BIRC proteins in the control of TNF signalling in pancreatic beta cells

Author:
Tan, Meijun Bernice

Publication Date:
2012

DOI:
https://doi.org/10.26190/unsworks/16000

PLEASE TYPE

THE UNIVERSITY OF NEW SOUTH WALES
Thesis/Dissertation Sheet

Surname or Family name: Tan

First name: MeiJun Other name/s: Bernice

Abbreviation for degree as given in the University calendar:

School: St Vincent's Clinical School Faculty: Medicine

Title: BIRC proteins in the control of TNF signalling in pancreatic beta cells

Abstract 350 words maximum: (PLEASE TYPE)

TNFα contributes to type 1 diabetes pathogenesis by impairing beta cell function and driving beta cell apoptosis. How TNFα directs these processes is not fully understood. Of note, baculoviral-IAP-repeat-containing (BIRC) proteins associate with the TNF complex to control down stream signalling but their function in beta cells is not known. Following TNF receptor ligation, Birc3 but not Birc1,2,4,5 or -6 was induced in mouse islets and MIN6 cells. Contact with TNFα triggers signalling cascades converging on the NF-κB, JNK, and p38 pathways that regulate inflammatory genes. The Birc3-promoter harboured three NF-κB and an AP-1 binding sites. Induction of endogenous Birc3 by TNFα was completely blocked by NF-κB inhibitors - thus NF-κB regulates Birc3 transcription. Further, BIRC3 gain-of-function induced activation of an NF-κB reporter and potentiated TNFα-induced NF-κB activation. Surprisingly, Birc3-/- islets showed delayed but functional NF-κB activation and exhibited dysregulated TNFα-induced Ccl2, Cxcl10 and Icam-1. Some data demonstrate BIRC2 can compensate for loss of BIRC3. Expectedly, Birc2-/-Birc3-/- islets showed markedly delayed IκBα degradation kinetics; suggesting that BIRC2/3 are crucial for NF-κB signalling. Remarkably, Birc2-/- and Birc3-/- islets showed unexpected phenotypes independent of NF-κB. Genetic analysis showed Birc2-/- islets exhibited increased basal Ccl2 and Cxcl10 expression. Cell signalling was further dysregulated by the addition of BIRC3 deficiency. Birc2-/-Birc3-/- islets exhibited increased basal A20, Icam-1, Ccl2 and Cxcl10, which were blocked by antagonizing JNK but not NF-κB or p38 pathway. Moreover, Birc2-/-Birc3-/- islets showed hyperphosphorylation of the JNK-target, c-Jun. BIRC proteins ubiquitinate substrates targeting them for proteosomal degradation. Proteosome inhibition in MIN6 cells mimicked the Birc2-/-Birc3-/- islet phenotype – increased basal Cxcl10, Ccl2 and Icam-1 expression with c-Jun-hyperphosphorylation. Birc2-/-Birc3-/- islets transplanted into diabetic-allogeneic recipients showed rapid loss of function indicative of dysregulated stress response. We propose that loss of BIRC2/3 uncoupled molecular control of inflammatory genes from TNF-signalling cascades. These present data demonstrate a novel, cell specific role for BIRC2 and BIRC3 as molecular rheostat that fine-tune NF-κB and JNK signalling to ensure transcriptional responses are appropriately matched to extra-cellular inputs. These pathways may be critical for beta cell stress responses.

Declaration relating to disposition of project thesis/dissertation

I hereby grant to the University of New South Wales or its agents the right to archive and to make available my thesis or dissertation in whole or in part in the University libraries in all forms of media, now or here after known, subject to the provisions of the Copyright Act 1968. I retain all property rights, such as patent rights. I also retain the right to use in future works (such as articles or books) all or part of this thesis or dissertation.

I also authorise University Microfilms to use the 350 word abstract of my thesis in Dissertation Abstracts International (this is applicable to doctoral theses only).

…… …… ……

Signature Witness Date

The University recognises that there may be exceptional circumstances requiring restrictions on copying or conditions on use. Requests for restriction for a period of up to 2 years must be made in writing. Requests for a longer period of restriction may be considered in exceptional circumstances and require the approval of the Dean of Graduate Research.

FOR OFFICE USE ONLY Date of completion of requirements for Award:

THIS SHEET IS TO BE GLUED TO THE INSIDE FRONT COVER OF THE THESIS

UNSW
THE UNIVERSITY OF NEW SOUTH WALES

BIRC proteins in the control of TNF signalling in pancreatic beta cells

Meijun Bernice Tan

A thesis submitted in fulfillment of the requirement for the degree of
Doctor of Philosophy

St Vincent's Clinical School
Faculty of Medicine

August 2012

Originality Statement

'I hereby declare that this submission is my own work and to the best of my knowledge it contains no materials previously published or written by another person, or substantial proportions of material which have been accepted for the award of any other degree or diploma at UNSW or any other educational institution, except where due acknowledgement is made in the thesis. Any contribution made to the research by others, with whom I have worked at UNSW or elsewhere, is explicitly acknowledged in the thesis. I also declare that the intellectual content of this thesis is the product of my own work, except to the extent that assistance from others in the project's design and conception or in style, presentation and linguistic expression is acknowledged.

Signed ……

Date ……

Copyright Statement

Authenticity Statement

'I certify that the Library deposit digital copy is a direct equivalent of the final officially approved version of my thesis. No emendation of content has occurred and if there are any minor variations in formatting, they are the result of the conversion to digital format.'

Signed ……

Date ……

Abstract

TNFα contributes to type 1 diabetes pathogenesis by impairing beta cell function and driving beta cell apoptosis. How TNFα directs these processes is not fully understood. Of note, baculoviral-IAP-repeat-containing (BIRC) proteins associate with the TNF complex to control down stream signalling but their function in beta cells is not known. Following TNF receptor ligation, *Birc3* but not *Birc1*,*2*,*4*,*5* or -*6* was induced in mouse islets and MIN6 cells. Contact with TNFα triggers signalling cascades converging on the NF-κB, JNK, and p38 pathways that regulate inflammatory genes. The *Birc3*-promoter harboured three NF-κB and an AP-1 binding sites. Induction of endogenous *Birc3* by TNFα was completely blocked by NF-κB inhibitors - thus NF-κB regulates *Birc3* transcription. Further, BIRC3 gain-of-function induced activation of an NF-κB reporter and potentiated TNFα-induced NF-κB activation. Surprisingly, *Birc3*$^{-/-}$ islets showed delayed but functional NF-κB activation and exhibited dysregulated TNFα-induced *Ccl2, Cxcl10* and *Icam-1*. Some data demonstrate BIRC2 can compensate for loss of BIRC3. Expectedly, *Birc2*$^{-/-}$ *Birc3*$^{-/-}$ islets showed markedly delayed IκBα degradation kinetics; suggesting that BIRC2/3 are crucial for NF-κB signalling. Remarkably, *Birc2*$^{-/-}$ and *Birc3*$^{-/-}$ islets showed unexpected phenotypes independent of NF-κB. Genetic analysis showed *Birc2*$^{-/-}$ islets exhibited increased basal *Ccl2* and *Cxcl10* expression. Cell signalling was further dysregulated by the addition of BIRC3 deficiency. *Birc2*$^{-/-}$*Birc3*$^{-/-}$ islets exhibited increased basal *A20, Icam-1, Ccl2 and Cxcl10*, which were blocked by antagonizing JNK but not NF-κB or p38 pathway. Moreover, *Birc2*$^{-/-}$*Birc3*$^{-/-}$ islets showed hyperphosphorylation of the JNK-target, c-Jun. BIRC proteins ubiquitinate substrates targeting them for proteosomal degradation. Proteosome inhibition in MIN6 cells mimicked the *Birc2*$^{-/-}$*Birc3*$^{-/-}$ islet phenotype – increased basal *Cxcl10, Ccl2* and *Icam-1* expression with c-Jun-hyperphosphorylation. *Birc2*$^{-/-}$*Birc3*$^{-/-}$ islets transplanted into diabetic-allogeneic recipients showed rapid loss of function indicative of dysregulated stress response. We propose that loss of BIRC2/3 uncoupled molecular control of inflammatory genes from TNF-signalling cascades. These present data demonstrate a novel, cell specific role for BIRC2 and BIRC3 as molecular rheostat that fine-tune NF-κB and JNK signalling to ensure

transcriptional responses are appropriately matched to extra-cellular inputs. These pathways may be critical for beta cell stress responses.

Acknowledgements

Firstly, I would like to thank my supervisor A/Professor Shane Grey for your patience and guidance in the last four years. Thank you for all the time you have spent on correcting my writing and oral presentation skills. More importantly, I would like to thank you for trusting and believing in me, and for fostering my passion for science.

I would also like to thank past and present members of Lab54 and people from the immunology department who had provided many stimulating scientific discussions and had assisted me with experimentations. Eliana – Your love for science rubbed off on me when I met you at the Garvan PhD Open Day 4 years ago. Thank you for your friendship and encouragement. Stacey – Thank you for all the help you have provided throughout my time at the Garvan. Thanks for providing a listening ear during times of crisis and for making sure that our lab orders go through no matter how busy you are. Jeanette – It was fun sharing the bench aisle with you. I am so glad you came back to finish your PhD, you will do great. Nathan – My sidekick in the lab, I will miss all the conversations we had. It was nice having someone to chat about NF-κB and JNK, and "cry" over Western blotting. Thank you so much for agreeing to help me with the islet transplantation work for this project, it made a big difference to this work. Eli – Thank you for all the wonderful discussions we had about TNF signalling, BIRCs and the TRAFs. I would also like to extend my thanks to Anita Weinberg, Andrew Yam, David Liuwantara and Peta Snikeris who were here at some point of my PhD for their friendship and assistance.

I would like to dedicate this thesis to my family and friends. This thesis would not be possible without your love and support. Mum, Dad and Bevan thank you for everything you have given me. I would not have gotten this far without you. Words cannot express my gratitude. To my dear friends Adrian, Ivy, Katja, Beatrice and Su-An who had not only offered me a place to stay, but had also provided me with the much-needed laughter, delicious meals and company - You are like a family away from home to me. I would also like to thank the Tsai family for their encouragement and comfort during the last four years. To Dessi, Doris, Nike, Angelica, Ellen, Heidi and Jeng Yie: Thank you girls for your friendship. I will miss you all heaps but I know we will always be in touch.

Most of all, I would like to thank my pillar of support, Louis, for believing in my abilities more than I believe in myself. Thank you for keeping me sane during experimental crisis, during manuscript and thesis writing. Thank you for your comfort, unconditional love, the surprises you have whipped up to cheer me up and for listening to all my scientific ideas without ever getting bored of it. Lastly, not forgetting our cat, Takeo, for his company and for trying to "edit" this thesis on several occasions by walking or sleeping on my keyboard.

Contribution of work

Chapter 1:

Chapter 2:

Chapter 3:

Chapter 4:

Chapter 5: Islet transplantation was performed by Mr Nathan Zammit.

Chapter 6:

Financial support

During my thesis I have received generous funding from:
Australian Postgraduate Award (APA), 2008-2011
Garvan Institute of Medical Research, 2008-2012

Publications arising from this thesis

Tan, B. M., Zammit, N. W, Slattery, R.M., Walters, S. N., E. Malle and Grey, S. T. BIRC proteins as molecular suppressors of JNK activation in pancreatic beta cells. Diabetologia. (Accepted: 19 Oct 2012)

List of abbreviations

AcD	Actinomycin D
A20 (TNFAIP3)	tumour necrosis factor alpha-interacting protein 3
AP-1	activating Protein 1
APC	antigen presenting cell
ASK1	apoptosis signal-regulating kinase 1
BIRC	baculovirus inhibitor of apoptosis protein repeating containing
bp	base pair
BSA	bovine Serum Albumin
cDNA	complementary DNA
CD4+	T-cell surface glycoprotein CD4
CD8+	T-cell surface glycoprotein CD8 alpha chain
COX-2	cyclooxygenase 2 (prostaglandin-endoperoxide synthase 2)
c-FLIP	cellular FLICE inhibitory protein / caspase 8 and FADD-like apoptosis regulator
CPH	Cyclophilin A
Cre	causing recombination / **C**yclization **re**combinase
DNA	deoxyribonucleic acid
DMSO	dimethyl sulfoxide
DKO	double knockout
ER	endoplasmic reticulum
FBS	fetal bovine serum
FADD	fas-associated death domain protein
FRT	flippase recombination target
FLP	flippase

HIP	human insulin promoter
IAP	inhibitor of apoptosis protein
ICAM-1/CD54	intercellular adhesion molecule 1
iNOS	inducible nitric oxide synthase
IP-10/CXCL10	Interferon gamma-induced protein 10
IκB	inhibitor of kappaB
IKK	inhibitor of kappa B kinase
IL	interleukin
IFN	interferon
JNK	c-Jun N-terminal kinase
kb	kilobase
Lox-P	locus of crossover in P1
LPS	lipopolysaccharide
mRNA	messenger ribonucleic acid
MAPK	mitogen-activated protein kinase
MEF	mouse embryonic fibroblast
MHC	major histocompatibility complex
MCP-1/CCL2	monocyte chemotactic protein-1
NF-κB	nuclear factor of kappa light polypeptide gene enhancer in B-cells
NOD	non-obese diabetic
NIK	NF-κB inducing kinase
PBS	phosphate buffered saline
PDTC	pyrrolidine dithiocarbamate
PCR	polymerase chain reaction

p53	protein 53
qRT-PCR	quantitative realtime PCR
RING	really interesting new gene
RIP	rat insulin promoter
RIPK1	Receptor-interacting serine/threonine-protein kinase 1
ROS	reactive oxygen species
SEM	standard error of the mean
STAT	signal transducers and activators of transcription
T1D	type 1 diabetes
T2D	type 2 diabetes
TNF	tumour necrosis factor
TNFR	TNF family receptor
TRAF	TNFR associated factor
TRADD	TNFR type 1-associated DEATH domain
Ub	Ubiquitin
WT	wild-type
kDA	kilodalton
h	hour (s)
min	minute (s)
s	second (s)
mmol/L	millimole per liter
U/mL	Units per milliliter
rpm	revolutions per minute
g	gram

°C	degrees centigrade
V	Voltage
v/v	volume per volume
w/v	weight per volume
5'-UTR	5' untranslated region

Table of Contents

List of Figures

List of Tables

1 Introduction

1.1 Overview

Diabetes mellitus is the most common endocrine disease and is a public health issue worldwide. It has been estimated that at least 346 million people in the world suffer from the disease (WHO, 2011). It is also the third leading cause of mortality by disease in the world (WHO, 2004). In 2007-2008, approximately 898,800 Australians had been diagnosed with diabetes. Global incidence of diabetes is anticipated to rise from 366 million in 2011, to 552 million by 2030 (IDF, 2011). Currently, the two major forms of the disease are type 1 and type 2 diabetes.

Type 1 diabetes (T1D) is an autoimmune disease where immune cells destroy pancreatic beta cells, resulting in insufficient insulin production to cope with the body's needs. T1D accounts for 5-10% of diabetes cases in the world (WHO, 2011) and is estimated to affect more than 122,300 people in Australia (JDRF, 2011). Genetic predisposition combined with environmental triggers can alter immune function, activate autoimmune beta cell destruction and contribute to the pathogenesis of T1D (Daneman, 2006; Jahromi and Eisenbarth, 2007; Kim and Polychronakos, 2005). Beta cell destruction involves immune cells, including T cells, B cells, macrophages and dendritic cells (Jansen et al., 1994; Noorchashm et al., 1997; Willcox et al., 2009). It has been demonstrated that cytotoxic T cells are the crucial players in the development and progression of T1D in human and animal models (Coppieters et al., 2012). Cytotoxic T cells induce beta cell death by activating key pathways that induce apoptosis. This leads to eventual demise of beta cells by extrinsic and intrinsic apoptotic pathways (Willcox et al., 2009), through caspase activation and mitochrondrial release of cytochrome C (Grunnet et al., 2009; Mathis et al., 2001).

Since the discovery of lymphocytic infiltrates within the islets of T1D patients (Gepts, 1965), our knowledge on the immunopathology of T1D have significantly improved (Coppieters et al., 2011; Phillips et al., 2009). Despite this, diagnosed

individuals are still destined to a lifetime of daily glucose monitoring and insulin injections to maintain glucose homeostasis. More importantly, exogenous insulin administrations cannot recapitulate the excellent glucose control provided by beta cells. Poor glucose control results in a number of deleterious diabetic complications (NEJM, 1993). Therapies for T1D includes methods to replenish beta cell mass such as islet transplantation and stem cell therapy (Cheung et al., 2000a; Robertson, 2004b) or using mechanisms that stimulate endogenous beta cell regeneration (Halban, 2004). While these techniques may treat T1D, the majority of islet transplant recipients still developed recurrent autoimmunity against the transplanted islets (Hilbrands et al., 2009; Roep et al., 1999). Therefore, unless the fundamental dysregulated immune response is resolved, replenishment of beta cell mass is not a long-term solution for the cure of T1D.

Insulin resistance occurs prior to the development of hyperglycaemia in type 2 diabetes (T2D) (Martin et al., 1992). It is accompanied by the progressive loss of beta cell function (Leahy, 2005; Poitout and Robertson, 2002), which eventually leads to disease pathogenesis. Beta cell dysfunction is central to the onset and progression of T2D (Weyer et al., 1999). This is also associated with decreased beta cell mass due to increased apoptosis (Butler et al., 2003). Factors such as mitochondrial dysfunction, oxidative stress, endoplasmic reticulum stress, dysfunctional triglyceride/free fatty acid cycling and glucolipotoxicity are the likely causes of early beta cell failure and death (Prentki and Nolan, 2006). Initially, individuals could control the disease with lifestyle changes alone. However, at late stage, diagnosed individuals also require daily exogenous injections of insulin for proper glucose control. Like T1D, patients are faced with diabetic complications and may require therapies to replenish beta cell mass. As hyperglycaemia proceeds, secondary processes that are associated with glucotoxicity such as O-linked glycosylation (Robertson, 2004a) and accumulation of islet amyloid polypeptide (O'Brien et al., 1993) can enhance beta cell dysfunction and beta cell apoptosis (Prentki and Nolan, 2006). In addition, emerging data suggest a link between islet inflammation and the progression of T2D (Donath et al., 2009; Donath et al., 2008; Ehses et al., 2009), thus placing further emphasis on the need to understand the role of the immune system on beta cell survival.

1.2 The pancreas

1.2.1 The islets of Langerhans

Islets of Langerhans are micro-organs that direct the endocrine physiology within the pancreas. Discovered in 1869 by German pathological anatomist Paul Langerhans (Morrison, 1937), the human pancreas contains about one million islets corresponding to around 1% of the total pancreas mass. Each islet comprised of about two thousand five hundred endocrine cells (Weir and Bonner-Weir, 1990). Typically, beta cells make up about between 48% and 59% of the human islets, depending on the region accessed (Cabrera et al., 2006) (Figure 1.1). The alpha cells representing approximately between 33% and 46% of the islet (Cabrera et al., 2006) (Figure 1.1), secrete glucagon for mobilising glycogen stores in the liver in response to low glucose. The delta-cells that make up between 8% and 12% of islet cells secrete somatostatin, known to exert inhibitory effects on other hormones including insulin and glucagon (Figure 1.1). The cells that secrete pancreatic polypeptide, known to stimulate gastric secretion, make up less than 2% of the islets (Cabrera et al., 2006).

It is important to note that the composition of beta and alpha cells in mouse islets, often used as a model for the study of islet biology, are quite different to the structure of human islets. Unlike human islets, mouse islets are made up of more beta cells (~77%) and less alpha cells (~18%) (Cabrera et al., 2006). Noticeably, the mouse islet architecture comprised of beta cells clustered within the core with the alpha cells surrounding the beta cells. Different to mouse islets, the alpha and beta cells are distributed throughout the human islet (Cabrera et al., 2006). Despite this, currently it is not known how the differences in islet architecture affect islet physiology between species.

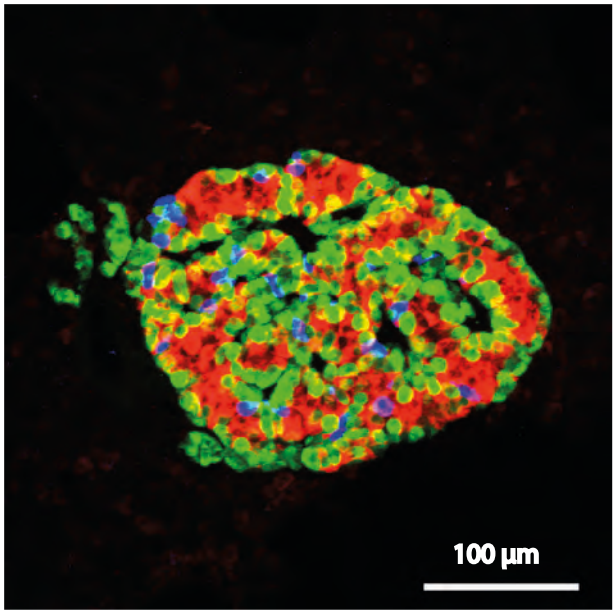


Figure 1.1. An image of a human islet *in situ*. Red represents insulin staining for beta cells, green represents glucagon staining for alpha cells and blue represents the somatostatin producing delta cells (Photo kindly provided by A/Prof. Shane Grey, Garvan Institute, NSW, Australia).

1.2.2 The function of insulin

Insulin is a peptide hormone crucial for several metabolic processes. In 1889, German scientists Minkowski and von Mering noted that total pancreatectomy in dogs led to the development of diabetes (Bliss, 1993; Dittrich, 1989). This led to their hypothesis that the pancreas secretes a substance that is important for metabolic control. In the 1920s, the insulin-secreting potential of pancreatic beta cells and the functional significance of insulin was discovered by Frederick Banting and Charles Best in pancreatectomized dogs (Banting and Best, 1990). They found that intravenous administration of chilled pancreas extracts led to reduced blood glucose levels in dogs made diabetic by removal of their pancreas. The first human treatment of diabetes occurred in 1922, on a 14-year old boy. The compound was named insulin. By early 1923, porcine insulin was commercially produced by Eli Lilly and made available for diabetes treatment.

Glucose levels are tightly controlled by pancreatic beta cells. In the fasting state, glucose homeostasis is maintained in equilibrium between glucose production by the liver and utilisation by insulin-dependent (e.g. liver, adipose tissue, muscle) and insulin-independent tissues (e.g. kidney and brain) (Cheatham and Kahn, 1995). In response to rise in blood glucose, beta cells secrete insulin to encourage its uptake into peripheral tissues and metabolism in cells (Meglasson and Matschinsky, 1986). Additionally, insulin also inhibits gluconeogenesis in the liver, kidneys and intestine to prevent glucose release from non-carbohydrate substrates. Further, insulin also participates in the regulation of carbohydrate, protein and lipid metabolism, ion and amino acid transport, glycogen synthesis, gene transcription and mRNA turnover, and DNA synthesis (Cheatham and Kahn, 1995; Lotspeich, 1949; Wilcox, 2005). For example, it causes muscle cells to take up amino acids, and inhibits the breakdown and release of lipids. Insulin also generates energy in the form of ATP as fuel for cells. Thus, insulin plays multiple roles in storage of ingested fuels and utilisation of fuels for cellular growth and differentiation.

1.3 Type 1 diabetes

1.3.1 Beta cell failure in T1D

It has been demonstrated that progressive beta cell failure occurs in both forms of diabetes (Cnop et al., 2005). For T1D, progressive beta cell destruction occurs as a consequence of immune-mediated attack of pancreatic beta cells. In T2D, there is functional defect in insulin secretion as well as a loss of beta cell mass later in the disease process. As a result of long-term poor glucose regulation, both forms of diabetes can lead to serious health complications including ketoacidosis, kidney failure, heart disease, stroke and blindness. The main focus of this thesis is on T1D. However, we believe that the lessons learnt from our study are transferrable between the two forms of diabetes.

1.3.2 Diabetic complications

At the time of diagnosis, most patients are at the end stage of the disease, as clinical symptoms only manifest once 70-100% of the beta cell mass are destroyed or dysfunctional (Matveyenko and Butler, 2008). At this stage, individuals present with clinical symptoms including polyuria, polydipsia and weight loss. When glucose is not available, the body utilises fat as an alternative energy source (Eisenbarth GS, 2008). The degradation of fat causes toxic ketones to build up in the blood and urine in a condition known as ketoacidosis (Eisenbarth GS, 2008). Ketoacidosis is one of the leading causes of mortality in T1D. It starts with nausea, vomiting and dehydration and results in coma if left untreated (Eisenbarth, 1986).

As a result of poor glucose control, both T1D and T2D individuals eventually develop secondary complications, the risk of which is related to the duration of diabetes and the degree of glycaemic control. Poorly managed glucose homeostasis over time can lead to irreversible and devastating changes on small and large blood vessels (Nathan et al., 2005; Nathan et al., 2003). Long-term diabetic patients eventually develop diabetic complications, which manifests as macrovascular diseases, nephropathy, retinopathy and neuropathy. Macrovascular complications in T1D include coronary artery disease, peripheral arterial disease, and stroke. It

has been reported that T1D individuals are more susceptible to fatalities of ischemic heart disease than the general population (Laing et al., 2003a; Laing et al., 2003b). As diabetes continues to impact on vascular injury, conditions such as heart attack and stroke can lead to early mortality in individuals (Laing et al., 2003a; Laing et al., 2003b; Paterson et al., 2007). Furthermore, poor glycaemic control also induce changes in microvascular properties, resulting in complications such as nephropathy leading to renal failure, retinopathy resulting in blindness and neuropathy leading to foot complications (e.g. gangrene), the major cause of amputation (Melendez-Ramirez et al., 2010).

The severe outcomes of diabetic complications are the motivation behind the ongoing search for new therapies to fine-tune glucose control and cure T1D. Some of these strategies include replacement of beta cell shortage through administration of drugs, such as analogs of the incretin hormone and glucagon-like-peptide-1 (GLP-1), to stimulate insulin secretion in the remaining undestroyed beta cells (Hadjiyanni et al., 2008; Pratley and Gilbert, 2008). There are also attempts to stimulate beta cell neogenesis and regeneration (Dungan et al., 2009; Rooman and Bouwens, 2004; Rooman et al., 2002; Rosenberg et al., 2004; Suarez-Pinzon et al., 2005), and the use of islets from cadaveric organ donors, stem cells or islets of xenogenic origins (mostly from pigs or transgenic pigs) to replenish beta cell mass (Cardona et al., 2006; Hering et al., 2006; Meier et al., 2006; van der Windt et al., 2009; Witkowski et al., 2006). Even though this thesis does not directly explore the possibilities of T1D treatment, one of the expected outcomes of this study is to improve our knowledge on how beta cells respond under inflammatory conditions of T1D, which would contribute to the creation of new therapeutic agents or improve current strategies to overcome this disease.

