATAGGATATGCCCAACCTC	
<i>AMPKα2</i>	Fwd: GCTTAGCACGTTACCCTGGATGG	62
	Rev: GTTATCAGCCCAACTAATTACAC	
<i>β-actin</i>	Fwd: CGAGTCGCGTCCACCC	58
	Rev: CATCCATGGCGAACTGGTG	
<i>Cre</i>	Fwd: TTCACTGGTTATGCGGCGG	60
	Rev: GCCAGATTACGTATATCCTGGCAG	
<i>FER</i>	Fwd: ACCTTCAGACCTTGGCGTTGGAGG	60
	Rev: ACCTTCAGACCTTGGCGTTGGAGG	
<i>iGlu Cre</i>	Fwd: ATGTCCAATTTACTGACCG	58
	Rev: CGCCGCATAACCAGTGAAAC	
<i>LKB1</i>	Fwd: GGGCTTCCACCTGGTGCCAGCCTGT	60
	Rev: GAGATGGGTACCAGGAGTTGGGGCT	
<i>tdRFP</i>	Fwd: CTGTTCTGGGGCATGGC	58
	Rev: CTACAGGAACAGGTGGTGG	

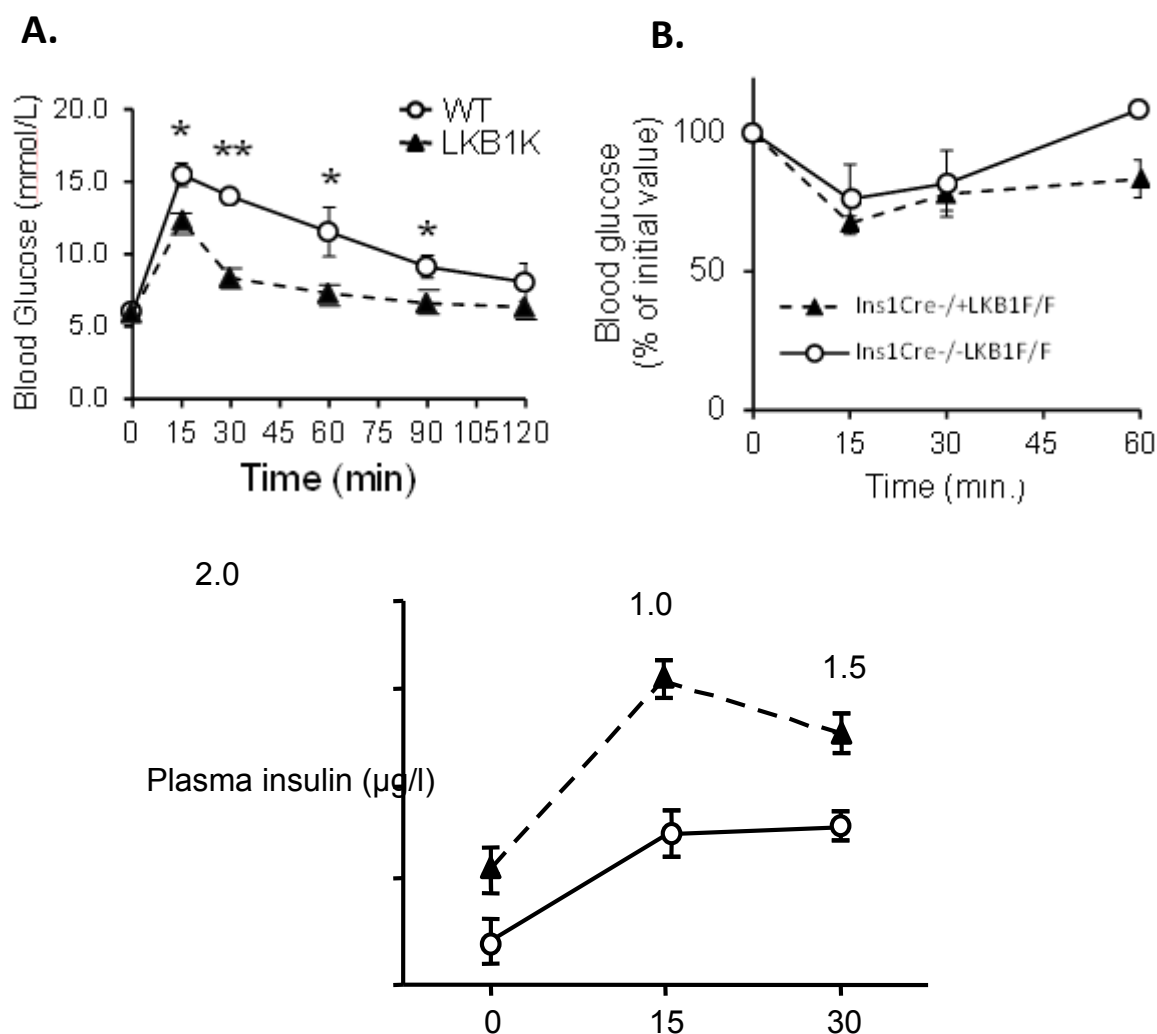
Appendix 3

Real time qPCR primers

Gene	Primer sequence
<i>Cyclophilin</i>	Fwd: AAGACTGAGTGGTTGGATGG Rev: ATGGTGATCTTCTTGCTGGT
<i>Gcg</i>	Fwd: CATTCAACCAGCGACTACAGCAA Rev: TCATCAACCACTGCACAAAATCT
<i>iCre</i>	Fwd: GCCGAAATTGCCAGAATCAG Rev: CAATGTGGATCAGCATTCTCC
<i>Ins1</i>	Fwd: GAAGCGTGGCATTGTGGAT Rev: TGGGCCTTAGTTGCAGTAGTTCT