1.3.3 Genetic disposition

It is well established that multiple genetic loci are involved in the susceptibility of T1D. Although a large number of diagnosed individuals do not have a family history of T1D, familial clustering shows higher risk among siblings with T1D of 6%, in contrast to 0.4 % frequency in the general US Caucasian population

(Spielman et al., 1980; Thomson et al., 1988). This trend was also seen in Japan, with a generally low frequency of T1D in the population. The risk of developing the disease is increased from 0.014% to 1.3-3.8% for those with siblings diagnosed with T1D (Ikegami and Ogihara, 1996). These studies suggest that T1D may require a genetic component to occur. Studies in the past years have progressed to successfully identify several genetic susceptibility loci that are associated with the risk to develop T1D.

1.3.4 Genetic susceptibility genes

One locus that strongly contributes to familial clustering lies within the major histocompatibility (*MHC*) region on chromosome 6p21, commonly termed the insulin-dependent diabetes mellitus (IDDM1) locus (Nerup et al., 1974; Rich, 1990; Singal and Blajchman, 1973). It has been demonstrated that the human leukocyte antigen (HLA) class II genes (*HLA-DRB1* and *-DQB1*) are the likely primary determinants of IDDM1 (Ronningen et al., 2001). For interest, other established T1D-associated genes include the insulin gene (*INS*) on chromosome 11p15 (Bell et al., 1984), cytotoxic T lymphocyte-associated 4 gene (*CTLA4*) on chromosome 2q33 (Nistico et al., 1996; Ueda et al., 2003), protein tyrosine phosphatase, non-receptor type 22 (*PTPN22*) on chromosome 1p13 encoding lymphoid protein tyrokine phosphatase (*LYP*) (Bottini et al., 2004; Smyth et al., 2004) and the interleukin-2 (IL-2) receptor-α (*IL-2RA*) gene (Lowe et al., 2007; Qu et al., 2007; Vella et al., 2005).

1.3.5 Environmental factors

Genetic predisposition does, to some extent, influence the incidence of T1D. Nonetheless, concordance rates between monozygotic twins is only 50% and between dizygotic twins a mere ~10% (Kyvik et al., 1995). Longer-term follow-up studies also show that identical twins of diagnosed individuals do eventually develop diabetes but with a delay of up to 30 years between the twins (Redondo et al., 2008; Redondo et al., 2001). These trends suggest that the environment may contribute to the development of the disease. Most attention to environmental factors has been paid to viral infections, with majority of the evidence implicating

the prevalence of enteroviruses (more precisely coxsackieviruses) as a leading candidate virus for the precipitation of T1D (Banatvala et al., 1985; Clements et al., 1995; Filippi and von Herrath, 2008; Gamble et al., 1969). However, the role of viruses as causative agents in T1D is yet to be confirmed and requires further examination (van Belle et al., 2011). Other environmental factors that may contribute to T1D include the composition of gut bacteria, early exposure to cow's milk, wheat proteins and vitamin D levels, although no consensus can be reached by evaluating current published data on these topics (van Belle et al., 2011).

1.3.6 Cell lines and animal models of T1D

Our current understanding on the events that occur during T1D progression in humans, largely relies on histological samples that were collected by Foulis and colleagues from patients with recent-onset of the disease, during a time when insulin was not available (Foulis, 1987; Foulis and Stewart, 1984; Willcox et al., 2009). This is because it is difficult to study the actual events during disease progression in humans. The clinical symptoms of T1D only occur years after the appearance of autoantibodies and by the time diagnosis is made, ~70-100% of the beta cells are already destroyed (Matveyenko and Butler, 2008). Hence our current understanding of T1D depends on *in vitro* and *in vivo* studies performed on a number of islet-derived beta cell lines and animal models of T1D. Common beta cell lines include MIN6 and β-TC_3 cells described in this thesis (Crisa et al., 1992; Delovitch and Singh, 1997; Efrat et al., 1988; Miyazaki et al., 1990), while animal models of T1D include the commonly used non-obese diabetic (NOD) mouse, biobreeding (BB) rat and rodent models of chemical and virus-induced diabetes. The NOD mice and BB rats spontaneously develop insulitis and T1D in a manner that closely matches the disease in humans (Bone et al., 1999; Crisa et al., 1992; Delovitch and Singh, 1997). However, it is important to bear in mind that there are limitations in studying human diseases in animal models. These include factors such as genetic homogeneity of the animals due to inbreeding compared to T1D in humans, which are heterogeneous and the differences in islet architecture between human and mouse. Despite these differences, the rodent models of T1D have significantly advanced our knowledge on the disease.

1.3.7 Immunopathology of T1D

Clinical diagnosis of T1D is described as irreversible destruction of pancreatic beta cells, involving cytokines and T lymphocytes specific for beta cell antigens (Nerup et al., 1988). The development of the disease involves complex multi-step processes with interactions between the beta cells and the innate and adaptive immune system, eventually leading to beta cell destruction by immune cells. T1D occurs in two stages: Insulitis occurs when lymphocytic infiltrates invade the islets and diabetes proceeds when large populations of the beta cells are destroyed leading to hyperglycaemia. The lymphocytic infiltrates participate in beta cell destruction by secreting inflammatory cytokines (Dahlen et al., 1998; Green et al., 1998; Jun et al., 1999a; Jun et al., 1999b; Pakala et al., 1999).

The first identification of immune infiltrates were made by Willy Gepts in pancreatic islets of children with T1D (Gepts, 1965). This significant finding marked T1D as an autoimmune disease. Following Gepts's discovery, subsequent studies confirmed and improved his original findings. Detailed examination of the inflammatory infiltrate in the pancreas of a newly diagnosed 12-year old child showed that the majority of the infiltrates are cytotoxic lymphocytes (Bottazzo et al., 1985). Later, further characterisation work showed that the major subset of cells that surround the islets were predominantly $CD8^+$ T cells (Hanninen et al., 1992; Somoza et al., 1994). Additionally, a study found that $CD8^+$ T cell numbers peaked with increasing beta cell death and disappear when all the functional beta cells are destroyed (Willcox et al., 2009). Similar to $CD8^+$ T cells, the study also showed that B lymphocytes follow the same patterns of appearance as T cells, while the macrophages and dendritic cells numbers remained unchanged during insulitis (Uno et al., 2007).

The sequence of lymphocytic cell invasion into the islets was determined in NOD mice and BB rats. The first cells to infiltrate the islets are macrophages and dendritic cells (Hanenberg et al., 1989; Jansen et al., 1994; Voorbij et al., 1989), followed by T lymphocytes, NK cells and B lymphocytes (Amano and Yoon, 1990). The effects of cytotoxic T cells in T1D pathology have been demonstrated. Transfer of $CD4^+$ and $CD8^+$ T cells from diabetic NOD mice into non-diseased newborn NOD

mice led to faster disease progression (Mueller et al., 1995). Furthermore, the prevalence of $CD8^+$ T cells within the insulitic lesions of T1D-diagnosed humans was recently confirmed by histology (Coppieters et al., 2012).

1.3.8 Production of cytokines by immune infiltrates

It has been shown that inactivation of macrophages in NOD mice or BB rats were sufficient to prevent diabetes progression (Lee et al., 1988a; Lee et al., 1988b; Oschilewski et al., 1985). B lymphocytes and dendritic cells were found to facilitate the activation of $CD8^+$ T cells into cytotoxic forms by presenting self-antigen on their MHC-I molecules to $CD8^+$ T cells (Jun et al., 1999b; Marino et al., 2012; Yoon and Jun, 2005). The activated $CD8^+$ T cells subsequently destroy beta cells (Jun et al., 1999b; Marino et al., 2012; Yoon and Jun, 2005). In addition, macrophages also contribute to beta cell destruction by secreting inflammatory agents such as nitric oxide and pro-inflammatory cytokines, including tumour necrosis factor alpha (TNFα), IL-1β and interferon gamma (IFNγ) (Appels et al., 1989; Mandrup-Poulsen et al., 1987; Pankewycz et al., 1995; Pukel et al., 1988). The expression of these proinflammatory cytokines are increased at the onset of diabetes (Held et al., 1990; Hirai et al., 2000; Hussain et al., 1996; Perez et al., 2004; Schloot et al., 2002).

1.3.9 The Copenhagen model

It is generally accepted that cytokines are implicated in the pathogenesis of T1D. The Copenhagen Model, proposed by Nerup *et al*, explained the sequence of events that lead to beta cell destruction (Nerup et al., 1988). Revisions of the original model were made in 1994 (Nerup et al., 1994) and again in 2003 (Bergholdt, 2004). The model suggests that cytokines are the major effectors of beta cell death in T1D. In this model, cytokine-induced free radical destruction is thought to precede cytotoxic T cells and/or auto-antibody-mediated beta cell death. This model hypothesizes that beta cell destruction in T1D starts with a cytokine-induction phase that does not involve an antigen or lymphocytic cells. Additionally, the model also assumes a latter phase that requires the participation of lymphocytes and autoantigens to amplify and perpetuate beta cell destruction, which ultimately results in beta cell death. While the exact sequence of events that occur during T1D

progression in humans remains unknown, the importance of cytokines in the pathogenesis of T1D has been demonstrated. Studies have shown that target deletion of cytokine signalling pathways in NOD mice can delay or even abrogate disease development. Moreover, increasing evidence also implicates the role of cytokines and inflammation as contributing factors to the pathogenesis of T2D. Therefore, in contrast to the Copenhagen model, cytokines do participate in the autoimmune destruction of beta cells.

1.4 The role of cytokines in diabetes

Increased cytokine production has been implicated in the pathogenesis of many autoimmune diseases (Brennan and Feldmann, 1992; Mandrup-Poulsen et al., 1996). In T1D, a number of cytokines including TNFα, IL-1β and IFNγ contribute to the pathogenesis of the disease (Baquerizo and Rabinovitch, 1990; McKenzie et al., 2006; Mehta et al., 1994; Mori et al., 2008; Ortis et al., 2008; Stephens et al., 1999; Thomas et al., 2004; Wachlin et al., 2003). The biological implications of these cytokines are two fold. Firstly, they can signal to beta cells through specific receptors expressed by beta cells (Hammonds et al., 1990; Hughes et al., 1990; Mori et al., 2008; Stephens et al., 1999; Walter et al., 2000). Secondly, they can induce the recruitment and effector functions of cells such as macrophages and T cells. Cytokines can also perturb beta cell function and induce apoptosis by activating signalling pathways and by inducing changes in gene transcription and protein synthesis.

1.4.1 TNFα biology

TNFα was discovered in 1975 as an endotoxin-induced factor that causes tumour regression through the induction of cell death (Carswell et al., 1975). The human form of this 157 amino acid homotrimer was cloned in 1984 (Pennica et al., 1984). The *TNF* gene lies on mouse chromosome 17 and on human chromosome 6, sharing the same locus as members of the TNF superfamily, lymphotoxin (*LT*) α and β genes (Browning et al., 1993; Carswell et al., 1975; Lawton et al., 1995; Nedospasov et al., 1986). The promoter region of *TNFa* is highly conserved among primates and lies in a region that is essential for transcriptional regulation (Leung

et al., 2000; Uglialoro et al., 1998). The pleiotropic cytokine influences cell proliferation, immune responses, inflammation and cell death and is expressed by a variety of cell types including macrophages, dendritic cells, T lymphocytes and pancreatic beta cells (Goldfeld et al., 1991; Sung et al., 1988; Uno et al., 2007).

The contributions of TNFα to T1D pathogenesis are multifaceted. Notably, *Tnfr1*-null islets are protected from destruction by T lymphocytes (Pakala et al., 1999). Further, TNFα is an early cytokine detected within the immune infiltrate surrounding islets (Dahlen et al., 1998). In the context of T1D, TNFα exerts detrimental effects including driving the expression of inflammatory genes in beta cells (Ortis et al., 2008), impairing insulin secretion (Zhang and Kim, 1995) and triggering beta cell apoptosis (Liuwantara et al., 2006). Studies on NOD mice showed the importance of TNFα in T1D. TNFα's ability to induce and perpetuate diabetes is clearly demonstrated as 3-week-old NOD mice administered with exogenous TNFα present with accelerated onset of diabetes and increased disease frequency (Yang et al., 1994). In contrast, diabetes progression can be ameliorated by anti-TNFα monoclonal antibody treatment (Yang et al., 1994). Moreover, TNFα production was reported to be increased with islet lymphocytic infiltration (Mueller et al., 1995) and can be prevented by treatment with anti-TNFα antibody (Brown et al., 1998). A great demonstration for the importance of TNFα in T1D comes from the *Tnfr1*-deficient NOD mice. It has been shown that *Tnfr1*-deleted NOD mice developed insulitis along with control NOD mice but were completely protected from diabetes (Kagi et al., 1999). Together, these studies supported the importance of TNFα in the progression of T1D. More information on the TNFα signalling pathway will be covered in the next section of this literature review.

1.4.2 IL-1β

The cytokine, IL-1β, is cytotoxic to beta cells. IL-1β is found to inhibit insulin secretion in rat islets (Hughes et al., 1990; Southern et al., 1990). Further, *in vivo* and *in vitro* studies showed that the cytotoxic effects of IL-1β on beta cells could be neutralised by antagonising the IL-1 receptor (IL-1R) (Dinarello, 1991; Nicoletti et al., 1994). IL-1β's involvement in T1D was further evidenced when *Il1r*-knockout

NOD mice were shown to have slower disease progression compared to its wild-type-NOD counterparts (Thomas et al., 2004). One of IL-1β's cytotoxic effects on beta cells is through the induction of inducible nitric oxide synthase (iNOS) production (Bergmann et al., 1992; Corbett et al., 1992; Delaney et al., 1993; Eizirik et al., 1992). IL-1β-induced nitric oxide production has been shown to directly cause apoptosis in RINm5F beta cell (Ankarcrona et al., 1994) and impair glucose-stimulated insulin release in rodent beta cells (Sandler et al., 1991). Together, these findings implicate a role for IL-1β in T1D progression.

1.4.3 IFNγ

The cytokine IFNγ also contributes to the development of T1D. It has been demonstrated that IFNγ can render the beta cell line, MIN6N8, susceptible to TNFα-mediated cell death *in vitro* (Kim et al., 2005). Further, IFNγ-stimulated β-TC_6-F7 and β-TC_3 cells showed decreased insulin secretion and increased MHC class I surface expression, suggesting that IFNγ can exacerbate beta cell inflammation during insulitis and hasten the development of T1D (Baldeon et al., 1998; Baldeon et al., 1997). Additionally, investigation on the role of IFNγ in NOD mice showed that cyclophosphamide treatment led to induction of diabetes in wild-type-NOD mice within 4 weeks of administration, while IFNγ receptor-knockout (*Ifngr*$^{-/-}$) and *Ifng*$^{-/-}$ NOD mice were resistant to cyclophosphamide-induced diabetes (Mori et al., 2008). These findings agreed with the data from *Ifngr*$^{-/-}$ NOD and *Ifngr*$^{-/-}$ BDC2.5 TCR tg mice, that harbour a mutation within the alpha-chain subunit of IFNR, demonstrating that IFNγ is an important causative agent for T1D (Wang et al., 1997).

However, conflicting results questioned the importance of IFNγ in T1D. In one study, *Ifng* gene inhibition delayed but did not prevent disease in NOD mice (Hultgren et al., 1996). Another study reported that *Ifngr-α-chain*-deficient NOD mice were protected from disease not because of the absence of IFNγ signalling *per se*, but rather from the presence of diabetes-resistant *Idd* allele that was transferred from the 129-mouse unto the NOD background (Kanagawa et al., 2000). Furthermore, it was reported in one study that *Ifngr-β-chain-subunit-*

deficient NOD mice were also susceptible to disease (Serreze et al., 2000), which questioned the importance of IFNγ in the pathogenesis of T1D. Moreover, experiments performed on NOD-transgenic mice expressing a dominant negative mutant *Ifngr* on beta cells showed that IFNγ plays a role in increasing MHC class I expression but not diabetes (Thomas et al., 1998). Thus, IFNγ may not directly cause disease but may enhance the degree of lymphocytic infiltration into the islets. Despite the controversies, it has been demonstrated that the cytotoxic effects of TNFα can be synergised with IL-1β and IFNγ (Cetkovic-Cvrlje and Eizirik, 1994; Hamaguchi et al., 1990; Mandrup-Poulsen et al., 1987; Pukel et al., 1988), suggesting that perhaps IFNγ does not exert direct effects on beta cells, but work with other cytokines such as IL-1β and TNFα to intensify the immune response in T1D.

1.4.4 Current therapies targeted at blocking cytokine signalling

Much effort has been placed in developing therapeutics aimed at preventing cytokine signalling to treat T1D. Blockade of IL-1R signalling with anakinra resulted in improved glucose control, insulin secretion and decreased systemic inflammation for T2D patients (Larsen et al., 2007a). This provided a proof-of-concept that IL-1β is detrimental to beta cell function and suggests that blockade of IL-1β signalling may preserve beta cell function and maintain glycaemic levels in T1D and T2D patients. The efficacy of anakinra is currently tested in clinical trials for the treatment of T1D (Pickersgill and Mandrup-Poulsen, 2009; Soumya Adhikani, 2009). To counteract the effects of TNFα in T1D, administration of a TNFα antagonist (etanercept) in a phase II T1D treatment trial, resulted in improved outcomes of insulin production and preservation of beta cell function (Mastrandrea et al., 2009). Despite this, opposing results were documented in separate trials, demonstrating the complexities of blocking TNFα. One trial showed that some patients treated with etanercept for rheumatoid arthritis developed T1D (Bloom, 2000; Boulton and Bourne, 2007; Tack et al., 2009), while antibodies to neutralise TNFα in rheumatoid arthritis, resolved T1D in some subjects (Arif et al., 2010). These results demonstrate the complexities of blocking TNF-signalling for diabetes treatment. An alternative method is to target multiple cytokine-signalling

pathways to resolve T1D. For example, the combination of anakinra plus etanercept for simultaneous blockade of TNFα and IL-1β signalling prior to islet transplantation, in an animal study and in a human clinical trial, resulted in better islet transplantation outcomes (Matsumoto et al., 2011; McCall et al., 2012). Overall, both animal research and clinical studies have demonstrated the concept that targeting cytokine signalling in beta cells may be beneficial for T1D treatment. However, more work is required to understand the molecular mechanisms of cytokine signalling to avoid the opposing outcomes seen with current drugs in trial. For our study, we have focused on understanding how TNFα signals on a beta cell.

1.5 TNF receptor signalling

1.5.1 The two TNF receptors

TNF receptor 1 (TNFR1/p55) and receptor 2 (TNFR2/p75) are responsible for binding TNFα and interacting with intracellular adaptor proteins to activate multiple downstream signalling processes (MacEwan, 2002; Varfolomeev and Ashkenazi, 2004). TNFR1 is constitutively expressed and TNFR2 expression is thought to be restricted to lymphocytes and endothelial cells (Brockhaus et al., 1990; Hohmann et al., 1989; Porteu et al., 1991). While TNFR1 is mainly responsible for mediating the cellular effects of the soluble ligand of TNF, TNFR2 is reported to signal the effects of its membrane bound ligand (Grell et al., 1995). TNFR1 is thought to regulate apoptosis while TNFR2 is responsible for regulating proliferation, but crosstalk between the two receptors can occur in some cell types (Luo et al., 2006; Luo et al., 2010; Mukhopadhyay et al., 2001; Weiss et al., 1998).

1.5.2 TNFR1 signals two distinct complexes

TNFR1 is also known as the death receptor, important for mediating apoptosis (Nagata, 1997). Upon stimulation of the pathway, the adaptor protein TNF receptor associated protein with a death domain (TRADD) is recruited to the cytoplasmic domain of TNFR1. TRADD assembles at least two distinct signalling complexes that initiate opposing signalling pathways (Hsu et al., 1996; Micheau

and Tschopp, 2003) (Figure 1.2). Complex I activates both anti-apoptotic and proinflammatory mediators, and is also involved in the activation of complex II-mediated cell death. Following TNFR ligation, complex I is recruited to the cytoplasmic domain of the TNFR. Activation of the TNF signalling cascade involves the binding of TRADD to TNFR, followed by the recruitment of (TNFR-associated factor) TRAF2/5 to TRADD (Hsu et al., 1996). BIRC2 and BIRC3 were first discovered as part of the TNFR2 signalling complex through their association with TRAF2 (Rothe et al., 1995). Receptor interacting protein (RIP) can be ubiquitinated by both Lys-63-linked and Lys-48-linked ubiquitination (Wertz et al., 2004). Some data show that TRAF2 is the E3 ligase responsible for targeting RIP for Lys-63-linked ubiquitination (Wertz et al., 2004). However, accumulating evidence suggest that TRAF2 may be important for facilitating the recruitment of BIRC2/3 to the TNFR complex (Mace et al., 2010; Vince et al., 2009) and that BIRC2/3 are the E3 ligases responsible for catalysing Lys-63-linked RIP ubiquitination (Bertrand et al., 2008; Haas et al., 2009; Mahoney et al., 2008; Varfolomeev et al., 2008; Zheng et al., 2010). In this complex, TNFα stimulation results in activation of the transcription factors *nuclear factor* kappa-light-chain-enhancer of activated B cells (NF-κB) and mitogen-activated protein kinase (MAPK) transcription factors and in some cases apoptosis (Liu and Han, 2001; Smith et al., 1994). These signalling cascades are imperative for the control of genes involved in immune response, inflammation and cell survival (Baeuerle and Baltimore, 1996; Liu and Han, 2001; Varfolomeev and Ashkenazi, 2004). Further, it is thought that RIP is essential for activating NF-κB (Kelliher et al., 1998; Ting et al., 1996) while TRAF2 is required for activation of the c-Jun N-terminal kinase (JNK) pathway (Habelhah et al., 2004).

Under some circumstances, a secondary complex referred to as complex II, comprising of Fas-associated death domain protein (FADD) and caspase-8/10 can trigger the pro-apoptotic cascade (Micheau and Tschopp, 2003) (Figure 1.2). Normally, TNFα-stimulated cells are protected from the pro-apoptotic force of complex II by the expression of NF-κB-induced genes including *A20* and *c-FLIP* regulated via complex I. *A20* and *c-FLIP* prevent activation of caspase-8 possibly at the level of complex II (Cottet et al., 2002; Liuwantara et al., 2006). Thus, TNFα

triggers a co-ordinated and complex cellular program regulating cell proliferation, inflammation and cell death.

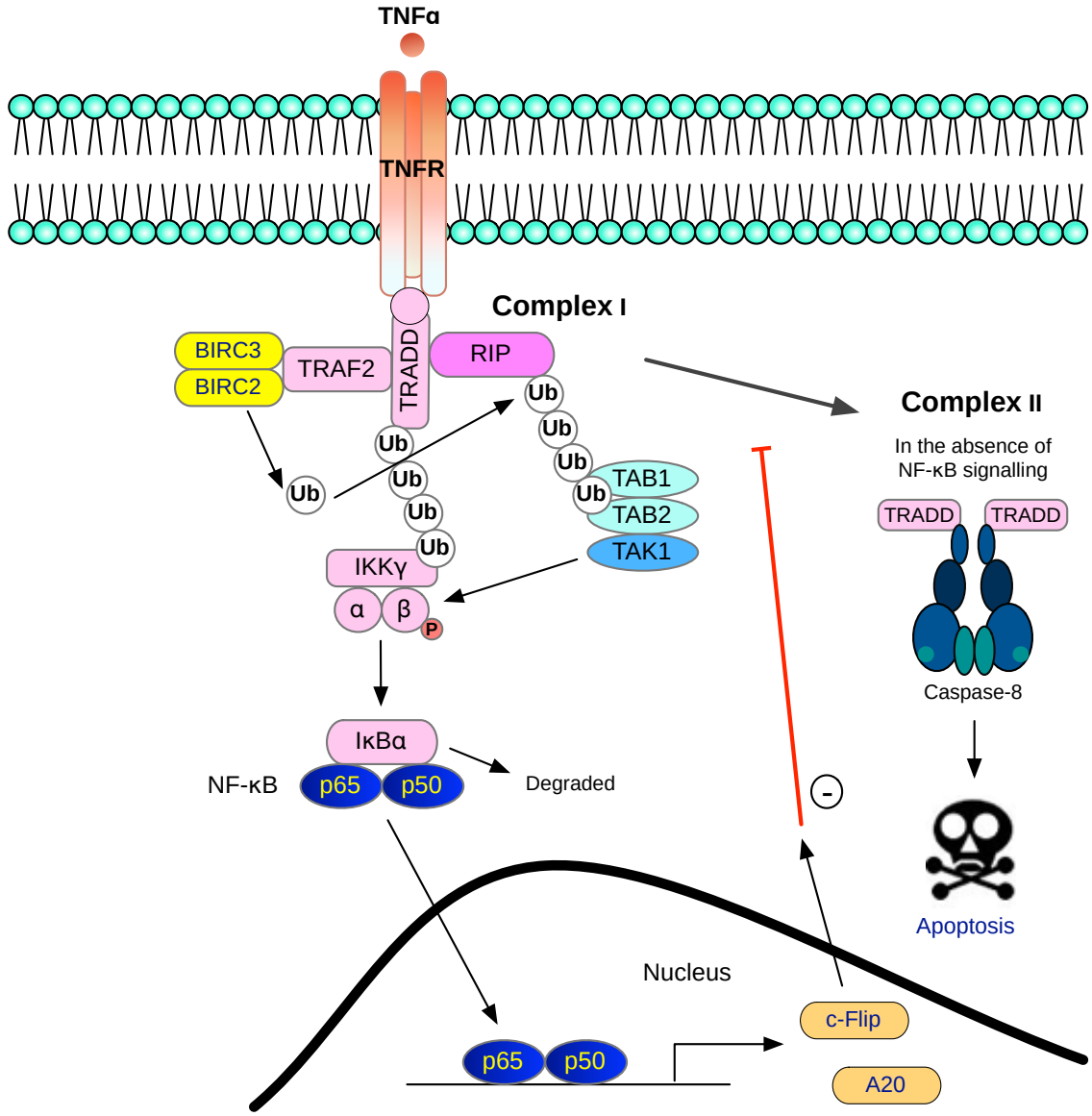


Figure 1.2. TNFR1 activation of the NF-κB signalling pathway. Activation of TNFR1 stimulates complex I formation comprising of TRADD, RIP, TRAF2, BIRC2 and BIRC3. BIRC2/3 targets RIP for Lys-63-linked ubiquitination, leading ubiquitin (Ub)-associated recruitment of TAK1-TAB2-TAB3 complex and IKKγ-IKKα-IKKβ complex. These cascades activate TAK1, which phosphorylates IKKβ and result in the degradation of the NF-κB inhibitor, IκBα. Once degraded, inhibition on NF-κB (consisting of p65/p50 subunits) is then lifted. The freed p65/p50 heterodimer translocates to the nucleus and activate NF-κB transcription. Under some circumstances, TNFα can activate the death-inducing complex II, consisting of FADD and caspase-8. Activation of complex II leads to apoptosis via the extrinsic pathway. Normally, this pathway is prevented by the expression of NF-κB-regulated anti-apoptotic genes such as *c-Flip* and *A20*. Diagram adapted and modified from (Gyrd-Hansen and Meier, 2010).

1.5.3 NF-κB signalling

The NF-κB superfamily of transcription factors is made up of two subfamilies - namely "NF-κB" proteins and "Rel" protein. The Rel subfamily proteins are RelA (p65), RelB and c-Rel, and the NF-κB subfamily comprise of precursor and ankyrin repeat-containing proteins, NF-κB1 (p105, processed to p50) and NF-κB2 (p100, processed to p52) (Gilmore, 2006; Perkins, 2007). Signal transduction via this pathway relies on NF-κB proteins interacting with Rel proteins. Depending on external signal, NF-κB can result in activation of the canonical or the non-canonical pathway (Bonizzi et al., 2004; Perkins, 2007). As these pathways are responsible for the expression of a large number of genes, their activation is tightly controlled by ubiquitin-dependent signalling cascades involving sequential assembly of protein complexes that lead to the activation of kinases. The two NF-κB signalling cascades are triggered by different receptor activation and require different inhibitor of kappa B (IκB) kinase (IKK) complexes, responsible for regulating downstream phosphorylation, degradation and inactivation of IκB proteins.

Normally, these transcription factors are activated upon stimulation of receptors or triggered by conditions of cellular stress such as DNA damage (Perkins, 2007). NF-κB plays a central role in regulating the expression of genes required for the body's innate and adaptive immune responses (Bonizzi and Karin, 2004; Gyrd-Hansen and Meier, 2010). Moreover, it is imperative for the survival and proliferation of some cell types. For example, the RelA subunit responsible for mediating the strong transcriptional activation potential of NF-κB (Schmitz and Baeuerle, 1991), is required for embryo survival and liver proliferation in mice. Without it, *Rela*-knockout mice suffer severe liver degeneration and die at the stage of embryo development (Beg et al., 1995; Bonizzi et al., 2004).

1.5.3.1 The canonical NF-κB pathway

Activation of the canonical pathway can occur with receptor stimulation by proinflammatory cytokines including TNFα and IL-1β. This triggers a signalling cascade involving the central activation complex comprising of IKKα (IKK1), IKKβ (IKK2) and the regulatory IKKγ (NEMO). This pathway is essential for the

regulation of inflammatory responses, lymphoid cell proliferation and apoptosis (Hoffmann and Baltimore, 2006). TNFα stimulation results in activation of the canonical NF-κB pathway and the ubiquitination of the RIP kinase (Kanayama et al., 2004; Legler et al., 2003) (Figure 1.2). Ubiquitinated RIP directs the assembly of two RIP-associated kinase complexes: transforming growth factor-β-activated kinase comprising of (TAK1)-TAB2-TAB3 and the IκB kinase (IKK) complex comprising of IKKγ-IKKα-IKKβ (Besse et al., 2007; Kanayama et al., 2004). The TAK1-TAB2-TAB3 complex results in activation of TAK1. Subsequently, TAK1 phosphorylates IKKβ, resulting in phosphorylation and degradation of the natural NF-κB inhibitor IkappaB-alpha (IκBα), responsible for sequestering NF-κB in the cytoplasm (Chen et al., 1995; Scherer et al., 1995). The heterodimer comprising of p50/p65 (RelA; NF-κB) then enters the nucleus and induces transcription of target proinflammatory and anti-apoptotic genes.

1.5.3.2 The non-canonical NF-κB pathway

Activation of the non-canonical NF-κB pathway occurs with stimulation of the TNFR by other members of the TNF receptor superfamily including CD40, B cell activating factor (BAFF), lymphotoxin and TWEAK (Bonizzi and Karin, 2004). This pathway is thought to be important for the development and maintenance of secondary lymphoid organs. NF-κB-inducing kinase (NIK) and IKKα are the kinases that regulate the activation of this pathway (Bonizzi and Karin, 2004; Zarnegar et al., 2008a). Activating this pathway involves phosphorylation and degradation of NF-κB2 (p100) into the active form RelB/p52 heterodimer (Hayden and Ghosh, 2008; Xiao et al., 2004). This pathway will be further described in the context of the function of BIRC proteins in section 1.7.4.3, in page 35.

1.5.4 The role of NF-κB in T1D

NF-κB regulates the beta cell's response to cytokine exposure and plays a central role in determining the beta cell's fate in T1D. On the one hand, NF-κB contributes to regulating the inflammatory response of beta cells. During insulitis, the lymphocytic infiltrate secrete cytokines and chemokines to attract migration of

immune cells into the islets, and beta cells can worsen the development of insulitis by secreting more inflammatory mediators regulated by NF-κB. Following cytokine exposure, beta cells express several NF-κB-regulated cytokines and chemokines including CXCL10 (IP-10), CCL2 (MCP-1) and ICAM-1 (Cardozo et al., 2001a). Suppression of CXCL10, CCL2 and ICAM-1 protected NOD mice from disease (Cameron et al., 2000; Martin et al., 2001; Morimoto et al., 2004), indicating that NF-κB does contribute to diabetes progression. In support of the pro-apoptotic role of NF-κB, some studies show that blockade of NF-κB protected beta cells from cytokine-induced apoptosis (Eldor et al., 2006; Ortis et al., 2008).

On the other hand, NF-κB also provides protection against TNFα-induced beta cell death. This was demonstrated in a study that found inhibition of NF-κB hastened apoptosis in beta cell lines and also in primary islets stimulated with TNFα and IFNγ (Chang et al., 2003). In addition, *RelA*$^{-/-}$ but not WT islets were sensitive to TNFα-induced apoptosis (Thomas et al., 2006). Particularly, NF-κB is also responsible for regulating the expression of key anti-apoptotic genes including *c-flip* and *A20* (Cottet et al., 2002; Grey et al., 1999; Liuwantara et al., 2006). The pro-survival role of c-FLIP lies in its ability to bind to complex II of TNF signalling and prevent activation of caspase-8 (Haag et al.). Further, the role played by A20 is both anti-apoptotic and anti-inflammatory. A20 negative regulates NF-κB signalling by blocking IκBα degradation and preventing the inflammatory response of NF-κB activation (Grey et al., 1999). Thus, the beta cell's fate in health and disease may lie in the balance between inflammatory and survival signals regulated by NF-κB, perhaps depending on the duration and strength of activation of this pathway (Zhao et al., 2011b).

1.5.5 Stress-activated MAPK signalling

MAPK signalling mediates diverse cellular functions including that of the regulation of gene expression, immune responses and apoptosis (Chang and Karin, 2001; Keshet and Seger, 2010; Rincon and Davis, 2009). The stress-activated protein kinase (SAPK) of the MAPK pathway includes the JNK and p38 families. These SAPK are activated under diverse conditions of stress including that of

ultraviolet light and irradiation, cytotoxic drugs, cold and heat shock, proinflammatory cytokines, shearing stresses and reactive oxygen species (ROS) production (Clerk et al., 1998; Davis, 2000; Derijard et al., 1994; Kyriakis et al., 1994; Raingeaud et al., 1995).

1.5.5.1 p38 signalling

The MAPK p38 pathway is activated by upstream kinases MAPK kinase (MKK)3 and MKK6 (Raingeaud et al., 1996). Apart from JNK activation, the kinase MKK4 can also activate p38 signalling (Derijard et al., 1994). Furthermore, p38 is also activated by autophosphorylation through interaction with TAB1 (Ge et al., 2002; Ge et al., 2003). TAB1 plays a role in p38 signalling by binding to and activating the upstream MAPKK kinase, TAK1 (Ge et al., 2002). Finally, the activated p38 phosphorylates and activates downstream transcription factors such as ATF-2 and Elk-1 (Raingeaud et al., 1995; Raingeaud et al., 1996).

1.5.5.2 The role of p38 in T1D

It has been demonstrated that TNFα stimulation can induce p38 activation in rat insulinoma cells (Saldeen et al., 2001). In a separate study, ectopic expression of TAB1 in the beta cell line, β-$TC_{6,}$, induced p38 phosphorylation and led to increased beta cell apoptosis (Makeeva et al., 2007). Conversely, inhibition of p38 signalling was found to prevent cytokine-induced apoptosis in rat (Saldeen et al., 2001) and human islets (Makeeva et al., 2006), implicating that p38 contribute to beta cell apoptosis in the presence of cytokine.

1.5.5.3 JNK signalling

JNK signalling directly modulates the function of some pro- and anti-apoptotic proteins. For example, JNK can activate the intrinsic apoptosis pathway (Lin and Dibling, 2002; Liu and Lin, 2005; Wang et al., 2000) by inducing cytochrome c release from the mitochondria (Tournier et al., 2000) in a process mediated by Bax and Bak (Lei et al., 2002). JNK can also induce apoptosis via the phosphorylation of Bim to generate jBID, which is later processed to Bid. Activated Bid induces the

release of SMAC from the mitochondria into the cytoplasm causing the induction of apoptosis (Deng et al., 2003; Lei and Davis, 2003).

The JNK family is encoded by JNK1, JNK2 and JNK3 (Tournier et al., 2000). While JNK1 and JNK2 are ubiquitously expressed and are the main mediators of the diverse functions of JNK in different cell types, the expression of JNK3 is restricted to the brain, heart and testis (Davis, 2000; Tournier et al., 2000). The upstream JNK signalling proteins, MKK4 and MKK7, are required for JNK1 and JNK2 activation. Activation of JNK involves phosphorylation on the Thr183 and Tyr185 directed by MKK4 and MKK7, which are activated by phosphorylation of a diverse group of upstream MAPKK kinases (MAPKKKs) such as ASK, MEKK and TAK1 (Manning and Davis, 2003). Slightly different to p38, the transcription factors activated by JNK are ATF-2, c-Jun, c-Fos and Elk-1 responsible for mediating the cellular effects of JNK (Gupta et al., 1996; Karin et al., 1997; Sluss et al., 1994). The downstream transcription factor activator protein-1 (AP-1) is formed from binding of transcription factors such as ATF-2, c-Jun and c-Fos as homo- or heterodimers (Karin et al., 1997).

1.5.5.4 Role of JNK in T1D

JNK is found to signal deleterious effects on pancreatic beta cells. Blockade of JNK signalling protected rodent and human islets from cytokine-mediated apoptosis (Aikin et al., 2004; Ammendrup et al., 2000). Consistent with these findings, are the studies that showed improved graft survival and function, with suppression of JNK activation prior to the islet transplantation (Noguchi et al., 2009; Noguchi et al., 2005). Notably, TNFα-mediated inhibition of insulin secretion is a result of JNK activation (Varona-Santos et al., 2008). Indeed, JNK was also found to induce endoplasmic reticulum (ER) stress and production of ROS, which result in beta cell inflammation. In addition, JNK can also enhance the production of some NF-κB-regulated inflammatory genes, contribute to increased lipolysis and suppress activation of peroxisome proliferator-activated receptor γ (PPARγ) in beta cells, all of which are implicated in T2D pathogenesis (Hotamisligil, 2005). Despite this, the

exact mechanism of JNK-induced cell death in beta cells is not well understood. Thus, further studies are required to understand JNK signalling in beta cells.

1.6 BIRC family proteins

Inhibitors of apoptosis proteins (IAP) are *bona fide* baculorviral IAP repeat (BIR) containing proteins (Figure 1.3). Also known as baculorviral IAP repeat containing (BIRC) proteins, the nomenclature between human and mouse IAPs is not uniform. For example human IAP2 (hIAP2) is also known as mouse IAP1 (c-IAP1). For consistency, we refer to these proteins by their BIRC names in this thesis. To date, eight mammalian BIRC proteins have been identified: neuronal apoptosis inhibitory protein (NAIP; also known as BIRC1); cellular IAP1 (c-IAP1; also known as BIRC2); cellular IAP2 (c-IAP2; also known as BIRC3); X chromosome-linked IAP (XIAP; also known as BIRC4); survivin (also known as BIRC5); BIR-containing ubiquitin-conjugating BIR domain enzyme apollon (also known as BIRC6); melanoma IAP (ML-IAP; also known as Livin or BIRC7); and IAP-like protein 2 (ILP2; also known as BIRC8) (Salvesen and Duckett, 2002; Vucic, 2008).

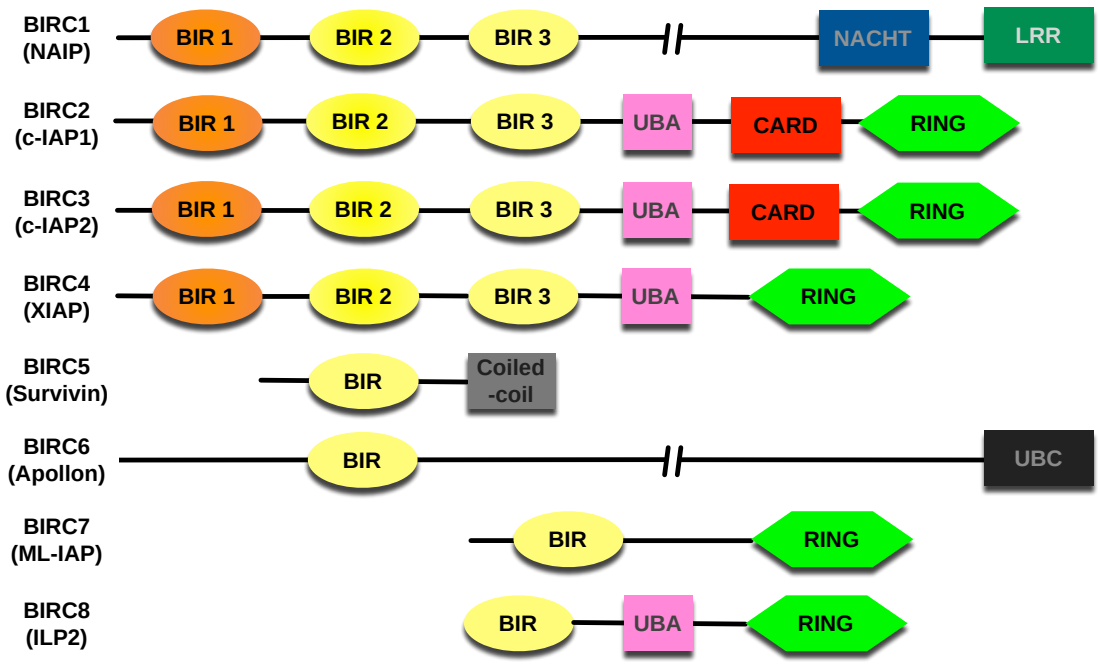


Figure 1.3. Eight BIRC proteins have been identified in humans. All BIRC proteins contain BIR motifs. Adapted from (Fulda and Vucic, 2012).

1.6.1 Structural features of BIRC protein

1.6.1.1 BIR domains

BIR domains mediate the protein-protein interactions and inhibition of apoptosis functions of BIRC proteins. BIRC proteins have up to three copies of 70 amino-acid conserved zinc-binding BIR domains (Figure 1.3) (Hinds et al., 1999; Sun et al., 1999; Sun et al., 2000). The BIR motif is made up of an invariant number of amino acids, which includes the conserved three cysteines and one histidine residue within the sequence $CX_2CX_{16}HX_{6-8}C$ (Uren et al., 1998). BIR domains comprise of a series of four or five α-helices and a three-stranded β-sheet, with a zinc ion held together by the conserved cysteine and histidine residues (Chai et al., 2001; Hinds et al., 1999; Huang et al., 2001; Riedl et al., 2001; Sun et al., 2000). BIRC1 to 4 contain three BIR domains, whereas BIRC5 to 8 only consist of one BIR domain in their structures. The function of BIR domains has been clearly elucidated in BIRC4. The anti-apoptotic function of BIRC4 will be addressed in the next section.

1.6.1.2 RING domains

In addition to BIR domain, BIRC2, 3, 4, 7 and 8 also have a carboxy-terminal RING domain capable of E3 ubiquitin ligase function (Figure 1.3) (Varfolomeev and Vucic, 2008; Vaux and Silke, 2005). Analysis of BIRC2 and BIRC4 showed that the RING domain is important for mediating their own ubiquitination (Yang et al., 2000b). The same domain in BIRC3 is found to be necessary for self-ubiquitination and for mono-ubiquitination of caspase-3 and -7 (Huang et al., 2000). Self-ubiquitination of BIRC proteins is important for maintaining their levels *in vivo*. It has been demonstrated that BIRC3 levels are increased in cells derived from *Birc2*-deficient mice. The increased is attributed to increased BIRC3 protein stability, as normally BIRC2 regulates BIRC3 levels by targeting it for ubiquitination and degradation (Conze et al., 2005). In addition, the RING domain is also important for catalysing ubiquitination of proteins that associate with BIRC proteins, including inhibition of caspases that bind to different BIR domains of BIRC proteins.

1.6.1.3 Ubiquitin-associated (UBA) domain

The BIRC proteins BIRC2, 3 4 and 8 also possess a ubiquitin-associated (UBA) domain facilitating the binding of these proteins to monoubiquitin and Lys(48)- and Lys(63)-linked polyubiquitin chains (Blankenship et al., 2009; Gyrd-Hansen et al., 2008). It is thought that the UBA domains of BIRC2 and BIRC3 are not required for their participation of TNF signalling or for ubiquitin ligase activity of BIRC2, but rather for proteosomal degradation of the BIRC proteins themselves (Blankenship et al., 2009). The UBA of BIRC2 is also found to be important for endothelial cell survival, for protecting cells from TNFα-mediated apoptosis and also for NF-κB activation by BIRC4 (Gyrd-Hansen et al., 2008).

1.6.1.4 Caspase recruitment (CARD) domain

Unique to BIRC2 and BIRC3 is the caspase recruitment domain (CARD) whose function was recently elucidated (Figure 1.3). It turns out that the function of the CARD domain of BIRC proteins is unconventional. Instead of facilitating recruitment or substrate binding, the CARD domain functions to stabilise the BIRC2 protein by staying in a "closed confirmation" (Figure 1.4), thus preventing autoubiquitination by the RING domain (Lopez et al., 2011; Oetjen and Duckett, 2011). During activation, the CARD domain undergoes conformational change, lifting its inhibition on the RING domain, thus allowing "RING dimerisation" and E3 ubiquitin ligase activation (Lopez et al., 2011).

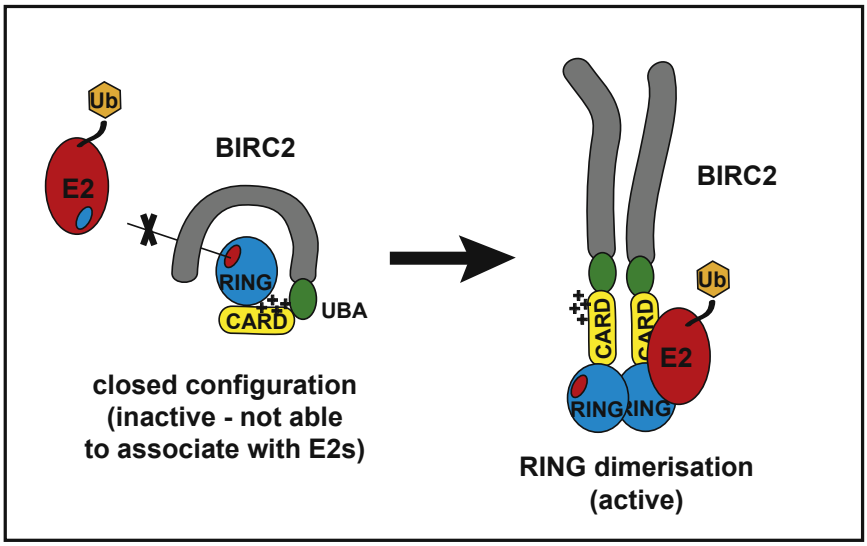


Figure 1.4. The CARD domain of BIRC2 inhibits E3 activity of RING domain to prevent auto-ubiquitination of the protein (inactive state). When activated, the CARD domain changes conformation and allows dimerisation of the RING domain and auto-ubiquitination of BIRC2 (active state). Reproduced from (Lopez et al., 2011).

1.6.1.5 Other domains in BIRCs

Additional domains unique to BIRC proteins include the ubiquitin-conjugating (UBC) domain of BIRC6, which has been implicated to catalyse the covalent attachment of ubiquitin to target proteins (Chen et al., 1999; Hauser et al., 1998). Also, the coiled-coil domain of BIRC5 (Jeyaprakash et al., 2007) and the nucleotide-binding and oligomerization domain (NACHT) [named after neuronal apoptosis inhibitory protein (NAIP), CIITA (MHC class II transcription activator), HET-E (incompatibility locus protein from Podospora anserina) and TP1 (telomerase-associated protein)] and the leucine-rich repeat (LRR) domains of BIRC1 (Damiano et al., 2004; Liston et al., 1996; Wilmanski et al., 2008). The functions of some of these domains have not been elucidated. The ones with known function will be mentioned in the context of BIRC proteins in the next sections.

1.7 Function of BIRC members

1.7.1 Background

BIRC proteins are a family of anti-apoptotic proteins that are conserved across several species (Roy et al., 1997). The first IAPs, Cp-IAP and Op-IAP, were discovered in baculovirus by virtue of their ability to enhance viral propagation in insect cells, by preventing the activation of defensive apoptosis in host cells (Birnbaum et al., 1994; Clem and Miller, 1994; Crook et al., 1993). Subsequently, other BIRC proteins were identified in insects and vertebrates (Duckett et al., 1996; Hay et al., 1995; Liston et al., 1996; Rothe et al., 1995; Roy et al., 1997; Uren et al., 1996; You et al., 1997). BIRC2, BIRC3 and BIRC4 are the most well characterised members of the mammalian BIRC proteins. It is now acknowledged that in addition to their role as inhibitors of apoptosis, BIRC proteins also participate in diverse cellular processes including signalling events that facilitate cell motility and metastasis (Dogan et al., 2008; Mehrotra et al., 2010), MAPK signalling, proliferation, mitosis, promotion of cell-cycle progression and ubiquitin-dependent signalling events such as regulation of NF-κB transcription factor, which mediates expression of genes that control inflammation, immunity, cell migration and cell survival (Gyrd-Hansen et al., 2008; Gyrd-Hansen and Meier, 2010; Rumble and Duckett, 2008). Their diverse functions may be attributed to the different domains found within their structures (Figure 1.3). Particularly, the highly conserved BIR domains within a single BIRC protein can have different functions (Eckelman et al., 2006; Srinivasula and Ashwell, 2008). One of the crucial contributions of BIRC proteins to cell survival and tumour formation lies within their role in NF-κB signalling and participation in the innate immunity (Gyrd-Hansen and Meier, 2010). In this thesis, the multitude of functions served by BIRC proteins is reviewed. These information will highlight the importance to examine the roles of BIRCs in pancreatic beta cells and what is already known about them in beta cells.

1.7.2 Apoptosis

Apoptosis is a highly regulated process of programmed cell death leading to biochemical events that cause changes in cell morphology and eventually demise of the cell. It is an important process since inhibition of apoptosis can result in a number of cancers, autoimmune diseases, inflammatory diseases, and viral infections. The key executioners of destruction in apoptosis are caspases. The four apoptotic pathways are (Fan et al., 2005): (1) the mitochondrial/intrinsic pathway involves the pro-apoptotic members of BCL2 proteins piercing into the outer mitochondrial membrane, releasing cytochrome c into the cytoplasm. Released cytochrome c then associates with APAF-1 to form apoptosomes, leading to sequential caspase-9 and then caspase-3 activation (Li et al., 1997; Zou et al., 1999); (2) the extrinsic pathway is initiated outside the cell through ligation of extracellular signals and the death receptors such as Fas ligand (FasL)/Fas, TNF/TNFR and (tumor necrosis factor-related apoptosis-inducing ligand) TRAIL/TRAIL-R on the cell membrane (Ozoren and El-Deiry, 2003). Activation of TNFR1 triggers DISC complex formation, activating caspase-8 and -10, which in turn activates the final death effectors, caspases-3 and -7 (Meier and Vousden, 2007; Micheau and Tschopp, 2003); (3) the endoplasmic reticulum (ER) stress-induced pathway leads to the activation of caspase-2 and caspase-9 (Cheung et al., 2006); and finally (4) granzyme B activation of caspase-3 and mitochondria pathway through exocytosis of granules by cytolytic T cells and natural killer cells onto target cells (Goping et al., 2003; Lord et al., 2003). These pathways are not mutually exclusive. Under some circumstances, activation of one pathway can involve the activation of another.

The anti-apoptotic function of BIRC proteins lies in their ability to inhibit caspase activation directly or indirectly by modulating upstream signalling pathways that control caspase activation. Figure 1.5 depicts the complex roles played by BIRC proteins in modulating three main apoptosis pathways triggered by ER stress, the extrinsic and intrinsic apoptosis pathways. The next section of this thesis provides a summary of the different signal transduction pathways or caspase inhibition mechanisms that BIRC proteins involve in to modulate apoptosis.