<i>Ins2</i>	Fwd: AGCCCTAAGTGATCCGCTACAA Rev: CATGTTGAAACAATAACCTGGAAGA
<i>LKB1</i>	Fwd: GCCGGGCAACCTGCTACT Rev: CAACACCGAGGTCGGAGATC
<i>Nptx2</i>	Fwd: GCTCCTTGCAAACCCTCAAG Rev: GAGCACCTCTCGGAAGTCG

Appendix 4

In vivo insulin secretion and glucose tolerance improves in Ins1LKB1KO mice

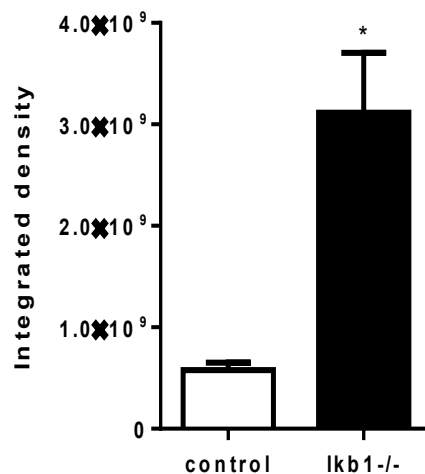
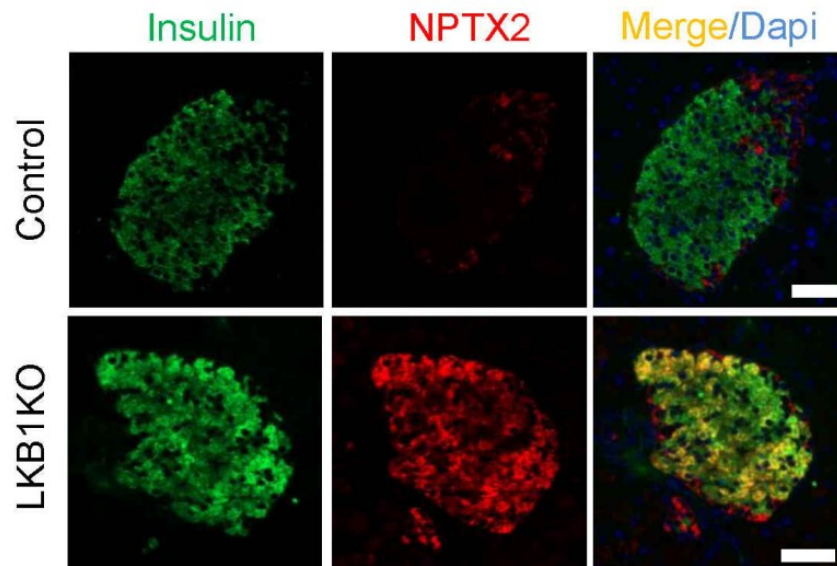


Appendix 4: Glucose tolerance and *in vivo* insulin secretion becomes enhanced in Ins1LKB1KO mice

(A) Insulin tolerance tests (0.75U/Kg insulin) and (B) intraperitoneal glucose tolerance tests (3g/Kg) were performed and insulin secretion during IPGTT measured (C) in 10-12 week old Ins1LKB1KO and WT mice. $N=3-5$ mice, * $P<0.05$, ** $P<0.01$, *** $P<0.001$, using 2-way ANOVA. Data are expressed as mean $\pm$ SEM [427]. Experiments were performed by Marina Kone [427].