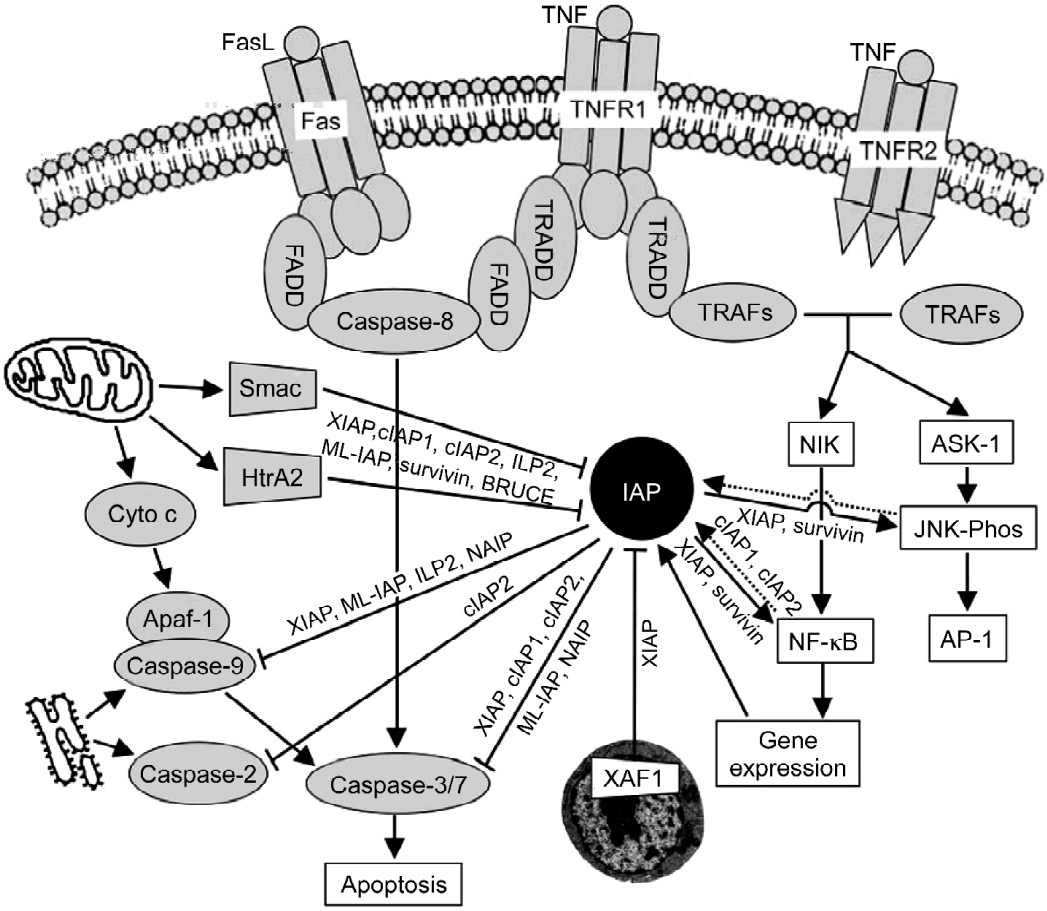


Figure 1.5. Complex roles played by BIRC (or IAP) proteins in apoptosis regulation. BIRC proteins are involved in preventing the activation of different caspases. Natural antagonists of BIRCs include second mitochondrial activator of caspases (Smac), high-temperature-regulated A2 (HtfA2) and X-linked IAP-associated factor 1 (XAF1). Additionally, BIRC proteins provide positive regulation on the NF-κB and JNK pathways. (NAIP/BIRC1, cIAP1/BIRC2, cIAP2/BIRC3, XIAP/BIRC4, Survivin/BIRC5, BIRC6/BRUCE/Apollon, BIRC7/ML-IAP and BIRC8/ILP2) Figure reproduced from (Wei et al., 2008).

1.7.3 The role BIRC1/NAIP in neural health and innate immunity

The first human IAP, BIRC1, was isolated based on its contribution to the neurodegenerative disorder, spinal muscular atrophy (SMA) (Roy et al., 1995). NAIP deletions and mutations correlated with individuals with the most severe form of SMA (Essawi et al., 2007; Roy et al., 1995). Loss of functional NAIP prevented the normal process of motor neuron apoptosis, and is associated with severity of the disease (Akutsu et al., 2002; Watihayati et al., 2009). Biochemical analysis showed that NAIP is able to inhibit the catalytic processing of caspase-9 within the apoptosome complex, preventing caspase-3 activation and apoptosis at its initiation stage (Davoodi et al., 2010). In addition, NAIP is also thought to contribute to cellular survival by activating JNK1 (Sanna et al., 2002). Apart from neuronal survival, NAIP is also known as the nucleotide oligomerization domain (Nod)-like receptor that plays a role in pathogen sensing and signal transduction in the innate immune response (Damiano et al., 2004). Further, NAIP is also reported to play a role in promoting the assembly of inflammasomes involved in activating cytokines such as IL-1β, IL-18 and inflammatory caspases (Chamaillard et al., 2003; Fritz et al., 2006; Martinon and Tschopp, 2007).

1.7.4 BIRC2 and BIRC3 are highly conserved anti-apoptotic proteins

1.7.4.1 Anti-apoptotic function of BIRC2 and BIRC3

The BIRC family proteins, BIRC2 and BIRC3, were identified based on sequence homology to a family of viral IAP proteins known to bind and inhibit caspase activity (Liston et al., 1996). It is thought that BIRC2 and BIRC3 may have arised from gene duplication, as they share highly conserved sequences and are found to reside in tandem within the human chromosome 11q22 (Roy et al., 1997). Both BIRC proteins are implicated to inhibit the intrinsic and extrinsic apoptotic pathways. In support of their anti-apoptotic function, BIRC2 and BIRC3 are found to bind to and inhibit the activity of caspase-3 and -7 *in vitro* (Burke et al., 2010; Roy et al., 1997). However, *in vivo* assays showed that they were weak caspase inhibitors (Eckelman and Salvesen, 2006). Despite these findings, it was recently demonstrated that although BIRC2 did not significantly suppress caspase activation, it is able to bind to and ubiquitinate caspase-3 and -7 at distinct steps

during caspase processing (Choi, Butterworth et al. 2009). Furthermore it was reported that the BIR2 and BIR3 domains of BIRC2 could sufficiently interfere with the caspase-9 apoptosome complex, preventing caspase-3 activation and mediation of apoptosis (Burke et al., 2010). Thus, BIRC2 and BIRC3 may inhibit apoptosis by hindering the process of caspase activation.

In fact, the RING domain was thought to confer the anti-apoptotic functions of BIRC2 and BIRC3. The RING domain targets specific substrates for proteosomal degradation (via K48-polyubiquitin linkages) or for participation in specific signalling pathways (through K63-polyubiquitin linkages) (Mahoney et al., 2008). *In vitro*, the RING domain of BIRC3 was sufficient for mono-ubiquitination of caspase-3 and -7 (Huang et al., 2000). Therefore BIRC2 and BIRC3 may contribute to apoptosis inhibition, possibly by maintaining the levels of active effector caspases within cells, by targeting them for ubiquitination and degradation (Choi et al., 2009). It has been reported that BIRC2 and BIRC3 are also able to inhibit activation of the pro-apoptotic molecule, Smac/DIABLO, by targeting them for proteosomal degradation (Hu and Yang, 2003). Moreover, increased BIRC2 or BIRC3 protected cells from radiotherapy, endoplasmic reticulum stress, tissue injury, TRAIL- and Fas-induced apoptosis (Cheung et al., 2006; Dong et al., 2003; Endo et al., 2005; Gagnon et al., 2003; Imoto et al., 2002; Nagata et al., 2011; Peng et al., 2005; Wang et al., 2005; Warnakulasuriyarachchi et al., 2004). Due to their nature to self-ubiquitinate, they are found to be weak inhibitors of apoptosis compared to the other BIRC proteins such as XIAP. In one study, it was reported that co-expression with TRAF2 prevented autoubiquitination by the RING domain of BIRC2, allowing it to suppress Smac/DIABLO and protect against cell death (Csomos et al., 2009). Collectively, these studies suggest that BIRC2 and BIRC3 can function as anti-apoptotic genes.

1.7.4.2 BIRC2 and BIRC3 in TNFα-mediated apoptosis and NF-κB signalling.

One of the key features of BIRC2 and BIRC3 are their participation in the canonical and non-canonical NF-κB signal cascade. Of interest, NF-κB activation can induce BIRC2, BIRC3 and BIRC4 expression, further suggesting that BIRC proteins participate in a positive feedback loop in TNFα-mediated NF-κB signalling (Gyrd-

Hansen and Meier, 2010) (Figure 1.6). BIRC2 and BIRC3 are thought to positively influence the canonical NF-κB signalling pathway but are required to exert negative regulation on the non-canonical pathway.

Indeed, BIRC2 and BIRC3 were first identified as proteins that bind to TRAF1 and TRAF2 in a complex associated with TNFR2 (Rothe et al., 1995; Uren et al., 1996). Within the signalling complex, BIRC2 is thought to be responsible for ubiquitination and degradation of TRAF2 during TNF signalling (Wu et al., 2005). BIRC2 and BIRC3 may function to poly-ubiquitinate and target RIP for degradation (Park et al., 2004), a step thought to be required for TNFα-mediated NF-κB signalling to occur (Kelliher et al., 1998) (Figure 1.6). Although a recent study showed that TNFα-mediated NF-κB signalling could still occur independent of RIP (Wong et al., 2010), thus the requirement of RIP in NF-κB activation in TNF signalling may be cell-type specific. As BIRC2 and BIRC3 have highly homologous structures, it is not surprising that they function redundantly in TNF signalling. Noticeably, studies on the *Birc2*- and *Birc3*-single-deleted mice showed no gross functional defects in TNFα-induced RIP ubiquitination and TNFα-mediated NF-κB signalling (Conte et al., 2006; Conze et al., 2005). However, deletions of both BIRC proteins significantly impaired TNFα-induced RIP ubiquitination and NF-κB activation (Bertrand et al., 2008; Haas et al., 2009; Mahoney et al., 2008; Varfolomeev et al., 2008), implicating that BIRC2 and BIRC3 plays a role in the canonical NF-κB signalling pathway.

It has been demonstrated that both BIRC2 and BIRC3 can function to protect cells from the death-inducing effects of TNFα (O'Donnell et al., 2007; Petersen et al., 2007; Wang et al., 2008). In the absence of BIRC2 and BIRC3, TNFα ligation induces RIP-dependent complex II formation and caspase-8 mediated apoptosis (Petersen et al., 2007; Wang et al., 2008) (Figure 1.6), suggesting that BIRC2/3 can mediate cell survival and protect cells from TNFα-mediated apoptosis. The anti-apoptotic ability of BIRC2 and BIRC3 are also demonstrated in their role in cell-cycle regulation. It has been reported that during the G2/M phase, NF-κB activation up-regulates BIRC3 expression, a step that is crucial for the survival of

cells in mitosis. In agreement to its anti-apoptotic function, the study also reported that loss of BIRC3 resulted in the death of mitotic-arrested cells (Jin and Lee, 2006).

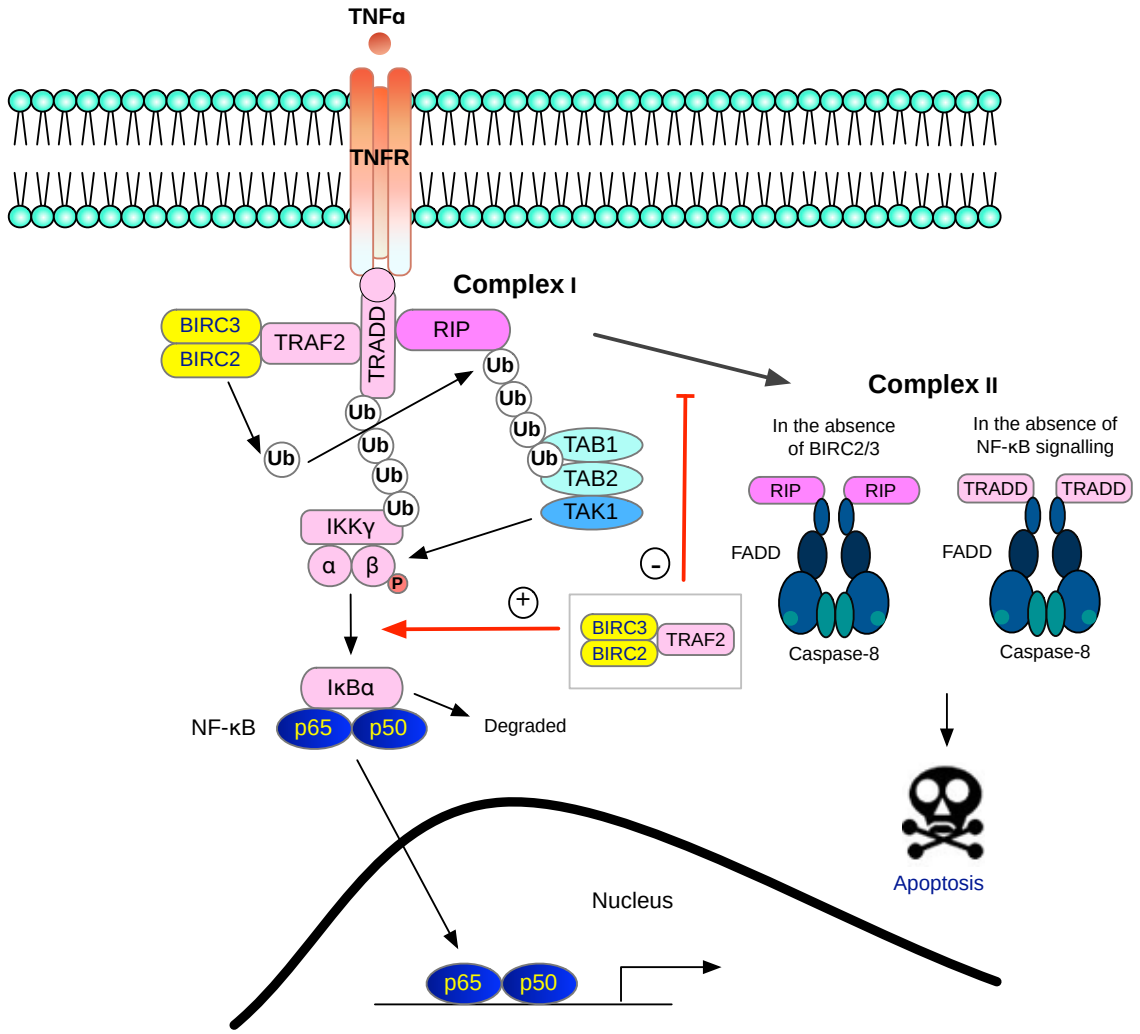


Figure 1.6. The function of BIRC2 and BIRC3 in TNFR1 activation of NF-κB signalling. Activation of TNFR1 stimulates complex I formation comprising of TRADD, RIP, TRAF2, BIRC2 and BIRC3. Both BIRC proteins are thought to suppress TNFα-mediated apoptosis by participating in the positive-feedback loop for TNFα-mediated NF-κB signalling and inhibit complex II formation. In the absence of BIRC2/3, complex II is formed by RIP and in the absence of NF-κB signalling, TRADD takes part in complex II formation. Adapted and modified from (Gyrd-Hansen and Meier, 2010).

1.7.4.3 The role of BIRC2 and BIRC3 in non-canonical NF-κB signalling and multiple myeloma

In addition, BIRC2 and BIRC3 also play an important role in the non-canonical NF-κB signalling pathway, particularly in B lymphocytes (Gardam et al.). In resting cells, activation of this pathway is suppressed by constitutive NIK degradation by the TRAF3-TRAF2-BIRC2/3 complex (Vallabhapurapu et al., 2008; Zarnegar et al., 2008b) (Figure 1.7A). In this complex, TRAF3 binds to NIK causing the TRAF3-NIK homodimer to bind with the TRAF2-BIRC2/3 complex. BIRC2 and BIRC3 are the E3 ligase required for degradation of NIK (Figure 1.7A) and are crucial in preventing unwanted constitutive activation of the non-canonical NF-κB pathway (Zarnegar et al., 2008b) (Figure 1.7B). Notably, spontaneous non-canonical NF-κB activation and mutations of BIRC2/3 were associated with the pathogenesis of multiple myeloma (Keats et al., 2007). This is because the loss of BIRC2 and BIRC3, the resultant NIK accumulation and constitutive non-canonical NF-κB activation are associated with the prolong survival and accumulation of B lymphocytes as well as conditions of malignancies (Gardam et al.).

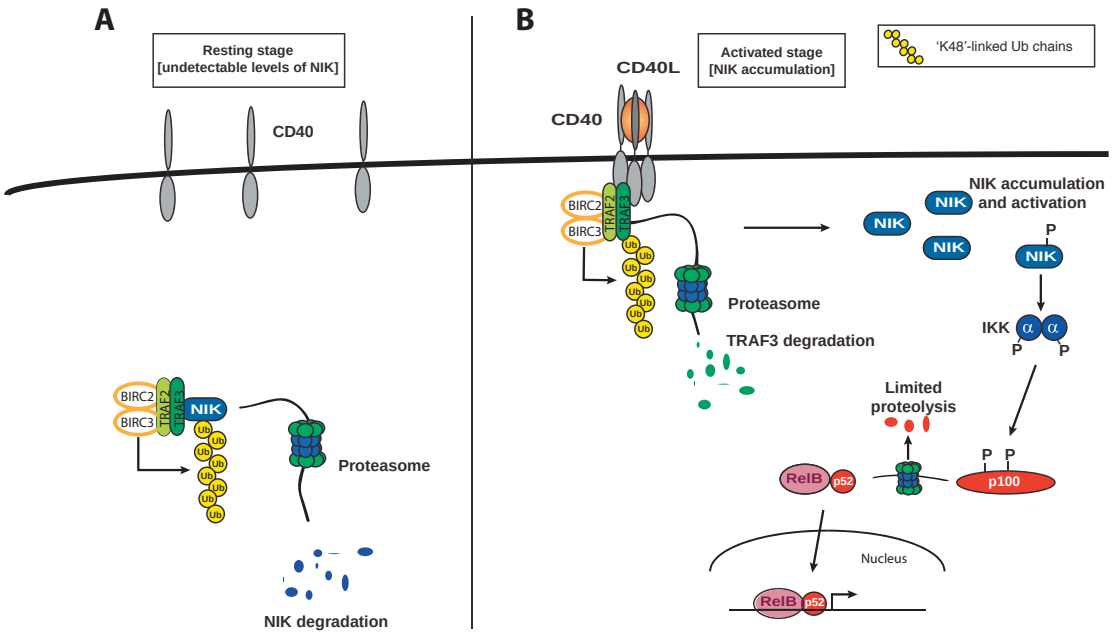


Figure 1.7. The non-canonical NF-κB signalling pathway. (A) Under resting conditions, the adaptor protein TRAF3 binds to NIK and recruits the TRAF2/BIRC complex. In this complex, BIRC2 and BIRC3 prevent activation of the non-canonical NF-κB pathway by targeting NIK for ubiquitination and degradation by the proteasome. (B) Following CD40 stimulation, the TRAF2/TRAF3/BIRC complex is recruited. BIRC proteins target TRAF3 for K48-linked polyubiquitination (Ub), leading to TRAF3 degradation by the proteosome. Without TRAF3, NIK levels accumulate, as it can no longer be recruited to the TRAF2/BIRC complex for degradation. The accumulated NIK becomes activated by autophosphorylation (P). Subsequently, the activated NIK in turn activates IKKα to phosphorylate p100 (NF-κB2), resulting in proteosome-dependent processing of p100 to generate p52. The released RelB-p52 complex then translocates to the nucleus and brings about transcription of target genes. Diagram adapted and modified from (Lopez and Meier, 2010).

1.7.4.4 BIRC2 and BIRC3 in JNK signalling

Apart from NF-κB signalling, BIRC2 and BIRC3 are also implicated in MAPK signalling (Varfolomeev et al., 2012). Their involvement in MAPK signalling was demonstrated in *Birc2*- plus *Birc3*-knockout B cells. CD40-induced JNK and p38 activation was impaired in *Birc*-deficient B cells (Gardam et al.). More recently, similar results were observed with BIRC antagonist (BV6)-treated cancer cells to induce degradation of BIRC2 and BIRC3. Like B cells, BV6-treated cancer cells displayed impaired NF-κB, JNK and p38 activation in responses the members of the TNF superfamily including TNFα, CD40, TWEAK, LIGHT and TL1A (Varfolomeev et al., 2012). Furthermore, the loss of signalling through these pathways correlated with reduced expression of downstream TNF-regulated genes, demonstrating that these proteins play a role in MAPK signalling pathways.

1.7.4.5 BIRC2 and BIRC3 in tumorigenesis, innate immunity and cell cycle regulation

In addition to TNFR signalling, the E3 ligase of BIRC2 and BIRC3 are also required for ubiquitination of substrates in other signalling pathways. For example, BIRC2 was found to encourage tumorigenesis by ubiquitinating the tumour suppressor and natural c-Myc antagonist, Mad1, and targeting it for degradation (Xu et al., 2007). Notably, BIRC2 and BIRC3 also contribute to innate immunity by ubiquitinating RIP2, allowing signalling through the NOD receptors (Bertrand et al., 2009). Their importance in this pathway is demonstrated in single *Birc2*- and *Birc3*-deficient mice, which fail to mount NOD receptor responses. Moreover, BIRC3 also contributes to the pathogenesis of mucosa-associated lymphoid tissue (MALT) lymphomas in a process of chromosomal translocation with MALT1, generating a chimeric fusion protein called BIRC3/MALT1 (Zhou et al., 2005). BIRC3/MALT1 protein is critical for tumorigenesis as it induces NEMO polyubiquitination and stimulates constitutive NF-κB signalling, leading to B cell malignancy and lymphoma progression.

1.7.5 BIRC4 as a direct caspase inhibitor

One of the key roles of BIRC4 (XIAP) is direct caspase inhibition. It has been shown that ectopic expression of XIAP can prevent caspase activation and protect cells from apoptosis mediated by both the extrinsic and intrinsic apoptosis pathways (Conte et al., 2001; Deveraux et al., 1998; Deveraux et al., 1997; Takahashi et al., 1998; Trapp et al., 2003; Wilkinson et al., 2004). XIAP-deficient cells were found to be sensitive to apoptosis induced by chemotherapy drugs, TRAIL and TNFα (Chawla-Sarkar et al., 2004; Cummins et al., 2004; McManus et al., 2004; Sasaki et al., 2000; Stehlik et al., 1998). XIAP exerts its anti-apoptotic activity by directly binding to and inhibiting the activation of caspase-3, caspase-7 and caspase-9. Mutagenesis studies showed that XIAP inhibits caspase-9 activity through its BIR3 domain (Sun et al., 2000), by sequestering the catalytic activity of caspase-9 in a monomeric state (Shiozaki et al., 2003) (Figure 1.8A). Studies have shown that the linker region upstream of its BIR2 domain is responsible for caspase-3 (Riedl et al., 2001; Sun et al., 1999) and caspase-7 inhibition (Chai et al., 2001; Huang et al., 2001) (Figure 1.8B). Further, the BIR2 domain also contains a small pocket that can bind to caspase-3 (Riedl et al., 2001). However, it has also been suggested that the anti-apoptotic activity of XIAP may stem from its ability to inhibit the activity of natural BIRC antagonist, Smac/DIABLO via its BIR3 domain (Liu et al., 2000) and not from caspase-3 and caspase-9 inhibition (Silke et al., 2002). In addition to the BIR domains, the RING domain of XIAP also contributes to apoptosis inhibition. It has been reported that inhibition of the RING domain rendered fibroblast cells sensitive to TNFα-mediated apoptosis (Jin et al., 2009; Schile et al., 2008). Additionally, XIAP can also protect cells from apoptosis by acting as an ubiquitin ligase for caspase-3 in the presence of its RING domain by directly binding to it (Suzuki et al., 2001). Together, these findings demonstrate that XIAP is a potent inhibitor of apoptosis.

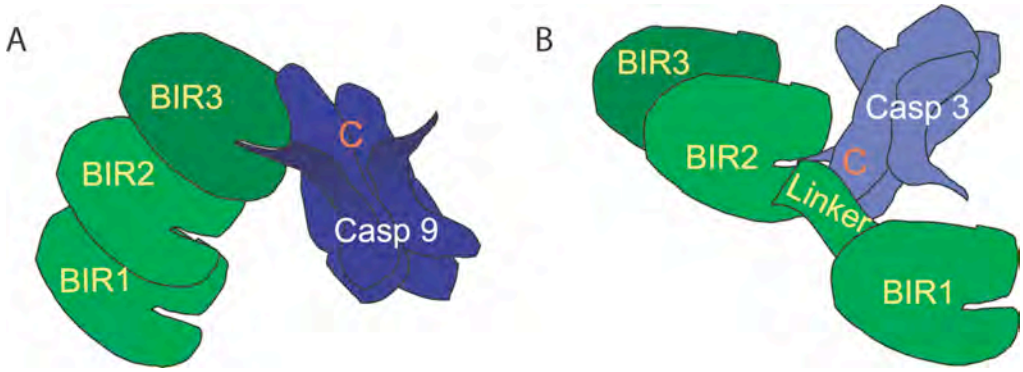


Figure 1.8. The BIR domains of BIRC4 are able to bind to and inhibit caspase activation. (**A**) Caspase-9 binding and inhibition occurs through the groove of the BIR3 domain of XIAP; C represents the catalytic cysteine residues of caspase. (**B**) Caspase-3 binding and inhibition occurs within the linker region upstream of the BIR2 domain of XIAP. This binding site is also required for caspase-7 binding. Modified from (Verhagen et al., 2001).

1.7.5.1 Other functions of BIRC4

Like BIRC2 and BIRC3, XIAP can also modulate NF-κB activation and inflammation. XIAP is thought to activate NF-κB signalling in response to DNA damage and bacterial infection (Bauler et al., 2008; Krieg et al., 2009; Schile et al., 2008). For NF-κB activation, XIAP is thought to mediate NF-κB activation by activating TAK1 and inhibiting the negative regulation on NF-κB by targeting COMMD1 for degradation. COMMD1 suppresses NF-κB transcription by repressing NF-κB-chromatin interactions. XIAP has also been reported to play a role in NOD receptor signalling by associating with RIP2 and participating in innate immune responses against *Listeria* infection. Additionally, XIAP is found to be involved in modulating JNK activation. Firstly, XIAP can activate JNK1 to protect against interleukin-1beta-converting enzyme (ICE)-induced apoptosis (Sanna et al., 1998). Secondly, XIAP is thought to prevent transforming growth factor beta 1 (TGF-β1)-mediated apoptosis by polyubiquitinating TAK1 and targeting it for degradation (Kaur et al., 2005). Suppressing TAK1 expression inhibits TGF-β1-induced JNK activation and apoptosis. These studies collectively demonstrate the diverse roles played by XIAP in mediating cell survival and innate immunity through various signalling pathways.

1.7.6 BIRC5 in foetal development, tumour survival and cell division

The main cellular processes played by Survivin (BIRC5) are in foetal development, cancer cell survival and cell division. The importance of Survivin in foetal development was demonstrated in mice. Survivin-deficient mice do not survive beyond early embryonic stage (Okada et al., 2004). A study found Survivin expression was not detected in differentiated cells but was highly up-regulated in human tumours and foetal tissues (Sah et al., 2006). In the context of cancer, Survivin expression was found to correlate with disease outcomes (Altieri, 2003; Duffy et al., 2007). Notably, Survivin is implicated as a suitable therapeutic target for cancer, because Survivin-inhibition resulted in increased sensitivity of cancer cells to chemotherapy drugs and death receptor-mediated apoptosis (Altieri, 2003). Currently, the drugs YM-155 (Astellas Pharma, Inc) and LY-2181308 (ISIS Pharmaceuticals, Eli Lilly & Company) that inhibit Survivin expression are being tested in phase I/II clinical trials for cancer treatment (http://clinicaltrials.gov). In addition, some studies have showed that Survivin also plays a crucial role in cell division (Altieri, 2006; Lens et al., 2006). This role has been clearly demonstrated in thymocytes, where *Survivin*$^{-/-}$ thymocytes show abnormal mitosis and eventually die from impaired cell division (Okada et al., 2004). This function of Survivin in mitosis largely lies with its role in directing CPC complex components during cell division. Thus, Survivin is crucial for embryonic development, tumorigenesis and cell division.

1.7.7 The role of BIRC6 in preventing cellular apoptosis

BIRC6 (BRUCE or Apollon) is a large IAP molecule (530 kDa) made up of a BIR domain and a UBC domain. The UBC domain of Apollon mediates its pro-survival function (Ren et al., 2005). Like Survivin, *Apollon*-deletion led to the death of embryos and neonates (Hao et al., 2004), suggesting its importance in mouse embryonic survival (Ren et al., 2005). The anti-apoptotic function of Apollon lies in its ability to bind to and target death inducers SMAC and caspase-9 for ubiquitination and degradation (Hao et al., 2004). In addition, Apollon is thought to promote cell survival by negatively regulating the tumour suppressor protein p53 and preventing it from activating mitochondrial apoptosis (Ren et al., 2005). These

data demonstrate that Apollon contributes to anti-apoptotic functions and is essential for the survival of mouse embryos.

1.7.8 BIRC7 in cancer cell survival

BIRC7 (or melanoma-IAP; ML-IAP) is a potent anti-apoptotic protein induced by the stimulation of death receptors and chemotherapeutic drugs. BIRC7 possesses a BIR2 domain that bears structural similarities to that of BIRC4 and is the domain responsible for conferring the anti-apoptotic functions of BIRC7 (Vucic et al., 2000). Its expression is associated with exacerbated disease outcomes (Dasgupta et al., 2010) and correlates strongly with the resistance of tumours to chemotherapeutic agents (Zhou et al., 2012). Like Survivin, BIRC7 is highly expressed in numerous cancer cells and in tissues undergoing development, but is not expressed in differentiated tissues, thus making it a suitable therapeutic target for cancer treatment.

1.7.9 BIRC8 in preventing cellular apoptosis

BIRC8 or IAP-like protein 2 (ILP-2) was cloned in 2001 (Richter et al., 2001). ILP-2 is normally only detected in the testis and is expressed by lymphoblastoid cells. ILP-2 expression is found be sufficient to protect cells from apoptosis mediated by ectopic expression of Bax, caspase-9 or Apaf-1, but has no effect on Fas-mediated apoptosis. The anti-apoptotic function of this BIRC member is thought to stem from its caspase-9 binding capacity, demonstrated in a cell-free system. However, *in vivo* examination showed that it binds weakly to caspase-9 (Shin et al., 2005). Hence the anti-apoptotic mechanisms mediated by ILP-2 require further examination.

1.8 Thesis aims

The precise molecular mechanism of inflammation on beta cell survival is not well understood. The broad objective of this thesis is to provide better understanding of beta cell biology under conditions of inflammation. To do this, the effects of cytokine signalling on beta cells function and survival are examined. A more detailed understanding of the immunopathology of T1D can aid in the appropriate design of therapies to curb the issue of islet death. This review summarises the important roles that BIRC proteins play in regulating apoptosis. They do this by mediating the degradation of death-inducing substrates such as caspases, Smac/DIABLO and MAD1. Importantly, BIRC proteins participate in diverse signalling pathways including those that regulate cell death machineries including that of TNFα-mediated canonical NF-κB signalling, JNK signalling and the non-canonical NF-κB signalling. In beta cells, BIRC proteins have been implicated to regulate cell survival and may participate in TNF signalling. It has been demonstrated that gain-of-function of XIAP and Survivin are linked to improved islet function and viability. Expectedly, up-regulation of these proteins led to better islet transplantation outcomes (Dohi et al., 2006; Emamaullee et al., 2005; Hui et al., 2005; Kim et al., 2005; Plesner et al., 2005; Plesner et al., 2010; Wu et al., 2010). Notably, Survivin plays a key role in beta cell expansion after birth (Liggins et al., 2003; Wang et al., 2010). Some studies have showed that beta cells express *Birc3*, and that *Birc3* expression is regulated by the TNFα-mediated NF-κB pathway (Liuwantara et al., 2006; Sarkar et al., 2009), while its binding partner *Birc2* has not been examined. These data suggested that in beta cells, BIRC3 may participate in the TNF-signalling feedback loop, though this idea has not been tested.

Our survey of the literature showed that BIRC proteins are key players of the TNFα-mediated NF-κB and JNK signalling pathways. Furthermore, both NF-κB and JNK signalling pathways are implicated to play an active role in determining the beta cell's fate in the pathogenesis of T1D. Thus, elucidating the function of BIRC proteins may provide valuable information on the TNF signalling network in beta cells.

The aims of this thesis are to:

(1) Determine the expression of BIRC family genes in response to inflammatory cytokines implicated in T1D;

(2) Study how BIRC gene expression is regulated in the presence of inflammatory cytokines in pancreatic beta cells;

(3) Elucidate the function of BIRC proteins in pancreatic beta cells:

   a) *By ectopic expression of Birc3 in pancreatic beta cells and*
   b) *By examining the requirements for BIRC3 and BIRC2 in TNF-signalling in the pancreatic beta cells of BIRC-knockout mice.*

2 Materials and methods

2.1 Animal work

2.1.1 Mouse lines

BALB/c, C57bl/6 and CBA WT mice were purchased from Australian BioResources (ABR) (Mossvale, NSW, Australia). All other mice were maintained as an inbred C57bl/6 genetic background at the ABR and experimental procedures were performed at the ages of between 8 to 16 weeks. *Birc2*$^{-/-}$ and *Birc3*$^{-/-}$ mice were a kind gift from Professor David Vaux (Walter and Eliza Hall Institute, Parkville, VIC, Australia) and Holly Carter helped with practical matters concerning these mice. *Birc3*$^{-/-}$ mice containing *Birc2*$^{loxP/lox\text{-}P}$ were crossed with either HIP-*Cre* mice (Kind gift from A/Professor Robyn Slattery, Monash University, Melbourne, VIC, Australia) or RIP-*Cre* mice [Cre driven by the rat insulin promoter; Strain B6.Cg-Tg(Ins2-cre)25Mgn/J; Jax mice, Bar Harbour, ME, USA] to generate beta cell specific double knockout *Birc3*$^{-/-}$*Birc2*Δβ-cell mice. Details of the genetic maps of *Birc2*$^{-/-}$ and *Birc3*$^{-/-}$ mice are provided in Chapter 4 and Chapter 5 of this thesis. The breeding strategies to generate *Birc3*$^{-/-}$*Birc2*Δβ-cell using HIP-*Cre* and RIP-*Cre* mice are described in chapter 4. Mice were maintained as heterozygous breeding pairs, so that WT littermates can be used as controls for experiments. All animal handling and experiment procedures were carried out according to the Australian Code of Practice for Care and Use of Animals for Scientific Purposes.

2.1.2 Intraperitoneal glucose tolerance test

Random blood sugar levels were determined using FreeStyle Lite (Abbott Laboratories. Abbott Park, Illinois, U.S.A.) for 12-week old, male, non-fasted mice. Approximately 0.3 µl of blood were collected from the tail vein for this measurement. For intraperitoneal glucose tolerance tests (IP-GTTs), 12-week old male mice were fasted overnight for ~16 h and were given free access to water. The next day, mice were weighed and fasting blood glucose levels were measured from the tail vein. Subsequently, mice were injected intraperitonally with 20%

dextrose (w/v; Sigma-Aldrich) to a final concentration of 2 g glucose per kilogram of body weight (2g/kg). Blood glucose levels were measured from the tail veins at 15, 30, 60, 90 and 120 min post glucose administration and the final readings were plotted as blood glucose concentration over time.

2.1.3 Isolation and primary culture of mouse islets

Mouse primary islets were isolated by perfusion of distended pancreas with 3 mL of 25 mg/mL Collagenase I/II Blend Research Grade (Roche, Indianapolis, IN, USA) in M199 media containing 4.16 mM $NaHCO_3$ (Sigma-Aldrich, St Louis, MO, USA) using a 30G needle. A maximum of 3 pancreata were placed in one 50 mL falcon tube on ice for processing. The tissue was then digested at 37 °C for 13 min. Tubes were placed on ice immediately and 30 mL of islet isolation media containing M199 + 10% bovine calf serum (BCS) (HyClone; Thermoscientific, Waltham, MA, USA) was added to stop digestion. Tubes were then shaken hard, vortexed to dislodge the acinar tissue from the islets and centrifuged at 314 rpm for 3 min at 4 °C. The supernatant was then discarded. This process was repeated twice and the resultant pellet was then re-suspended in 20 mL islet isolation media. To remove undigested pancreatic tissue, the mixture was then passed through a 425 micron sieve (US standard sieve series, A.S.T.E. E-11 specifications dual MFG, Co., Chicago, IL, USA). The emptied tube was then rinsed with 30 mL of islet isolation media and passed through the sieve. The sieved solution was then spun down at 314 rpm for 3 min at 4 °C and the supernatant was discarded. Tubes were placed upside down on bench coat to drain off excess supernatant. The pellet was then resuspended in 10 mL of Ficoll-Paque Plus (GE Heathcare, Rydalmere, NSW, Australia) by vortexing. Additional 10 mL of Ficoll-Paque Plus was then added to rinse the sides off the 50 mL tube. Next, 10 mL of serum-free M199 (Sigma-Aldrich) was added carefully down the sides of the 50 mL tube to overlay the top of the ficoll layer. The tubes were then spun down at 1612 rpm for 22 mins at 4 °C without rotor acceleration or deceleration. Intact islets would be found in the interface between Ficoll and serum-free M199. For maximum islet recovery, the supernatant containing whole islets was tipped into the fresh 50 mL falcon tube without disturbing the cell pellet. This supernatant was then diluted into 100 mL of islet

isolation media and centrifuged at 314 rpm, 4 °C for 3 min. The supernatant was then discarded and isolated islets were counted. For *in vitro* experiments, approximately 100-150 islets were resuspended in 1 mL of primary islet culture media made up of Roswell Park Memorial Institute (RPMI) medium containing 20% Fetal Bovine Serum (FBS), 2 mM L-glutamine and 12.5 mM HEPES buffer from Gibco-Invitrogen in 24-well tissue culture plates (Corning Costar). Islets were rested overnight at 37 °C in 5% CO_2 and 95% air overnight prior to experimentation.

2.1.4 Islet transplantation

In some cases, isolated islets were used immediately for islet transplantation. Five days prior to operation, recipient mice were injected with 300 mg/kg of streptozotocin (STZ) (Sigma-Aldrich) in 10 mM citrate buffer (pH 4.2) to induce diabetes. Blood glucose levels were monitored using the FreeStyle Lite glucometer (Abbott Laboratories, Abbott Park, Illinois, U.S.A). Mice were deemed diabetic if their blood glucose level was ≥16 mM for two consecutive days. On the day of transplantation, approximately 150-200 islets isolated using the above protocol were transplanted from donor C57BL/6 (H-2^b) mice into STZ-induced CBA (H-2^k) diabetic mice (allogeneic model: three-donor mice per recipient). For transplantation, a left flank incision was made on the recipient mouse to access the kidney. A small nick was then made in the kidney capsule at the inferior renal pole and donor islets were transplanted under kidney capsule of the recipient mouse. Graft function was examined by monitoring the blood glucose level starting from 24 hr post-transplantation.

2.2 Genotyping

2.2.1 DNA extraction from tail clips

Genomic DNA (gDNA) for genotyping was obtained by digesting ~2 mm tail tips in 200 µL of 1X isolation buffer [10X isolation buffer recipe - 670 mM Tris, pH 8.8, 166 mM $(NH_4)_2SO_4$, 65 mM $MgCl^2$, 10% β-Mercaptoethanol (w/w), 5% Triton X-

100 (w/w) and 23 mL of nuclease-free water; Sigma-Aldrich] containing 5 µL of proteinase K (50 mg/ml; Promega, Madison, WI, USA) and incubated at 65 °C overnight. Tails are placed in -20 °C for long-term storage.

2.2.2 PCR for genotyping

To screen mouse genotypes, 2 µL of gDNA was added to 12.5 µL of GoTaq® Hot Start Green Master Mix, 2X (Promega) with 0.5 µL of 10 µM sense (F) and 0.5 µL of 10 µM anti-sense (R) primers and made up to 25 µL with nuclease-free H_20 in 8-well PCR strips (ABgene, Epsom, Surrey, UK). PCR cycling conditions were set according to manufacturer's guide. The number of cycles and annealing temperatures are dependent on specific primer sets and these are described below. PCR was performed on the PTC-200 DNA Engine Cycler (Biorad Laboratories, Gladesville, NSW, Australia).

2.2.2.1 Birc3$^{-/-}$ mice

Table 2-1 Genotyping primers for *Birc3*$^{-/-}$ mice

Primer	Sequence (5'-3')
***Birc3* F**	CCCTCAGGGTGTGTGGCTTA
***Birc3* R**	TGTGCATGGCTTCTGGTCGG
Flp'd R	CACTGGCTGTTCTTTCAGAGA

Table 2-2 Expected results for *Birc3*$^{-/-}$ mice genotyping; numbers refer to expected product sizes

Primer combination	Wildtype (WT)	Heterozygous (*Birc3*$^{+/-}$)	Homozygous (*Birc3*$^{-/-}$)
***Birc3* F + *Birc3* R**	275 bp	275 bp	No band
***Birc3* F + Flp'd R**	No band	231 bp	231 bp

Specific PCR conditions:

*Birc*3 F + *Birc*3 R = 30 cycles, annealing temperature of 50 °C for 20 s

*Birc*3 F + Flp'd R = 26 cycles, annealing temperature of 55 °C for 20 s

2.2.2.2 Birc2$^{-/-}$ mice

Table 2-3 Genotyping primers for *Birc2*$^{-/-}$ mice

Primer	Sequence (5'-3')
***Birc2* F**	TAGACCCTTTCTAAGTAGACTGGTT
***Birc2* R**	TTATAGAAATGTTCAGATGTC
Cre'd R	TCAATCCTCCATGCCACAATAAACAT

Table 2-4 Expected results for *Birc2*$^{-/-}$ mice genotyping; numbers refer to expected product sizes.

Primer combination	Wildtype (WT)	Heterozygous (*Birc2*$^{+/-}$)	Homozygous (*Birc2*$^{-/-}$)
***Birc2* F + *Birc2* R**	231 bp	231 bp	No band
***Birc2* F + Cre'd R**	No band	177 bp	177 bp

Specific PCR conditions:

*Birc*2 F + *Birc*2 R = 30 cycles, annealing temperature of 50 °C for 20 s

*Birc*2 F + Cre'd R = 26 cycles, annealing temperature of 55 °C for 20 s

2.2.2.3 Birc3$^{-/-}$Birc2Δβ-cell

Mice were kept as homozygous for *Birc3*$^{-/-}$*Birc3* $^{loxP/lox\text{-}P}$ (*Birc3*$^{-/-}$) and crossed with either HIP-*Cre* or RIP-*Cre* mice. The *Cre* lines were kept as heterozygous. The genotypes for these mice were tested as described.

Table 2-5 Genotyping primers for *Birc3*$^{-/-}$*Birc2Δβ-cell* mice

Primer	Sequence (5'-3')
***Cre* F**	TGCCAAGAACCTGATGGACA
***Cre* R**	TGCTAACCAGCATTTTCGTTCTGC
Insulin F	CGAGCTCGAGCCTGCCTATCTTTCAGGTC
Insulin R	CGGGATCCTAGTTGCAGTAGTTCTCAAG

Table 2-6 Expected results for *Birc3*$^{-/-}$*Birc2Δβ-cell* mice genotyping; numbers refer to expected product sizes.

Primer combination	Wildtype (WT)	*Birc2Birc3* double knockout (DKO)
***Cre* F + *Cre* R**	No band	600 bp
***Insulin* F + *Insulin* R**	400 bp	400 bp

Specific PCR conditions:

For both primer sets = 40 cycles, annealing temperature of 62 °C for 30 s.

2.2.3 Agarose gel electrophoresis to visualise PCR products

Amplified cDNA fragments (<1kb) were separated using 1.5-2% (w/v) agarose (Probiogen Biochemicals, Quantum Scientific, QLD, Australia) dissolved in TBE (Tris/Borate/EDTA) buffer. A suitable DNA dye such as SYBR Safe DNA gel stain (Invitrogen, Carlsbad, CA, USA) or ethidium bromide (Sigma-Aldrich) was added to the TBE running buffer in order to visualize PCR product bands under UV light. In some cases, PCR product was mixed with a loading dye (Promega, Maddison, WI, USA) before loading onto gel.

2.3 Cell culture conditions

2.3.1 Cell culture of MIN6 cells

MIN6 cells were generated by Miyazaki *et. al* (Miyazaki et al., 1990). The cells are derived from the pancreatic beta cells of transgenic mice, MIN6 cells are immortalised by transduction with the T antigen gene of simian virus 40 (SV40). MIN6 cells has been well characterised on the basis of glucose metabolism and glucose-stimulated insulin secretion compared to mouse islets (Ishihara et al., 1993). Cells were maintained in Dulbecco's modified Eagle's medium (DMEM; Gibco-Invitrogen, Carlsbad, CA, USA) supplemented with 10% FBS, 2 mM L-glutamine and 12.5 mM HEPES and incubated at 37 °C in 5% CO_2 and 95% air. Passage 32 to 39 cells were used for experiments.

2.3.2 Cell culture of β-TC_3 cells

β-TC_3 cells are derived from insulinoma cells that arise in the pancreatic beta cells of transgenic mice expressing the SV40 T antigen under the control of the rat insulin II promoter (RIP) (Efrat et al., 1988; Hanahan, 1985). These cells were cultured in RPMI media containing 10% FCS, 4 mM L-glutamine and incubated at 37 °C in 5% CO_2 and 95% air.

2.3.3 Cell culture of NIH-3T3 cells

The mouse embryonic fibroblast cell line established from Swiss mice, NIH-3T3 (Todaro and Green, 1963) was kindly provided by Louis Tsai (The Garvan Institute of Medical Institute, Darlinghurst, NSW, Australia). These cells were maintained in DMEM containing 10% FCS and 4 mM L-glutamine and incubated at 37 °C in 5% CO_2 and 95% air.

2.3.4 Cell culture of 2F2B cells

The mouse 2F2B endothelial cell line (American Tissue Culture Collection, Bethesda, MD, USA) was a kind gift of Dr Lindsay Wu (The Garvan Institute of Medical Research, NSW, Australia). Cells were maintained in RPMI containing 10% FCS and 4 mM L-glutamine and incubated at 37 °C in 5% CO_2 and 95% air.

2.3.5 Cell culture of HEK 293 cells

Human Embryonic Kidney 293 (HEK 293) cells were established from transformed cultures of normal human embryonic kidney cells with sheared adenovirus 5 DNA (Graham et al., 1977). These cells were used for amplification of recombinant adenoviruses. Cells were maintained in DMEM containing 10% FCS, 4 mM L-glutamine, 100 U/mL Penicillin (Gibco-Invitrogen) and 100 μg/mL Streptomycin (Gibco-Invitrogen) and incubated at 37 °C in 5% CO_2 and 95% air.

2.4 Cytokine stimulations

Cultured islets, MIN6, NIH-3T3 and 2F2B cells were stimulated with either 200 U/ mL of recombinant human TNFα (R&D Systems, Minneapolis, MN; 210-TA), mouse IL-1β (R&D Systems; 401-ML) or rat IFNγ (R&D Systems; 585-IF) at times indicated. Cells were then prepared for RNA isolation for quantitative real time PCR (qRT-PCR) or lysed whole cell protein lysates for western blot analysis. For most of the experiments unless stated otherwise, cytokine stimulation for RNA quantification were for 4 h and for reporter assays for 8 h to allow accumulation of the luciferase protein.

2.5 Molecular inhibitors

In some cases, islets or MIN6 cells were pre-treated with pharmacological inhibitors: Actinomycin D (AcD), SP600125, pyrrolidine dithiocarbamate (PDTC; Sigma-Aldrich, St Louis, MO, USA) and SB203580 (Cell Signalling Technology, Beverly, MA, USA) in cultured media and incubated at 37°C for 1 hr prior to cytokine stimulation for 4 h. Cells were then lysed for RNA or protein analysis.

For epigenetic screening, MIN6 cells were treated with 25-200 ng/mL of HDAC inhibitor, trichostatin A (TSA), overnight (~16 h) at 37 °C prior to TNFα stimulation for 4 h. Subsequently, cells were stimulated with TNFα for 4 h and lysed for RNA isolation and gene expression analysis.

2.6 Molecular cloning of genetic material into plasmids

Molecular cloning was used to create tools for this project:

1. Cloning of the 5' untranslated region (5'-UTR) of *Birc3* from gDNA to create an assay-based system to study the promoter region of the gene.
2. Cloning of the coding sequence (CDS) of *Birc3* from mRNA into an over-expression plasmid construct to facilliate *in vitro* study of its function.

2.6.1 Cloning of the Birc3 5'-UTR

2.6.1.1 DNA precipitation

Genomic DNA was extracted from tail tips obtained from BALB/c mice tails and digested as described in Section 2.2.1. Protein was pelleted by centrifugation at 13,000 rpm for 10 min and the supernatant was transferred into a fresh tube. DNA was precipitated in 2.5M final concentration of sodium acetate (pH 7.0) and 2 vol of cold absolute ethanol and incubated at -80 °C overnight or for 2 h on dry ice. Precipitated DNA was then recovered by centrifugation at 13,000 rpm for 10 min at 4 °C. The supernatant was decanted carefully. The resultant pellet was washed with cold 70% (w/v) ethanol and centrifuged again at 13,000 rpm for 10 min at 4 °C. The ethanol was then removed carefully, DNA pellet was left to air dry for 5-10 min and resuspended in 30 – 50 µL nuclease free H_2O.

2.6.1.2 Amplication of the Birc3 5'-UTR by PCR

For promoter cloning, putative transcription factor binding sites in the *Birc3* proximal promoter were identified using PROMO3.0 (Farre et al., 2003). The 5'-UTR of mouse *Birc3* was amplified from gDNA extracted above using the primers to target the region containing transcription-factor-binding-sites of interest (Table 2-7). The PCR reaction was performed using the KOD Hot Start DNA Polymerase (Merck KGaA, Darmstadt, Germany) according to the protocol provided. Briefly, 1 uL of gDNA was added to the PCR mix and made up to a final volume of 50 µL using nuclease-free H_2O. Cycling conditions for the primer pair were: 94 °C for 2 min, 94 °C for 20 s, annealing temperature of 58 °C for 10 seconds and 70 °C for 10 seconds for 40 cycles. The resultant PCR product was purified using the CloneWell system (Invitrogen) prior to ligation into the target plasmid.

Table 2-7. Primers for *Birc3* 5'-UTR cloning

Primer	**Sequence (5'-3')**
Forward	CTTAAGGCGGAACTCACAGG
Reverse	TGGCGCGATACCTTTAAATC

2.6.1.3 Purification of PCR product using the CloneWell system

Gel purification of PCR products and other DNA material such as products of restriction enzyme digests involves the separation of the product of interest by their base pair length during electrophoresis. This can be done by two methods: (1) tradition gel purification method involving DNA material being separated on an agarose gel using electrophoresis, the desired band is excised from the gel and extracted using commercial kits such as Gel Extraction Kit (Qiagen, Valencia, CA, USA) or (2) an alternative commercial electrophoresis system designed for gel purification known as the E-Gel® CloneWell™ system (Invitrogen). This system uses the SYBR Safe™ DNA Gel Stain 0.8 % agarose gel, ideal for separating bands of <5 kb. The samples are loaded onto the gel and the separated band of interest is captured at the bottom well.

2.6.1.4 Addition of 3'-A overhangs and ligation into pCRII TOPO vector

The purified promoter PCR product was then cloned into a TA overhang vector (pCRII-TOPO TA vector; Invitrogen; Figure 2.1) before being subcloned into the luciferase reporter (pGL3 basic vector luciferase reporter; Promega; Figure 2.2). To facilitate ligation into pCRII TOPO vector (Figure 2.1), 3'-A overhangs were added to the purified *Birc3*-5'-UTR PCR product using GoTaq Flexi DNA Polymerase (Promega). The A'-overhang reaction was set up using 4 μL of purified *Birc3*-5'-UTR PCR product, 4 μL 5X Colourless GoTaq Flexi Buffer, 2.5 mM MgSO4, 0.2 mM dATP, 5U Promega GoTaq Polymerase and was made up to 20 μL with nuclease-free H_2O. This mixture was incubated at 72 °C for 15 min. The resultant 3'-A-tailed product was then ligated into the pCRII TOPO TA vector (Invitrogen). Briefly, 4 μL of purified 3'-A overhang PCR product was added to the pCRII-TOPO ligation mixture containing 1 μL of TOPO vector, 1.2 M of NaCl and 0.06 M of $MgCl_2$. For each cloning reaction an insertless control reaction was performed in parallel. The mixture was mixed gently and incubated at room temperature for 30 mins before transformation into Top 10 competent *E. coli* cells.

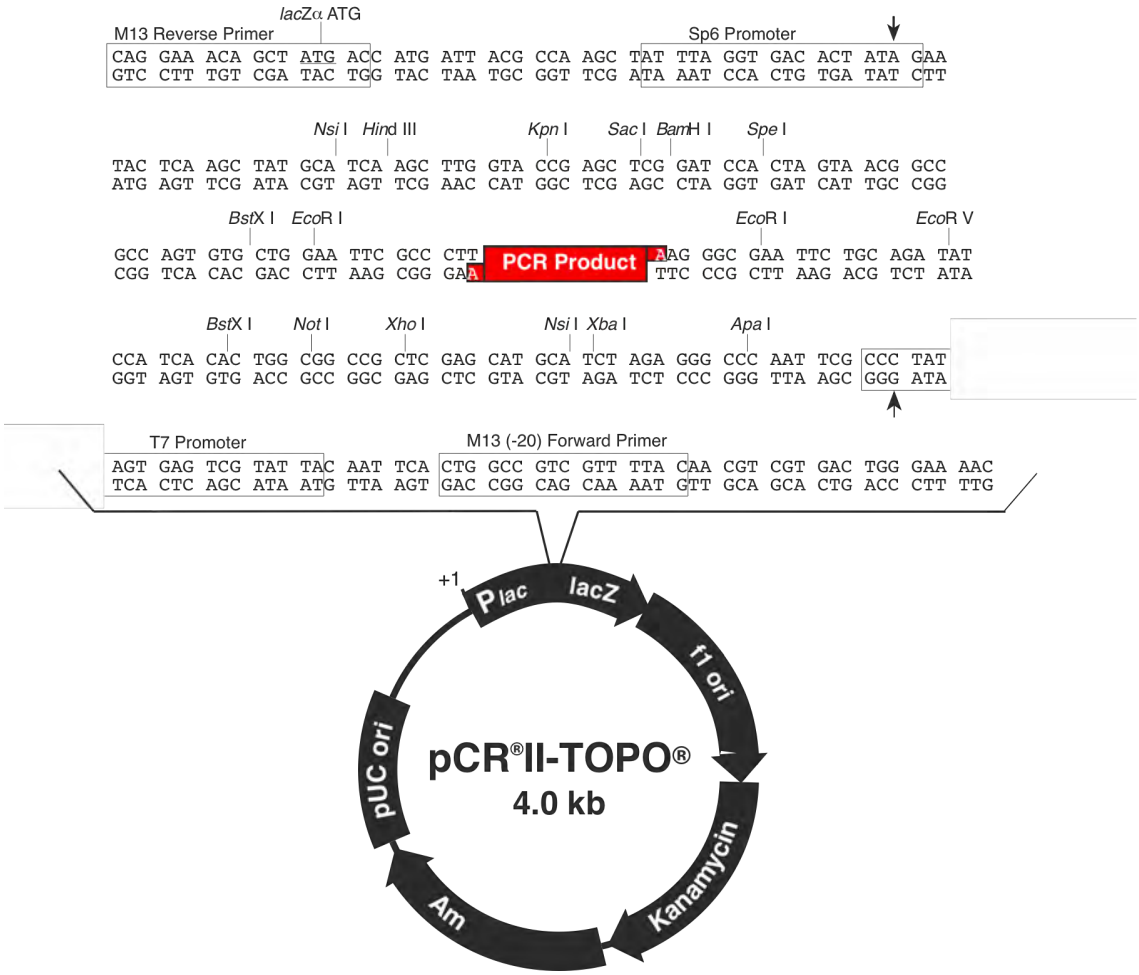


Figure 2.1. The plasmid map of pCRII-TOPO TA cloning vector. The A'-overhangs of PCR product (coloured in red) was ligated with the T'-overhangs present in vector.