Appendix 5

Nptx2 expression is up-regulated in islets of *Ins1LKB1KO* mice

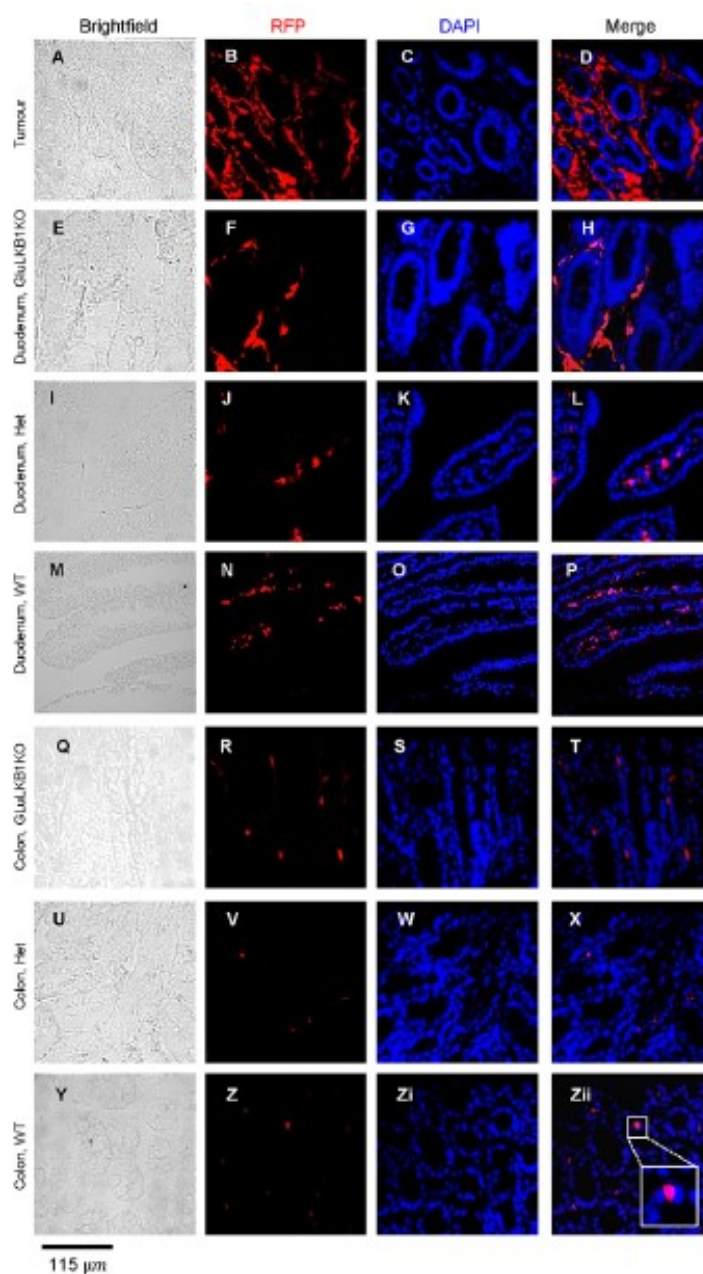


Appendix 5 - Protein expression of *Nptx2* is up-regulated in *Ins1LKB1KO* mice

(A) IHC of pancreatic slices stained with insulin (green) and *Nptx2* (red) and (B) density of *Nptx2* in islets was significantly enhanced in *Ins1LKB1KO* versus WT pancreas. Scale bar = 50μm, *n*=6-7 mice/genotype, **P*<0.05 by unpaired t-test. Data are expressed as mean ± SEM [427]. Experiments were performed by Dr Marie-Sophie Nguyen-Tu [427].

Appendix 6

Recombination in enteroendocrine L-cells was demonstrated by staining for RFP in GluLKB1KO mice



RFP recombination in proglucagon-expressing cells and polyps

IHC of frozen sections using an anti-RFP antibody of polyp, duodenum and colon of WT (M-P, Y-Zii), GluLKB1het (I-L, U-X) and GluLKB1KO mice (A-H, Q-T), brightfield, RFP, DAPI (nuclei localisation) and merged RFP/ DAPI images are shown. The insert in Zii shows an enlarged image of an RFP stained cell within a crypt. Experiments were performed by Dr Sagen Zac-Varghese [448].

List of Publications

Original (peer reviewed) articles:

1. **Sayers S**, Frank Reimann, Fiona M. Gribble, Helen Parker, Sagen Zac-Varghese, Stephen R. Bloom, Marc Foretz, Benoit Viollet and Guy A. Rutter. Proglucagon promoter Cre-mediated AMPK deletion in mice increases circulating GLP-1 levels and oral glucose tolerance. In Submission.
2. Swisa A, Granot Z, Tamarina N, **Sayers S**, Berdeesy N, Philipson L, Hodson, Wikstrom JD, Rutter GA, Leibowitz G, Glaser B, Dor Y. Loss of LKB1 in beta cells enhance glucose-stimulated insulin secretion despite profound mitochondrial defects. J Biol Chem. 2015 July
3. Kone M, Pullen TJ, Sun G, Ibberson M, Martinez-Sanchez A, **Sayers S**, Nguyen-Tu MS, Kantor C, Swisa A, Dor Y, Gorman T, Ferrer J, Thorens B, Reimann F, Gribble F, McGinty JA, Chen L, French PM, Birzele F, Hildebrandt T, Uphues I, Rutter GA. LKB1 and AMPK differentially regulate pancreatic β -cell identity. FASEB J. 2014 Nov;28(11):4972-85. doi: 10.1096/fj.14-257667. Epub 2014 Jul 28.
4. Zac-Varghese S, Trapp S, Richards P, **Sayers S**, Sun G, Bloom SR, Reimann F, Gribble FM, RutterGA. The Peutz-Jeghers kinase LKB1 suppresses polyp growth from intestinal cells of a proglucagon-expressing lineage in mice. Dis Model Mech. 2014 Nov;7(11):1275-86