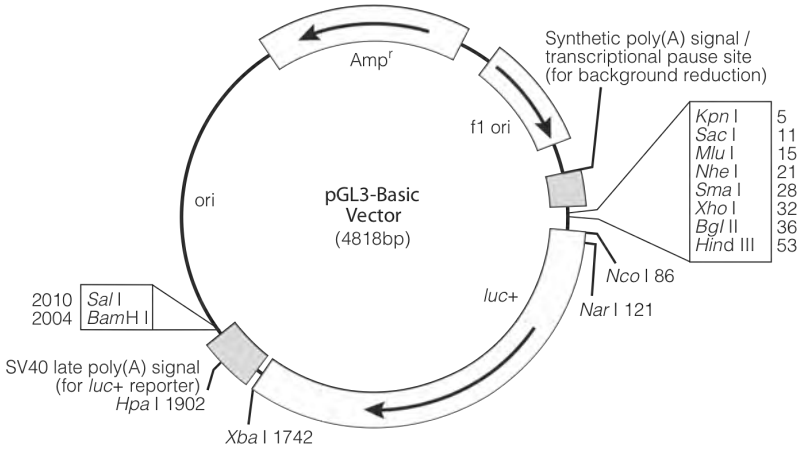


Figure 2.2. The plasmid map of pGL3-Basic Vector by Promega. The 5'-UTR of *Birc3* was inserted into the multiple cloning site between the Kpn I and Xho I restriction enzyme sites.

2.6.1.5 Transformation of plasmid into chemically competent E. coli

pCRII-TOPO TA cloned products were transformed into the One Shot TOP10 (Invitrogen) chemically competent *E. coli* cells. To perform transformation, 2µL of pCRII-TOPO vector containing the purified PCR product was added and mixed gently. The mixture was then placed on ice for 30 minutes and then heat shocked at 42°C for exactly 30 seconds in a water bath. Immediately, 250 µL of S.O.C media was added to the reaction and was left in a shaking incubator at 225 rpm, at 37 °C for 1 h. The bacterial mixture was then plated at different volumes onto Luria broth (LB) agar plates containing 100 µg/mL carbenicillin (Sigma-Aldrich) and 1.6 mg of X-Gal for selection. The LB plates were then incubated at 37 °C for 16 h.

2.6.1.6 Analyzing transformants

Bacterial colonies were then picked and screened for pCRII-TOPO vector that contain *Birc3*-5'-UTR PCR insert by PCR. To do this, white colonies were picked from LB plate and inoculated into PCR reaction containing the primers that were used to clone the *Birc3* promoter (Table 2-7). The PCR reactions were set up using GoTaq® Hot Start Green Master Mix, 2X (Promega) and visualised on 1.5% agarose/TBE gels as described above in the PCR for genotyping and agarose gel electrophoresis sections respectively. All positive PCR products were inoculated into liquid LB media with 100 µg/mL carbenicillin, grown overnight at 37 °C and used for plasmid extraction using the PureYield™ Plasmid Miniprep System (Promega). Restriction digest by EcoR I was used to confirm the size of the insert. Inserts with the right size were then sent for gene sequencing to confirm the sequence of the *Birc3*-5'-UTR PCR and the direction that the product was inserted into pCRII-TOPO vector.

2.6.1.7 Digesting and dephosphorylating the final plasmid for ligation

To successfully ligate a DNA fragment into a plasmid of interest, it must be digested with restriction enzymes that are compatible with the insert. Restriction digests is performed in specific buffers and conditions that are suitable for the specific enzymes. After restriction digest, the linearised plasmid must be dephosphorylated to prevent self-ligation. This is simply performed by the

enzymatic removal of the 5'-phosphate groups on the "open" ends of the vector. For our experiment, the pGL3 basic vector was linearised by sequential digestion of Kpn I and Xho I restriction enzymes and dephosphorylated by incubation at 65 °C for 5 min with antartic phosphatase (New England Biolabs, Ipswich, MA, USA) and buffer included in kit.

2.6.1.8 Ligation of purified DNA fragment into plasmid

The *Birc3*-5'-UTR PCR in pCRII-TOPO vector was excised out of the vector using the restriction sites that compliment the sites present in the multiple cloning site (MCS) of the final vector of interest. For this we used the Kpn I and Xho I restriction enzymes sites (New England Biolabs) to allow for directional cloning of the *Birc3* promoter fragment into the MCS of the pGL3 basic vector (Figure 2.2). The insert was CloneWell purified and the molar ratio of insert to vector for ligation is determined using this formula:

$$\frac{\text{size of insert (kb)} \times \text{50 ng of plasmid}}{\text{size of vector (kb)}} = \text{ng of insert}$$

Usually, for inserts <3 kb in size, a ratio of 3:1 (insert:vector) is a good starting ratio. While inserts >3 kb, a start with ratio of 2:1 is recommended. For our vector, we started with a ratio of 3:1 but did not achieve any successful ligation. Through optimization and proper controls, we found that the molar ratio of 5:1 allowed successful ligation of the *Birc3* 5'-UTR insert into the pGL3 basic vector. The ligation was set up using T4 DNA ligase (New England Biolabs) according to the manufacturers protocol. Ligation reactions were incubated in a 16 °C waterbath overnight. The resultant ligated DNA were then transformed into TOP10 competent *E. coli* and analysed as described in section 2.6.1.5. The *Birc3* 5'-UTR insert in pGL3 vector was confirmed by gene sequencing. This vector is called pGL3-*Birc3* in our results chapter.

2.6.2 Cloning of the coding region of *Birc3*

The cloning of the coding region of *Birc3* were similar to the steps used for cloning the *5'-UTR* of *Birc3.* In short, the coding region of *Birc3* was cloned using cDNA from cytokine-stimulated mouse islets as a template. The PCR was performed using the Expand High Fidelity PCR system (Roche) and primers that were design to amplify the transcription start and stop codon of *Birc3* (Table 2-8). Primer designs were made using the MacVector software (MacVector, Inc, Cary, NC, USA). The Expand High Fidelity PCR system generated PCR products with sticky 3'-A overhangs without having to perform a separate reaction to add the A-tails. A gradient PCR was set up to determine the best annealing temperature for this primer set. The annealing temperatures for gradient PCR was set between 57 °C to 70 °C, using the primer melting temperature (in this case 62 °C) as the median temperature setting. To confirm size, 5 µl of PCR products were ran on 0.7% agarose gel at 60V for 2 h. Once the size of the product was checked, wells with bands of the right size were combined, precipitated to concentrate DNA volume and CloneWell purified.

The resultant purified PCR product was then ligated into pCRII-TOPO vector and the sequence was confirmed by analyzing restriction digested inserts using agarose gel electrophoresis to confirm product size. At this stage, selected vectors with inserts are then sent for gene sequencing. The vector with the right insert was then selected for a second PCR. The second PCR was set up using the Expand High Fidelity PCR system with a forward primer that includes a 6X his-tag and Bgl II restriction enzyme sites upstream of the *Birc3* transcription start site (Table 2-9). In addition, the reverse primers adds Sal I restriction enzyme sites to *Birc3* (Table 2-9). The PCR product was CloneWell purified and then ligated into pCRII-TOPO vector. Positive clones were confirmed by gene sequencing and were selected for the final subcloning into the pIRES2-eGFP vector (Clontech Laboratories, Inc, Mountain View, CA, USA; Figure 2.3). pIRES2-eGFP were linearised by restriction digest at the Bgl II and Sal I sites (New England Biolabs) and dephosphorylated. The PCR insert in pCRII-TOPO vector was excised using Bgl II and Sal I restriction enzymes. The final insert containing the CDS of *Birc3* + 6x His-tag on the 5' end was

then ligated into pIRES2-eGFP vector and its sequence was confirmed by sequencing. This vector is called the pIRES2-eGFP-*Birc3* (eGFP-*Birc3*) in our results chapter.

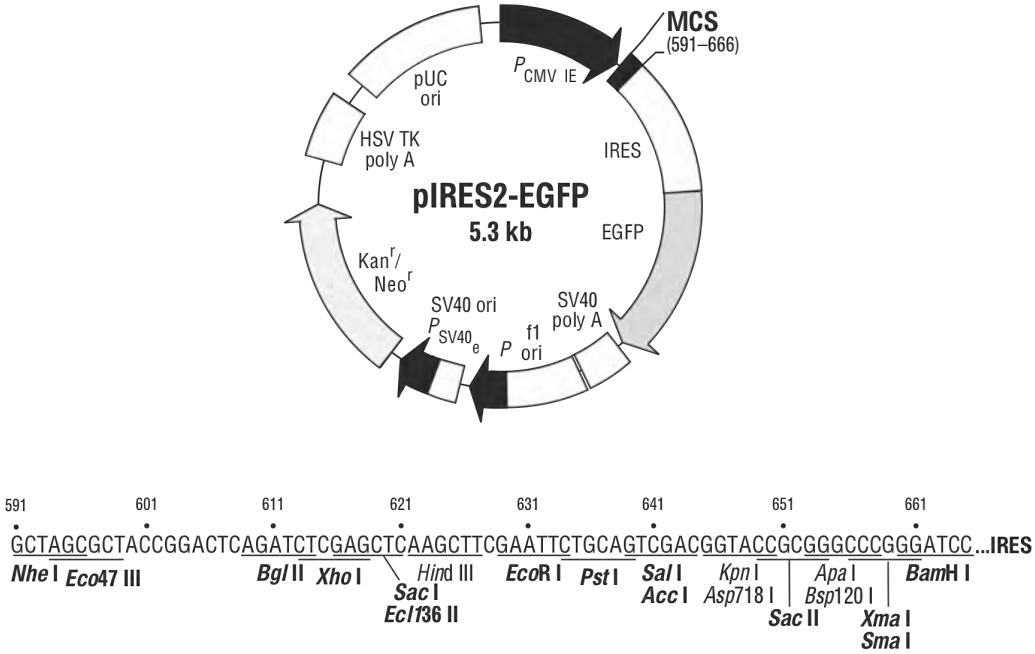


Figure 2.3. The plasmid map of pIRES2-eGFP vector by Clontech. The entire coding sequence of *Birc3* was inserted into the multiple cloning site using Bgl II and Sal I restriction enzyme sites.

Table 2-8. First set of primers used for cloning the CDS of *Birc3* from mouse islets

Primer	**Sequence (5'-3')**
mBirc3.START F	CATGAACATGGTTCAAGACAGCG
mBirc3.STOP R	CTTGTTCAGGAGAGAAATGTGCG

Table 2-9. Second set of primers used for cloning the CDS of *Birc3* with restriction enzyme sites and 6X his-tag sequences added into them.

Primer	**Sequence (5'-3')**
mBIRC3(AA2).BglII.His	ATTAAGATCGCCGCCACCATGCATCACCATCACCATCACAA
Forward	CATGGTTCAAGACAGCGCC
mBIRC3.SalI Reverse	AATGTCGACTCAGGAGAGAAATGTGCGCACTG

2.6.3 Acquired and purchased plasmids

The *A20*.Luc luciferase reporter constructs were a kind gift of Professor Rivka Dikstein (The Weizmann Institute of Science, Rehovot, Israel) (Ainbinder et al., 2002) and the expression plasmids encoding *RelA/p65* and *Traf2* were kind gifts from the Beth Israel Deaconess Medical Center - Harvard Medical School (Boston, MA, USA) (Anrather et al., 1997). The NF-κB.Luc and pRSV-β-galactosidase reporter were purchased from Promega and were previously described (Liuwantara et al., 2006).

2.6.4 Plasmids amplification

For amplification, plasmids were transformed into TOP-10 chemically competent *E. coli* cells and grown in 150 mL cultures of LB broth. Plasmids were purified using HiSpeed Plasmid Maxi Kit (Qiagen) according to manufacterer's protocol. For longterm storage and future use of plasmids without having to perform transformation reactions, 500 µL of the transformed plasmid broth were added with glycerol to a final volume of 25 % glycerol and stored in -80 °C.

2.7 Transient transfection of cell lines

2.7.1 Transfection of β-TC_3 cells

The transfection of β-TC_3 cells with *A20*.Luc and *RelA/p65* were performed as previously described (Grey et al., 1999; Liuwantara et al., 2006). Briefly, $1.5x10^6$ of β-TC_3 cells were plated into 6-well tissue culture plates and cultured overnight for cells to attach. Transient transfections were performed using Lipofectamine 2000 reagent (Gibco-Invitrogen) with a total of 1 µg DNA per well. Transfections were set up according to protocol provided by Invitrogen. Alternate wells were then stimulated with TNFα and incubated for 8 h at 37 °C. Luciferase activity (Luciferase Assay System; Promega) were normalised to β-galactosidase activity (Galacto-Star; Applied Biosystems, Bedford, MA, USA) for relative luciferase activity (RLA). Luminosity was assayed using the FLUOstar OPTIMA luminometer (BMG Labtech GmbH, Allmendgruen, Ortenberg, Germany).

To test the effects of increased *RelA/p65* on the endogenous gene expression, β-TC_3 cells were transfected with 0.2 μg of *RelA/p65* and made up to 1 μg with pcDNA3.1+ empty vector (Invitrogen). Cells were washed in phosphate buffer saline (PBS; 136mM NaCl, 2.68mM KCl, 10.14mM Na_2HPO_4, 1.76mM KH_2PO_4) twice and lysed in buffer RLT for RNA isolation.

2.7.2 Transfection of MIN6 cells

For transfection of MIN6 cells with pGL3-*Birc3,* $1x10^6$ of MIN6 cells were plated into 6-well tissue culture plates and cultured overnight for cells to attach. Transient transfections were performed using 7.5 μl of Lipofectamine 2000 reagent with a total of 3 μg DNA per well. Transfections were set up according to the protocol provided by Invitrogen. To test the *Birc3* reporter activity, MIN6 cells were transfected with 0.1 μg of pRSV-β-galactosidase reporter to account for transfection efficiency, 2 μg of pGL3-*Birc3* and made up to 3 μg with the pcDNA3.1+ empty vector. Alternate wells were then stimulated with either TNFα, IL-1β, IFNγ or a mixture of all three cytokines and incubated for 6 h at 37 °C or at indicated times. Luciferase activity and β-galactosidase activity were analysed and calculated as mentioned above.

To study the effects of increased *Birc3* on NF-κB activity in beta cells, MIN6 cells were transfected with 2 μg of NF-κB.Luc, 0.3-0.6 μg of eGFP-EV or eGFP-*Birc3* (cloning was described above) and made up to 3 μg using pcDNA3.1+ vector. Subsequently, transfected cells were either left untreated or stimulated with TNFα for 4 h. Transfected cells were assayed for luciferase and β-galactosidase activity as described. In some cases, MIN6 cells were transfected with 0.3-0.6 μg of eGFP-EV or eGFP-*Birc3* and made up to 3 μg using pcDNA3.1+. The transfected cells were then lysed in buffer RLT for RNA isolation and gene expression analysis.

2.7.3 Transfection of NIH-3T3 cells

Transfection of NIH-3T3 was also performed using Lipofectamine 2000. A day before transfection, $0.75\ x10^6$ cells were plated onto 6-well plates to allow

attachment. To study the effects of increased *Birc3* on NF-κB activity, NIH-3T3 cells were transfected with 2 μg of NF-κB.Luc and either 1 μg of EV or eGFP-*Birc3*. To study the effects of increased TRAF2 on NF-κB activity, NIH-3T3 cells were transfected with 2 μg of NF-κB.Luc, 0.9 μg of TRAF2 or pcDNA3.1+ empty vector and made up to 3 μg with pcDNA3.1+. Transfected cells were then stimulated to TNFα for 8 h. Cells were assayed for luciferase and β-galactosidase activity as described above. In some cases, NIH-3T3 cells were transfected with 1 μg of eGFP-EV or eGFP-*Birc3*, 0.9 μg pcDNA3.1+ empty vector or TRAF2 and were made up to 3 μg with pcDNA3.1+ and processed for gene expression analysis.

2.8 Adenovirus transductions

Prior to experiments, recombinant adenoviruses (rADs) were propagated by infecting HEK293 cells in six T175 vented flasks (Corning CoStar). Cells were lysed and adenoviruses were extracted using the Adenopure Kit (PureSyn Inc, Malvern, PA, USA) according to instructions provided. The resultant viruses were titrated and quantified in HEK293 cells using the Adeno-X Rapid Titer Kit (Clontech) according to manufacturer's instructions. For our experiments, MIN6 were transduced with either a rAD expressing GFP (rAD-GFP) as a control or with rAD-IκBα. The rAD-GFP was a kind gift from Beth Israel Harvard Medical School, Boston, MA, USA. About 1 x10^6 MIN6 cells were re-suspended in 1 mL of serum-free DMEM containing 50 mM HEPES and infected with adenovirus at a multiplicity of infection (MOI) of 100 viral particles per cell and incubated at 37 °C for 2 h. After which, 1 mL of DMEM containing 20% FCS and 8 mM L-glutamine was added to each well and incubated overnight.

2.9 Determination of transfection and transduction efficiency

To determine transfection or transduction efficiency, eGFP-transfected or rAD-GFP-transduced MIN6 cells were place on ice, washed twice in PBS and resuspended in cold FACS buffer containing PBS with 1 % BSA (Gibco-Invitrogen), 0.1 % Sodium Azide (Amersham Pharmacia Biotech, Bucks, UK), 2 mM EDTA. The number of cells that expressed GFP protein was acquired on the BD FACSCanto

(BD Biosciences, San Jose, CA, USA) and analysed using FlowJo software (Tree Star Inc, Ashland, OR, USA). In some cases, the rAD-GFP-transduced or eGFP-transfected MIN6 cells were observed under a Zeiss inverted fluorescence microscope to determine transfection efficiency (Carl Zeiss Inc., Jena, Germany).

2.10 RNA analysis

2.10.1 RNA isolation

Total RNA was extracted from cell lines, primary mouse and human islets using the RNeasy Mini Plus Kit (Qiagen, Valencia, CA, USA). Cells were first washed in cold PBS. Cells exposed to the relevant treatment conditions were then resuspended in Buffer RLT combined with β-mercaptoethanol (100:1). The cell suspension was then homogenised in the QIAshredder (Qiagen, CA, USA) column by centrifugation for 2 min at 13000 rpm. The resultant flow through was transferred into the gDNA Eliminator Spin Columns and centrifuged for 30 s at 13000 rpm. Next, equal volumes of 70% ethanol (w/v) was added and mixed to the flow through. This suspension was then transferred into an RNeasy spin column and centrifuged for 30 s at 13000 rpm. The flow through was discarded and 700 µL of Buffer RW1 was added, followed by centrifugation for 30 s at 13000 rpm. After discarding the RW1 flow through, RPE buffer (500 µL) was added to cells and spun down for 30 s at 13000 rpm. The previous step was repeated and spin columns were replaced with fresh collection tubes provided in kit and spun down for a further 2.5 min to remove excess ethanol. The resultant RNA was then collected in 30-50 µL of RNase-free water (usually primary islets in 30 µL and $1x10^6$ MIN6 cells in 50 µL water). RNA concentration was measured using the NanoDrop Spectrophotometer ND-1000 (ThermoFisher Scientific, Waltham, MA, USA). RNA quality was estimated by $A_{260/280}$ ratio with a range of 1.8-2.2 being acceptable or confirmed on the RNA 6000 Nano Chip (Agilent, Santa Clara, CA, USA) using 2100 Bioanalyser (Agilent).

2.10.2 Ethanol precipitation of RNA

RNA with an $A_{230/260}$ of 1.8 or less is considered contaminated with chaotropic salts or guanidine isothiocyanate from RNA extraction, which can interfere with

downstream enzymatic reactions in cDNA synthesis step. Therefore ethanol precipitation to clean up carry over of salts in RNA is important. RNA was precipitated in 2 µL of molecular grade glycogen diluted 1:4 (Roche), 2M final concentration of ammonium acetate (Sigma-Aldrich) and 2.5 vol of cold absolute ethanol (Sigma-Aldrich). The mixture was then incubated at -80 °C for 16 h or on dry ice for 2 h. Precipitated RNA was then recovered by centrifugation at 13 000 rpm for 30 min at 4 °C. The supernatant was decanted carefully, the pellet washed in cold 70% (w/v) ethanol and centrifuged again at 13 000 rpm for 10 min at 4 °C. The ethanol was then removed carefully and RNA pellet was left to air dry for 5-10 min and resuspended in 20–30 µL nuclease free H_2O. RNA concentration and quality was then determined using the NanoDrop Spectrophotometer ND-1000.

2.10.3 cDNA synthesis

RNA was used for reverse transcription for cDNA synthesis using the QuantiTect Reverse Transcription Kit (Qiagen, Valencia, CA, USA). RNA was first treated with DNase to remove any DNA carryover during RNA extraction. 50-100 ng of RNA was added to 2 µL of 7X gDNA Wipeout Buffer and made up to a total of 14 µL with RNase-free water. The mixture was incubated at 42 °C for 2 min and then placed on ice. When the mixture is cooled, 4 µL of 5X Quantiscript RT Buffer, 1 µL of Quantiscript Reverse Transcriptase and 1 µL of RT Primer mix were added and the resultant mixture was further incubated at 42 °C for 30 min followed by 95 °C for 3 min. The cDNA product was then stored in -20 °C until required.

2.10.4 Quantitative real time PCR

RNA was converted into cDNA in a 20 µL reaction as described above. The cDNA was diluted 5-fold and 2.5 µL of the diluted cDNA was used as the template for the qRT-PCR mix. Real time PCRs were performed using FastStart SYBR Green Master (Roche). Negative controls for each real time PCR run included a non-template (water) control and a reaction without reverse transcriptase added, to control for PCR contamination or genomic DNA contamination. The real time PCR mixture contains 5 µL of FastStart SYBR, 0.5 µL of 10 µM sense (F) + anti-sense (R) primers

and 2 μL of nuclease-free water (Table 2-10). To determine whether *Birc2* and *Birc3* were suppressed in islets isolated from *Birc2*$^{-/-}$ and *Birc3*$^{-/-}$ mice respectively, another set of PCR primers were designed to target the region of exon 1-2, where the genes were deleted (Table 2-10). Amplification was performed using the LightCycler 480 (Roche) according to instructions provided for FastStart SYBR Green Master. The real time PCR conditions were: Initial denaturation: 95 °C for 10 min, a three step cycling conditions for 40 cycles of 95 °C for 15 s, 63 °C for 30 s and 72 °C for 30 s. The threshold cycle (Ct) was set by the LightCycler 480 Software v1.5.0. Relative expression for each gene was analysed using the comparative Ct method and normalised to the expression of the housekeeping gene, Cyclophillin A (*Cph*) (Livak and Schmittgen, 2001; Schmittgen and Livak, 2008). Fold changes for genes were calculated using the $2\Delta\Delta^{CT}$ method comparing the relative expression between control and treatment groups.

Table 2-10. Primer list for qRT-PCR using the FastStart SYBR Green Master (Roche) on the LightCycler 480 (Roche).

Name	Sequence (5'-3')	T_m (°C)
Birc1a	F: GCTTTGAAGCCATGCCCTTT	67.3
	R: TCTCCAGTGGGAACGAGCAG	67.9
Birc1b	F: AAATTGCCAAGGGAGCAACC	67.0
	R: GGCAGGTTGTCCAGGACTTG	66.9
Birc1e	F: GATCCTGCCAAGCCTTCCAT	67.4
	R: GGTGTTCGAGGCAGCTCTCA	68.0
Birc1f	F: GGTGACGACCCAATGGAAGA	67.2
	R: TCCATGGCTTCTGGAAGTGC	67.5
Birc2	F: TGGCTTGAGATGTTGGGAACC	68.2
	R: CATGAACAAACTCCTGACCCTTCA	68.1
Birc3	F: CACTGCAGCAACCTCATTCAGA	67.6
	R: TGCAATGTCATCTGTGGGAAGA	67.7
Birc4	F: ATCCAAACATCCGGGAGCA	67.7
	R: AGGCGCCTTAGCTGCTCTTC	66.9
Birc5	F: GGGCCTCCTAGCAGGATCTTGAG	67.9
	R: GTCCACGTCACAATAGAGCAAAGC	67.7
Birc6	F: AGGAAAGGGTTCAGCGTTGC	67.5
	R: CTGTGACCTGCCCATCATCTG	68.0
A20	F: CCTGTCACCAACGCTCCAAG	68.1
	R: ATTTCCAGTCCGGTGGCAAG	68.2
Ccl2	F: GGTCCCTGTCATGCTTCTGG	67.0
	R: CCTGCTGCTGGTGATCCTCT	66.9
Cxcl10	F: GACGGGCCAGTGAGAATGAG	67.1
	R: GTGTGTGCGTGGCTTCACTC	67.1
Icam-1	F: ATCACGAGGCCCACAATGAC	68.1
	R: CCATGGGAATGTCACCAGGA	67.2
Cyclophilin A	F: TGGACCAAACACAAACGGTTCC	65.1
	R: ACATTGCGAGCAGATGGGGTAG	69.2
Birc3	F: GGGGACGATTTAAAGGTATCG	59.0
(Spans Exon1-2)	R: TCGGTTTTACTGCTAGGCTGA	60.0
Birc2	F: TGATGGTGGCTTGAGATGTT	59.0
(Spans Exon1-2)	R: CCCTTCATCCGTATCAAGAACT	59.0

2.11 Protein analysis

2.11.1 Collection of whole cell protein lysates

Prior to lysis, MIN6 cells were washed twice in cold PBS. Cells were then lysed in 100 µL per well of cold lysis buffer containing 50 mM of HEPES, 150 mM NaCl, 10% v/v Glycerol, 1% v/v Triton X-100, 1.5 mM $MgCl_2$, 1 mM Ethylene glycol-bis(2-aminoethylether)-N,N,N′,N′-tetraacetic acid (EGTA), 10 mM Sodium pyrophosphate tetrabasic, 100 mM NaF, 1x PhosSTOP Phophastase Inhibitor (Roche) and 1x complete mini protease inhibitor cocktail (Roche). The monolayer cells were lifted using a cell scraper, transferred into 1.5 mL tubes, vortexed and kept on ice for 1 h before transfer to -80 °C for storage until required. Cells were allowed to go through one freeze-thaw cycle before use.

For primary islets, 100-150 islet cells were washed three times with PBS and lysed in 30-40 µL of cold lysis buffer. Cell lysates were vortexed quickly and passed through a 0.5 ml BD Micro-Fine™ insulin syringes (BD, Franklin Lakes, NJ USA) for 12-15 times, until all the cells are lysed. The cell lysates were incubated on ice for 1 h and placed in -80 °C for storage until required. Cells were allowed to go through one freeze-thaw cycle before use.

2.11.2 Western blot analysis

Crude cell lysates were then spun down at 13, 000 rpm for 10 min at 4 °C. Protein assay was performed using the Bradford Assay (Bradford, 1976) purchased from Biorad, to determine the protein concentration of the of the cell lysates. Cell lysates (10-30 µg) were mixed with 6x Laemmli sample buffer (0.35 M Tris-HCl; pH 6.8, 10% SDS, 30% glycerol, 0.175 mM Bromophenol Blue and 6% v/v β-mercaptoethanol) and then denatured by heating at 100 °C for 5 min. Samples were separated in 10% SDS-PAGE gels for 1.5-2 h at 120 V and the proteins transferred onto Immobilon-P Polyvinylidene fluoride (PVDF) membrane (Millipore, MA, USA) at 120V for 1.5 h in a standard western blotting transfer buffer containing 10% methanol. The PageRuler™ Prestained Protein Ladder (Fermentas Inc; Thermoscientific, Waltham, MA, USA) was used as a protein

marker reference to determine band size. The resultant membranes were then blocked for at least 1 h with Tris Buffered Saline (15.35 mM Tris HCl, 0.5 M NaCl) with 0.1% Tween (TTBS) and 5% bovine serum albumin (BSA). The membranes were then incubated overnight at 4 °C either with IκBα (9242L) or phospho-c-Jun (Ser73, 9164S) (1:1000; Cell Signalling Technology) overnight. In some cases, membranes were incubated for 2 h in β-actin antibody (clone AC15; Sigma-Aldrich). After incubation, the blots were washed three times with TTBS and incubated in appropriate peroxidase-conjugated secondary antibody (1:5000; Pierce, Rockford, USA) for at least 2 h. Blots were then washed in TTBS thrice prior to development of the blots on film using the ECL chemiluminescence detection method (Amersham Pharmacia Biotech, Bucks, UK). The resultant films were scanned and the relative intensities were determined by ImageJ densitometry software (National Institutes of Health, Bethesda, USA).

2.12 Statistical analysis

Results are expressed as mean ± standard error mean (SEM). Statistical comparisons between groups were made by single unpaired t-test between two groups or analysis of variance (ANOVA) with pairwise multiple comparisons made using an unpaired t-test. Analyses were performed using GraphPad Prism version 5.0d (GraphPad Software Inc, CA, USA). A value of $p<0.05$ was considered significant.

3 The expression and regulation of BIRC family genes in pancreatic islets and beta cells

3.1 Introduction

The hallmark of T1D is the chronic and progressive destruction of insulin-producing pancreatic beta cells by accumulated mononuclear infiltrate surrounding the islets (Gepts, 1965; Gepts and Lecompte, 1981; Kay et al., 1991; O'Reilly et al., 1991). T1D takes place in two stages: Insulitis occurs when lymphocytic infiltrate invades the islets and diabetes proceeds when large populations of the beta cells are destroyed, leading to hyperglycaemia. These lymphocytic infiltrates participate in beta cell destruction by secreting inflammatory cytokines (Dahlen et al., 1998; Green et al., 1998; Jun et al., 1999a; Jun et al., 1999b; Pakala et al., 1999). The proinflammatory cytokines, TNFα, IL-1β and IFNγ are directly linked to the pathogenesis of T1D. Over the years, linkage analysis and congenic mapping have identified at least twenty insulin-dependent diabetes (Idd) loci in NOD mice that contribute to the development of T1D (Maier and Wicker, 2005). Of relevance, the *Tnfa* gene within the Idd16 locus is implicated as a candidate gene for T1D in the NOD mouse (Babaya et al., 2002). The Idd4 region implicated a role for IFN responses in the pathogenesis of T1D in NOD mice (Ivakine et al., 2006) while the *Il-1b* gene is found to be located in the mouse Idd13 region (Burren et al., 2011). Studies have shown that blocking individual cytokine pathways in NOD mice can abrogate or slow the progression of T1D. For example, *Tnfr1*$^{-/-}$ NOD mice were completely protected from diabetes (Kagi et al., 1999) and *Il1b*-deleted NOD mice exhibited delayed disease progression (Thomas et al., 2004). Similarly, the *Ifngr*$^{-/-}$ and *Ifng*$^{-/-}$ NOD mice were protected from cyclophosphamide-induced diabetes (Mori et al., 2008). Together, these studies demonstrate that the cytokines TNFα, IL-1β and IFNγ contribute to the pathogenesis of T1D. In this thesis, we are interested in examining the role of TNFα in T1D.

Broadly, TNFα mediates a multitude of cellular responses including cell proliferation, programmed cell death and activates downstream NF-κB, p38, and JNK pathways that play pivotal roles in beta cell inflammation (Hsu et al., 1995; Yuasa et al., 1998). In the context of T1D, TNFα is one of the first cytokines that is detected in the immune infiltrate of islets during insulitis (Dahlen et al., 1998). Importantly, TNFα contributes to the pathogenesis of T1D by inducing inflammatory gene expression in beta cells (Ortis et al., 2008), by directly impairing the insulin secretion function of beta cells (Zhang and Kim, 1995) and by causing beta cell destruction (Liuwantara et al., 2006). A characteristic feature of diabetes is the infiltration of the pancreatic islets with T cells. More specifically, a study found *TnfrI*-null islets are resistant to destruction by T lymphocytes (Pakala et al., 1999). Thus, these studies collectively supported the importance of TNFα to the progression of T1D. However, despite the importance of TNFα in the pathogenesis of T1D, the exact molecular machinery governing the TNF signalling cascade in islets and beta cells is not fully understood.

BIRC proteins are implicated in the regulation of down-stream TNF signalling networks (Dupoux et al., 2009; Gentle et al.; Mahoney et al., 2008; Varfolomeev et al., 2008). A key cellular function of BIRC proteins is the regulation of apoptosis. While BIRC proteins were thought to primarily act as regulators of apoptosis, this function has not been established for all its members; it is now recognised that BIRC proteins are also involved in the regulation of diverse cellular functions including that of cell development, cell cycle regulation, regulation of signalling pathways and inflammation. As an example, the BIRC member, Survivin, not only supports cell survival which results in better islet transplantation outcomes (Dohi et al., 2006) but is also required to support beta cell expansion after birth (Liggins et al., 2003; Wang et al., 2010). One way that BIRC proteins may regulate such cellular processes is by acting as signalling molecules (Rothe et al., 1995).

Beta cells respond to cytokines by mediating gene transcription to increase the expression of genes that influence beta cell survival, apoptosis signals and beta cell function (Liuwantara et al., 2006; Ortis et al., 2008; Sarkar et al., 2009). Cytokines can induce the expression of anti-apoptotic gene such as *A20*, *c-flip* and *Bcl2*, which

are known to protect beta cells from TNFα-mediated apoptosis (Allison et al., 2000; Cottet et al., 2002; Grey et al., 1999; Iwahashi et al., 1996; Liu et al., 1996a; Liuwantara et al., 2006). In addition to the protective genes, cytokines can also induce the expression of deleterious inflammatory genes such as inducible nitric oxide synthase (*iNos*) and *Cox2* which impair beta cell function, as well as inflammatory chemokines and adhesion molecules such as *Cxcl2, Cxcl9, Cxcl10, Rantes, Ccl2* and *Icam-1* to exacerbate beta cell inflammation and cause beta cell apoptosis (Bradley et al., 1999; Camacho et al., 2001; Chen et al., 2001; Corbett et al., 1993a; Corbett and McDaniel, 1995; Corbett et al., 1993b; Eizirik et al., 1993; Frigerio et al., 2002; Ortis et al., 2008; Prieto et al., 1992). These findings highlight the importance of the beta cell's response to cytokine, where they may participate in their own destruction by inducing genes that exacerbate inflammation. Hence the key to the beta cell's fate under inflammatory conditions in T1D may lie in the balance between the expression of genes that signal for life and death (Figure 3.1).

Some studies have shown that members of the BIRC family are inducible by TNFα (Furusu et al., 2001; Stehlik et al., 1998; Wang et al., 1998). Further to this, it has been demonstrated that *Birc3* is induced following TNFα and IL-1β-stimulation in mouse and human islets (Liuwantara et al., 2006; Sarkar et al., 2009). As BIRC proteins are thought to function as regulators of apoptosis and are implicated to participate in TNF signal transduction, they may take part in modulating the beta cell's response to TNFα and influence the fate of a beta cell in the context of T1D. The major aims of this chapter are to examine in pancreatic islets and beta cells (1) how *Birc* family genes are expressed in response to cytokines, (2) how *Birc* family gene expression is regulated in response to cytokines and (3) to determine their function in beta cells.

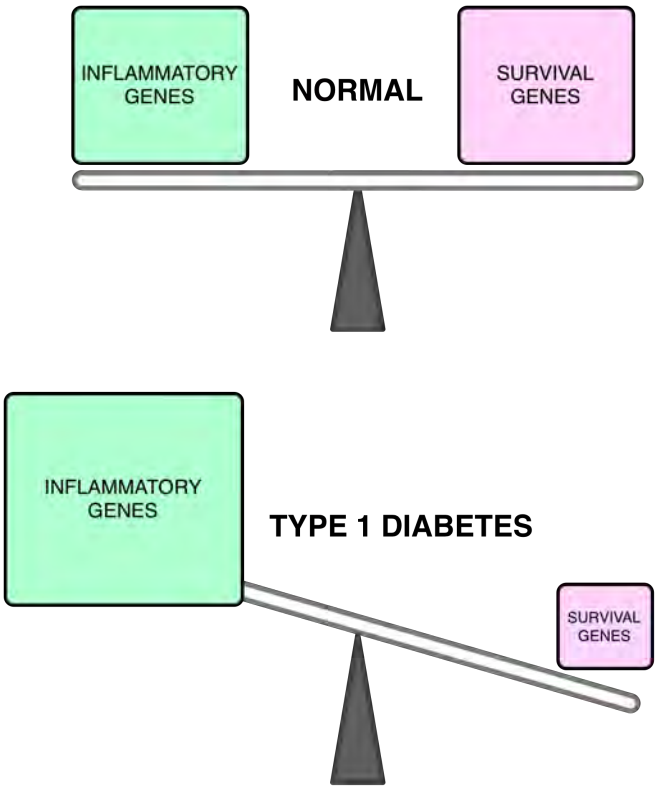


Figure 3.1. The beta cell's fate under normal and T1D conditions may rely on the balance between the expression of inflammatory and survival genes.

3.2 *Results*

3.2.1 BIRC family gene expression in pancreatic islets and beta cells

The levels of *Birc1-6* expressed in untreated or TNFα-stimulated mouse islets and beta cells were analysed by qRT-PCR. In the steady state, *Birc3* was highly expressed (Figure 3.2A) and was further increased by TNFα in mouse islets (Figure 3.2B). Islets are a heterogeneous tissue (Kim et al., 2009); as beta cells are the target of inflammatory attack in T1D (Foulis, 1987), we were interested to determine how TNF-signalling controls BIRC expression in beta cells. Different to intact islets, the beta cell line, MIN6 cells, exhibited low basal levels of *Birc1*, *2* and *3* but expressed high levels of *Birc4*, *5* and *6* (Figure 3.2C). Like intact islets, *Birc3* but not *Birc1, 2, 4-6* mRNA was increased by 5-fold in TNFα stimulated MIN6 cells (Figure 3.2D). These data demonstrated selective regulation of *Birc3* in response to TNFα in pancreatic islets and MIN6 cells.

3.2.2 The kinetics of *Birc3* expression in mouse islets and MIN6 cells

The kinetics of *Birc3* expression in mouse islets and MIN6 cells were studied. *Birc3* was rapidly induced and its expression levels were maintained up to 8 h post TNFα stimulation (Figure 3.2E). Similar to intact islets, MIN6 cells also showed rapid and then sustained expression levels of *Birc3* (Figure 3.2F). Thus *Birc3* is an early immediate response gene and its expression is maintained up to 8 h in mouse islets and beta cells.

3.2.3 Expression of *Birc3* in response to cytokines implicated in type 1 diabetes

The inflammatory cytokines, IL-1β and IFNγ, are also implicated in T1D pathogenesis (Wachlin et al., 2003). In this study, the effects of IL-1β and IFNγ on BIRC family gene expression in islets and beta cells were also tested. The study revealed a hierarchy in cytokine responsiveness, such that for mouse islets, IL-1β was the strongest inducer of *Birc3* (9-fold) compared to TNFα (5-fold) and IFNγ (4-fold) (Figure 3.2G). This pattern was also seen for MIN6 cells (Figure 3.2H). However, different to mouse islets, IFNγ did not induce a change in *Birc*3

expression in MIN6 cells. The cytokines TNFα and IL-1β are known to synergise with IFNγ and increase gene expression in islets (Wachlin et al., 2003). We found when combined, TNFα + IL-1β + IFNγ synergised *Birc3* expression three times more than IL-1β-induced *Birc3* levels in mouse islets and twice more than IL-1β-induced *Birc3* levels in beta cells. Collectively, our data showed that the overall expression pattern and regulation of *Birc3* in mouse islets and MIN6 cells are similar, with the exception of IFNγ.

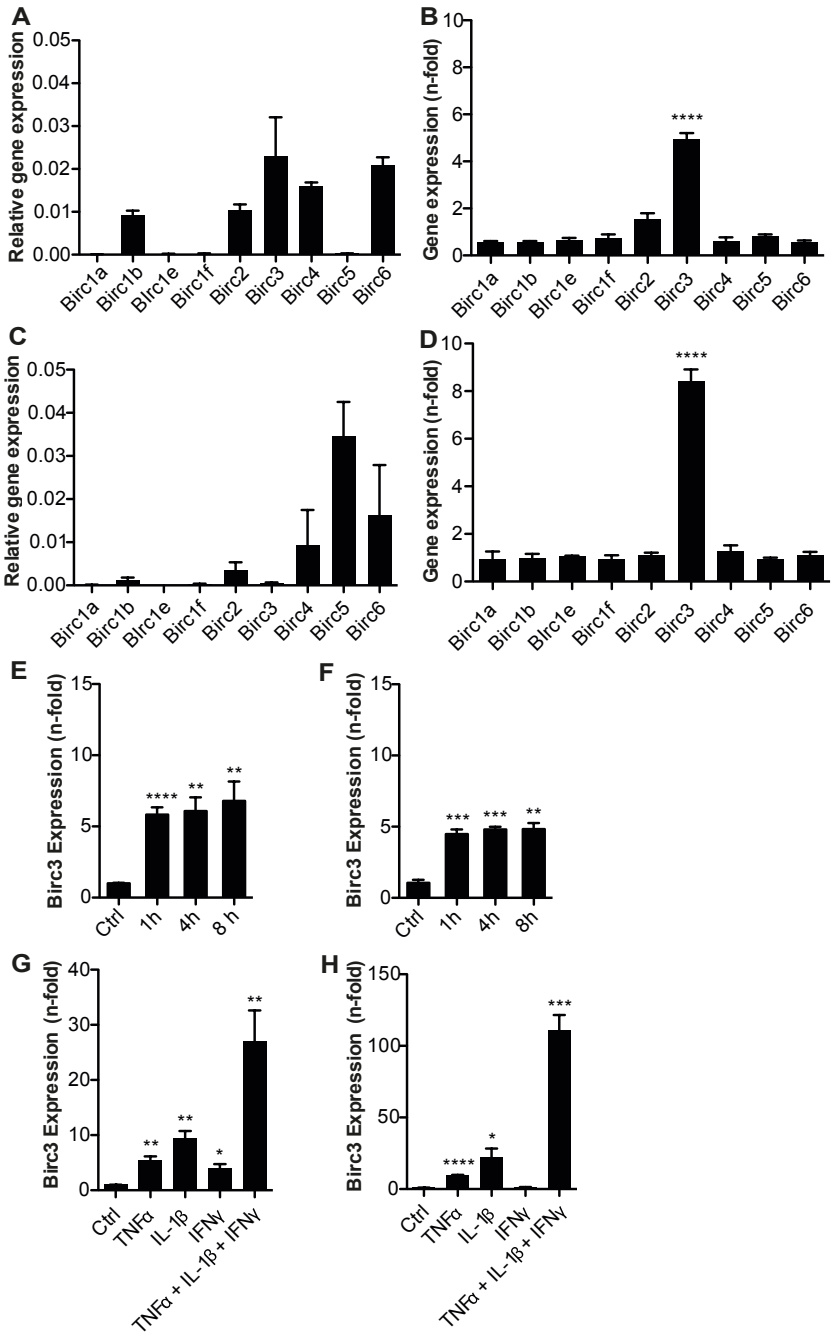


Figure 3.2. Expression of *Birc* family genes in islets and beta cells. Expression of *Birc1-6* analysed by qRT-PCR in mouse islets left untreated (A) or stimulated with TNFα (200U/mL) for 4 h (B) and MIN6 cells left untreated (C) or stimulated with TNFα (200U/mL) for 4 h (D). Data represent mean ± SEM from at least three independent experiments. Expression of *Birc3* in mouse islets (E) and MIN6 cells (F) stimulated with TNFα (200U/mL) for 1-8 h analysed by qRT-PCR. Data represent mean ± SEM from three independent experiments. Expression of *Birc3* in mouse islets (G) and MIN6 (H) either left untreated or stimulated with TNFα (200U/mL), IL-1β (200U/mL), IFNγ (200U/mL) or TNFα + IL-1β + IFNγ (200U/mL each) for 4 h, analysed by qRT-PCR. Data represent mean ± SEM from three independent experiments. Statistical comparisons were made by the analysis of variance (ANOVA) with pair-wise multiple comparisons made using an unpaired t-test or single unpaired t-test between two groups. All differences are significant. ($*p \leq 0.05$, $**p \leq 0.01$, $***p \leq 0.001$ and $****p \leq 0.0001$).

3.2.4 Cytokine-induced *Birc3* is regulated by *de novo* transcription

TNFα and IL-1β can regulate gene expression via transcription or through post-transcriptional modifications (Lentz et al., 1991; Vincenti et al., 1994). To test how TNFα mediate changes in *Birc3* expression, mouse islets and beta cells were treated with the general transcription inhibitor, Actinomycin D (AcD) (Goldberg et al., 1962), prior to TNFα stimulation. The optimal dose of AcD on these cell types were first determined by treating MIN6 cells with AcD in the range of 0.01 μM to 10 μM. Pre-treatment with AcD of 1 μM and 10 μM resulted in ~75% suppression of TNFα-induced *Birc3* expression in MIN6 cells (Figure 3.3A). Similarly, treatment of 1 μM AcD resulted in ~85% suppression of TNFα-induced *Birc3* expression in mouse islets (Figure 3.3B). Like TNFα stimulation, pre-treatment of 1μM AcD on MIN6 cells and mouse islets also prevented IL-1β-induced *Birc3* expression by >90% (Figure 3.3C and Figure 3.3D). These data demonstrated that cytokines such as TNFα and IL-1β regulate *Birc3* expression through *de novo* gene transcription in primary islets and beta cells.

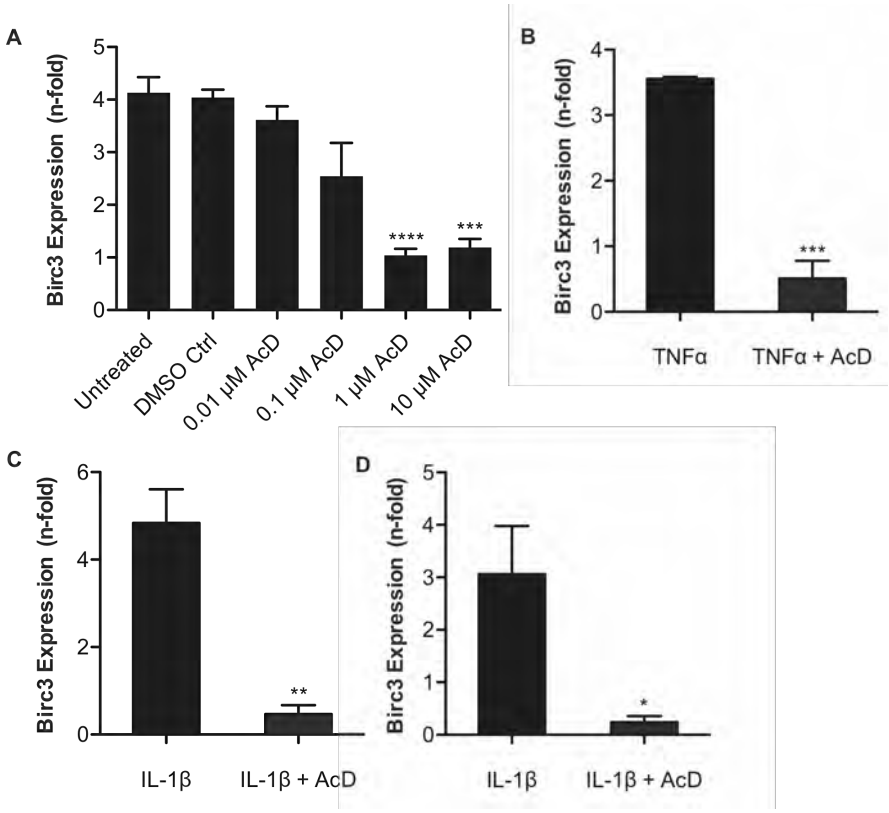


Figure 3.3 *Birc3* expression is regulated by *de novo* transcription in mouse islets and beta cells. (A) MIN6 cells were either left untreated, treated with DMSO or with 0.01-10 µM of AcD 1 h prior to TNFα (200 U/mL) stimulation for 4 h. *Birc3* expression was measured by qRT-PCR. Data is expressed as mean ± SEM for 3 independent experiments. (B): Mouse islets were either left untreated or treated with 1 µM of AcD 1 h prior to TNFα (200 U/mL) stimulation for 4 h. *Birc3* expression was measured by qRT-PCR. Data is expressed as mean ± SEM for 3 independent experiments. MIN6 cells (C) or mouse islets (D) were either left untreated or treated with 1 µM AcD 1 h prior to IL-1β (200 U/mL) stimulation for 4 h. *Birc3* expression was measured by qRT-PCR. Data is expressed as mean ± SEM for 3 independent experiments. All differences are significant. Statistical comparisons were made by single unpaired t-test between two groups. (∗p<0.05. ∗∗p<0.01, ∗∗∗p<0.001 and ∗∗∗∗p<0.0001).

3.2.5 The *Birc3* promoter has cytokine responsive elements

The 5'-untranslated region (5'-UTR) of the *Birc3* promoter sequence was obtained from the GenBank database (Benson et al., 2011) and examined for putative transcription factor binding sites that are responsive to TNFα using the PROMO 3.0 software (Farre et al., 2003; Messeguer et al., 2002). Within the 5'-UTR of the *Birc3* proximal promoter, three putative consensus NF-κB-binding sites and a TATA-like box with the sequence "TTTAAA" were identified (Figure 3.4). In addition, potential AP-1, Signal Transducers and Activators of Transcription 1 (STAT-1) and protein 53 (p53) putative binding sites were also identified (Figure 3.4). Thus, the *Birc3* proximal promoter harboured a region rich in putative transcription factor binding sites that are responsive to cytokines.

The region marked by the arrows "forward primer" and "reverse primer" was cloned into the pGL3-basic vector luciferase reporter and its cytokine-responsiveness was studied (Figure 3.4A). To clone the *Birc3* reporter, primers that span this *Birc3* promoter region amplified a PCR product that was 305 bp long from mouse gDNA (Figure 3.5A-lane 3). This product was subsequently ligated into the TOPO-TA cloning vector and sub-cloned into the pGL3 luciferase reporter. The final product of 395 bp was verified by adding restriction enzymes that recognise the sequences of KpnI and XhoI sites in the *Birc3* reporter (Figure 3.5B- Lane3) and by gene sequencing. The cloned *Birc3* promoter controls the expression of the luciferase reporter.

To test the effects of cytokines on the *Birc3* reporter, MIN6 cells were transfected with the *Birc3* reporter and stimulated with different combinations of cytokines. The *Birc3* reporter showed kinetics reminiscent of the expression of endogenous *Birc3*. Like its endogenous gene expression (seen in Figure 3.2), the *Birc3* reporter showed ~2-fold increase with TNFα, was more strongly activated by IL-1β (4-fold) and IFNγ alone did not induce any reporter activity (Figure 3.5C). The cytokine cocktail of TNFα + IL-1β + IFNγ showed a strong additive effect (6.5-fold). Further, the *Birc3* reporter was induced at 4 h and sustained up to 8 h (Figure 3.5D). These results indicated that the *Birc3* proximal promoter contained cytokine responsiveness elements that direct *Birc3* gene expression.

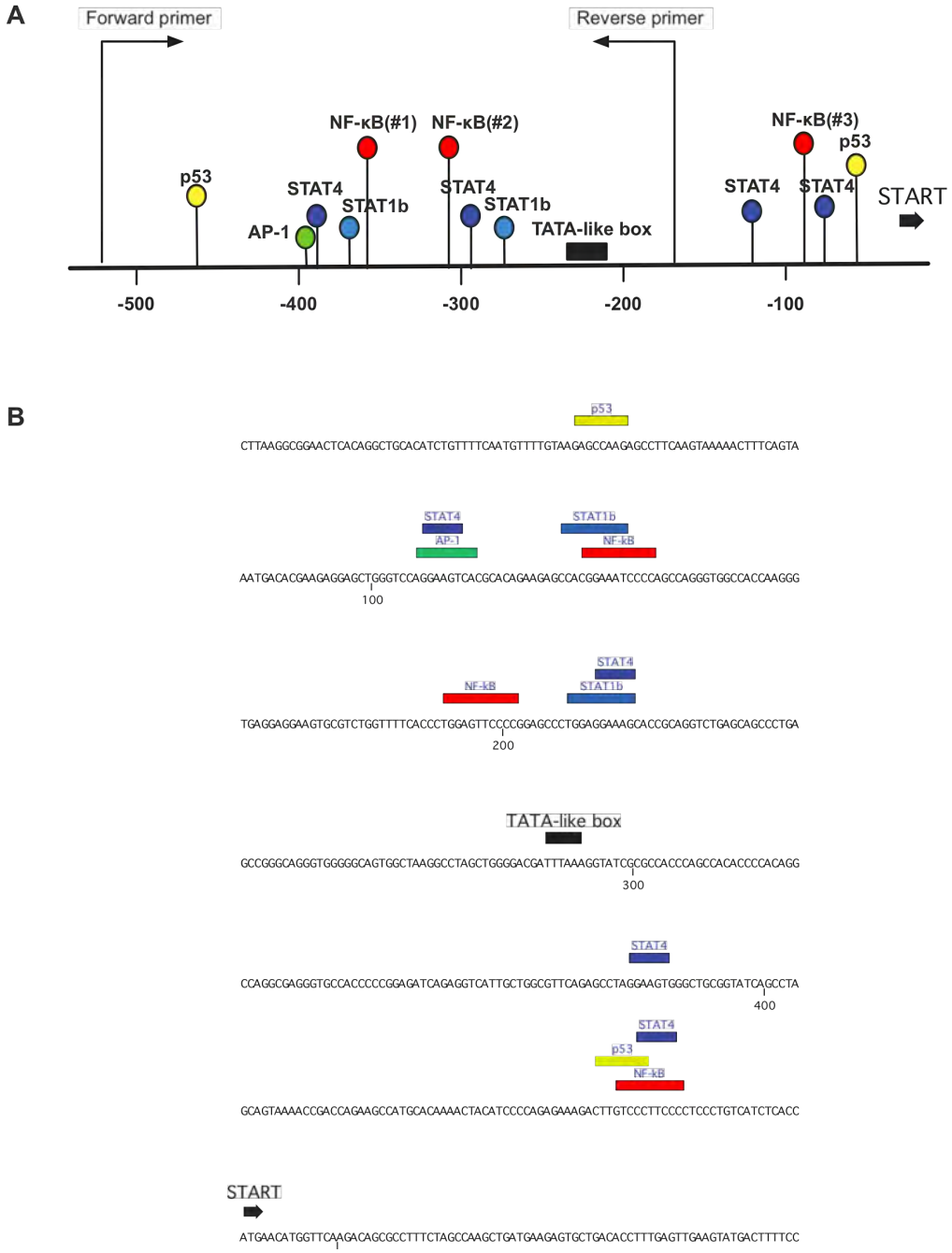


Figure 3.4. Analysis of the 5'-UTR of *Birc3* for putative transcription elements using PROMO3.0. (A) A condensed version of the *Birc3* proximal promoter shown in (B). The "forward primer" and "reverse primer" indicate the cloned region for promoter analysis. Putative NF-κB (red circles #1, #2 and #3), TATA-like box (black rectangle), AP-1 (green circle), STAT1b and STAT4 (blue circles) and p53 (yellow circle) binding sites are shown. Bold arrow marks the *Birc3* transcription start site. (B) A diagram depicting the sequence of *Birc3* proximal promoter ~500 bp upstream of the *Birc3* transcription start site (black arrow with "START" label). The putative transcription factor binding sites that were identified using PROMO3.0 are shown as follows: p53 (yellow box), STAT1b/4 (blue), AP-1 (green) and NF-κB (red).

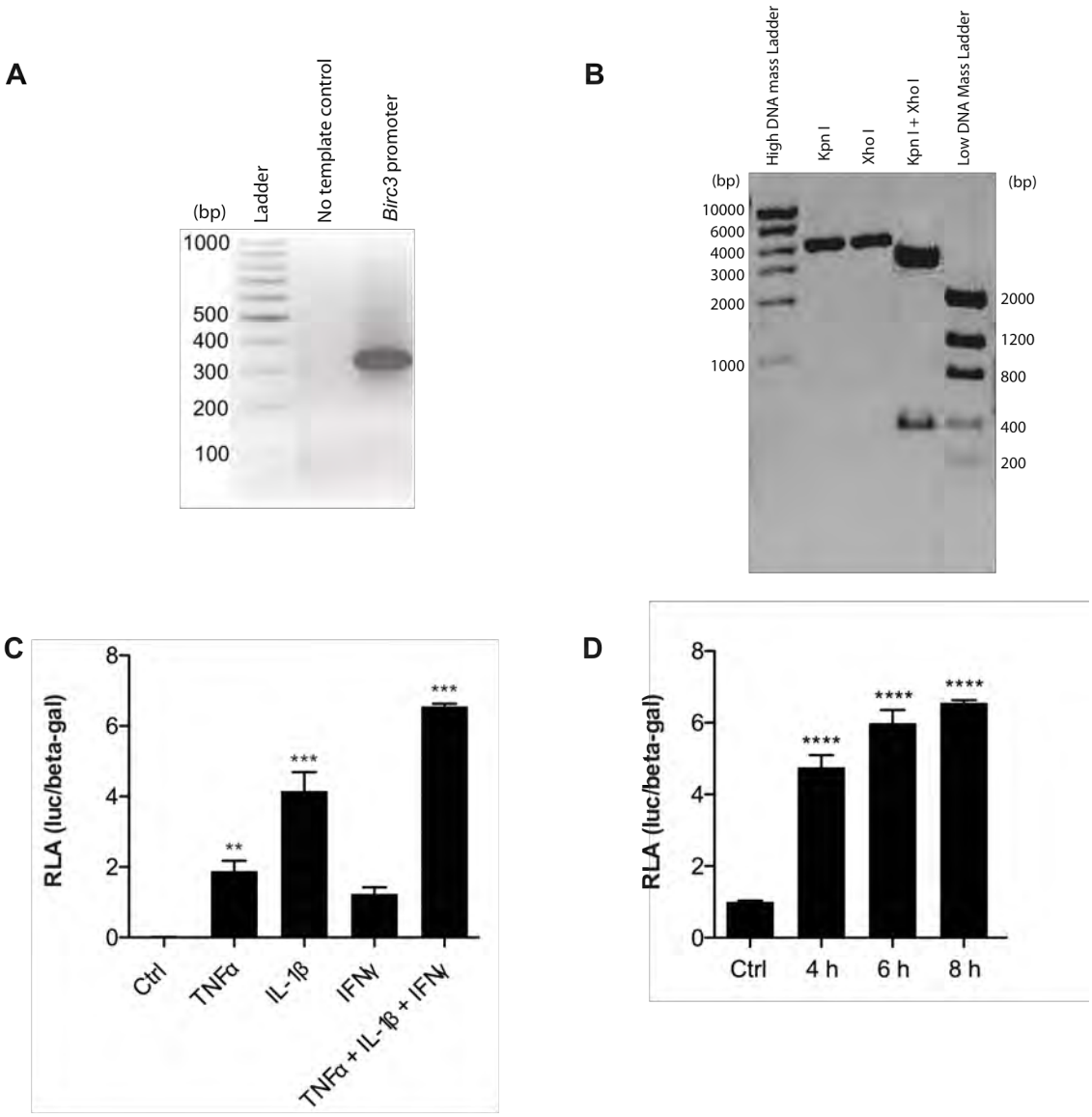


Figure 3.5. The *Birc3* 5'-UTR has cytokine responsive elements. (A) 100 bp ladder (Lane1), no template control (Lane2) and the 305 bp *Birc3* promoter fragment obtained by PCR (Lane 3). (B) High molecular mass ladder (Lane1), TOPO vector ligated to the *Birc3* promoter following KpnI (Lane 2) or XhoI (Lane 3) single cuts, the 305 bp *Birc3* promoter fragment released from TOPO vector following KpnI and XhoI digest (Lane 4- bottom band) and low DNA mass ladder (Lane 5). (C) Induction of the *Birc3* reporter in MIN6 cells by TNFα, IL-1β, IFNγ or TNFα + IL-1β + IFNγ analysed by luciferase reporter assay. RLA, relative luciferase activity. Data represent mean ± SEM from three independent experiments. (D) Induction of the *Birc3* reporter in MIN6 cells by TNFα + IL-1β + IFNγ for 4-8 h, analysed by luciferase reporter assay. RLA, relative luciferase activity. Data represent mean ± SEM from three independent experiments. Data represent mean ± SEM from three independent experiments. Statistical comparisons were made by single unpaired t-test between two groups. All differences are significant. (∗∗p≤0.01, ∗∗∗p≤0.001 and ∗∗∗∗p≤0.0001); Relative luciferase activity (RLA)

3.2.6 NF-κB signalling is necessary for TNF-induced *Birc3* expression

TNFα activates the JNK, p38 and NF-κB signalling pathways (Figure 3.6A) (Hsu et al., 1995; Yuasa et al., 1998). To determine the molecular mechanisms that drive *Birc3* transcription, MIN6 cells were either left untreated or pre-treated with the pharmacological inhibitors SP600125 (50 μM), SB203580 (20 μM) or PDTC (50 μM) to selectively target the JNK, p38 or NF-κB pathways respectively, prior to TNFα stimulation. The effectiveness of each inhibitor in blocking activation of JNK and p38 were assessed by Western blot analysis; the effective concentration of 50 μM PDTC was previously determined in our laboratory (Liuwantara et al., 2006). *Birc3* levels were measured by qRT-PCR. TNFα stimulation led to JNK activation and increased phosphorylation of JNK1 (46 kDa) and JNK2 (54 kDa) (Figure 3.6B). SP600125 of ≥50 μM significantly suppressed TNFα-induced JNK activation in MIN6 cells (Figure 3.6B). Similarly, TNFα stimulation led to the phosphorylation and activation of the p38 signalling pathway (43 kDa) (Figure 3.6C) and this was prevented by the treatment of ≥10 μM of SB203580 in MIN6 cells (Figure 3.6C).

When TNFα-induced *Birc3* levels were examined, neither SP600125 (Figure 3.6D) nor SB203580 (Figure 3.6E) pre-treatment at their optimal concentrations inhibited TNFα-induced *Birc3* expression, demonstrating that TNFα stimulated *Birc3* was not regulated through the p38 or JNK pathways. In contrast, TNFα-stimulated *Birc3* expression was suppressed by ~90% ($p \leq 0.05$) for MIN6 cells (Figure 3.6F) and ~75% for mouse islets ($p \leq 0.05$) (Figure 3.6G) when pre-treated with PDTC. These data showed that NF-κB is necessary for TNFα-induced *Birc3* expression.

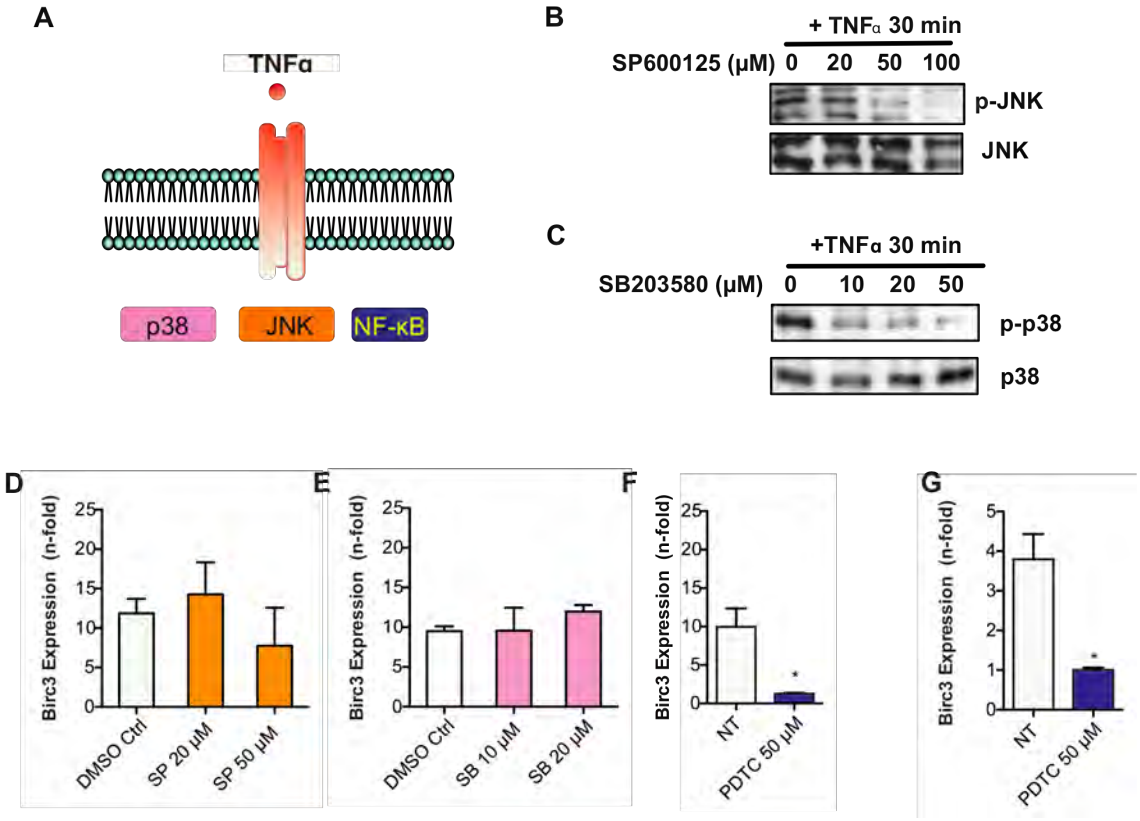


Figure 3.6 Analysis of signalling pathways involved in TNFα-induced expression of *Birc3* using pharmacological inhibitors. (A) A schematic of signalling pathways activated by TNFα. (B) Western blot analysis of the effects of 0-100 μM of SP600125 on JNK activation measured by JNK phosphorylation in MIN6 cells following TNFα stimulation. Total JNK levels were used as a loading control. Representative blots are shown. Duplicate experiments in each case generated similar results. (C) Western blot analysis of the effects of 0-50 μM of SB203580 on p38 activation determined by p38 phosphorylation in MIN6 cells following TNFα stimulation. Total p38 levels were used as a loading control. Representative blots are shown. Duplicate experiments in each case generated similar results. (D-F) Expression of *Birc3* in MIN6 cells with TNFα, either left untreated or treated with SP600125 (SP), SB203580 (SB) or PDTC. Data represent mean ± SEM from three independent experiments. (G) Expression of *Birc3* in mouse islets stimulated with TNFα, either left untreated or pre-treated with PDTC. Data represent mean ± SEM from three independent experiments. Statistical comparisons were made by single unpaired t-test between two groups. All differences are significant. ($*p \leq 0.05$).

3.2.7 The effects of IκBα overexpression on *Birc3* expression

IκBα, a natural inhibitor of NF-κB, binds to the cytoplasmic p65/NF-κB and prevents the nuclear translocation and activation of p65/NF-κB (Beg et al., 1992; Ganchi et al., 1992; Kumar and Gelinas, 1993). The role of NF-κB in modulating TNFα-induced *Birc3* expression was further tested by the overexpression of IκBα. MIN6 cells were transduced with recombinant adenovirus expressing either GFP (rAD.GFP) or IκBα (rAD.IκBα) prior to TNFα stimulation for 4 h and *Birc3* expression was measured by qRT-PCR. The optimal ratio of viral particle to MIN6 cells (multiplicity of infection; MOI) was determined by flow cytometry analysis. We achieved a maximum of ≥42% transduction efficiency with the MOI of 100:1 for MIN6 cells (Figure 3.7A). In agreement with the data from Figure 3.6, TNFα-induced *Birc3* expression is regulated by the NF-κB pathway in beta cells; as compared to non-infected cells (NI), TNFα-induced *Birc3* expression was suppressed in MIN6 cells expressing high levels of IκBα but not in GFP-expressing cells (Figure 3.7B). Similar to TNFα-induced *Birc3,* IL-1β- and TNFα + IL-1β + IFNγ-induced *Birc3* expression were also regulated by NF-κB (Figure 3.7C and Figure 3.7D). Therefore in beta cells, inflammatory cytokine can activate NF-κB signalling and induce *Birc3* transcription.

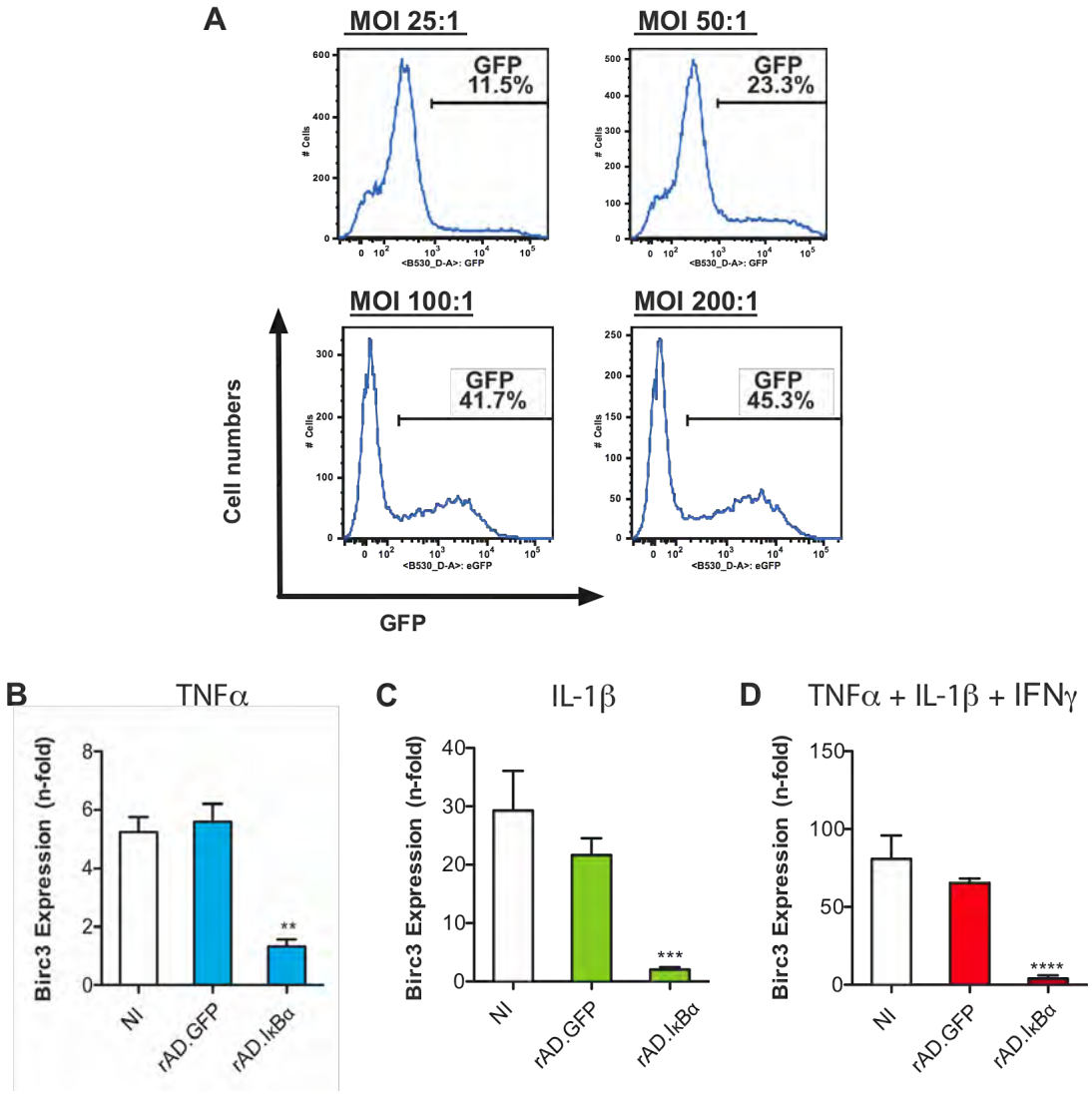


Figure 3.7. Examining the role of NF-κB signalling in TNFα-induced *Birc3* expression by overexpression of IκBα. (A) Flow cytometry analysis of transduction efficiency of rAD.GFP with the MOI of between 25:1 to 200:1 in MIN6 cells. Representative histograms are shown. Duplicate experiments in each case generated similar results. The x-axis shows GFP expression and y-axis represents total cell numbers. (B-D): Expression of *Birc3* in non-infected (NI) MIN6 cells or cells transduced with rAD.GFP or rAD.IκBα prior to TNFα or IL-1β or TNFα+IL-1β+ IFNγ stimulation. Data represent mean ± SEM from three independent experiments. Statistical comparisons were made by single unpaired t-test between two groups. All differences are significant. (∗∗p≤0.01, ∗∗∗ p≤0.001 and ∗∗∗∗ p≤0.0001).

3.2.8 NF-κB is sufficient to drive *de novo Birc3* expression

To determine whether NF-κB was sufficient to drive *de novo Birc3* expression, MIN6 cells were transfected with the *Birc3* reporter and a *RelA/p65* expression vector. The *Birc3* reporter showed 2-fold ($p \leq 0.001$) increase in luciferase activity (Figure 3.8A), demonstrating that NF-κB is sufficient to drive *Birc3* transcription. Conversely, MIN6 cells transfected with *Birc3* reporter and an *IκBα* expression construct showed 10-fold ($p \leq 0.05$) reduction in reporter activity compared to EV-transfected cells, when stimulated by a cytokine cocktail containing TNFα + IL-1β + IFNγ (Figure 3.8B). These results demonstrated that NF-κB is required for the activation of *Birc3* transcription by direct and positive interaction with its promoter.

To determine whether *RelA*/*p65* was sufficient to drive endogenous *Birc3* gene expression, another beta cell line, β-TC_3 cells, were transfected with the *RelA*/*p65* expression vector and *Birc* family gene expression was determined by qRT-PCR. This experiment was performed on β-TC_3 cells because the conditions for transfection of p65 were previously optimised in these cells (Liuwantara et al., 2006). Similar to TNFα-stimulated *Birc3* expression in mouse islets and MIN6 cells (Figure 3.2B and Figure 3.2D), *Birc3* was selectively induced by overexpression of p65 (~6-fold, $p \leq 0.05$) when compared to other BIRC family members (Figure 3.8C). Together, these data allowed us to conclude that NF-κB is both necessary and sufficient to drive *de novo* transcription of *Birc3*.

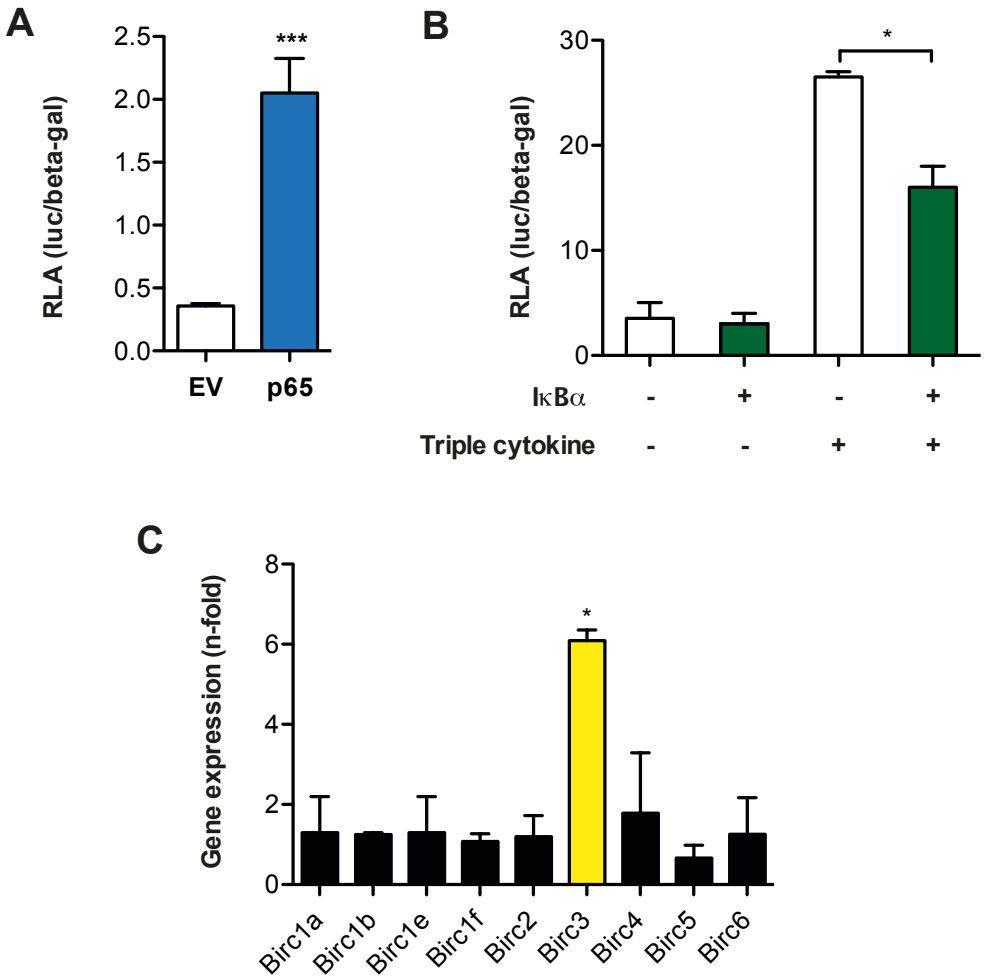


Figure 3.8. NF-κB is sufficient to drive *de novo Birc3* expression. (A) Induction of the *Birc3* reporter in MIN6 by 0.6 μg EV (white bars) or 0.6 μg *RelA/p65* (blue bars) analysed by luciferase reporter assay. RLA, relative luciferase activity. Data represent mean ± SEM from three independent experiments. (B) *Birc3* reporter activity in MIN6 transfected with 0.3 μg EV or 0.3 μg IκBα, in the absence (white bars) or presence of TNFα + IL-1β + IFNγ (triple cytokine; 200U/mL each; green bars) analysed by luciferase reporter assay. RLA, relative luciferase activity. Data represent mean ± SEM from three independent experiments. (C) Induction of endogenous *Birc1-6* mRNA in β-TC_3 by RelA/p65. Data represent mean ± SEM from three independent experiments. Statistical comparisons were made by single unpaired t-test between two groups. All differences are significant. ($*p \leq 0.05$ and $***p \leq 0.001$).

3.2.9 Specific expression of BIRC3 is not unique to pancreatic islets and MIN6 cells

Our data so far demonstrated that TNFα induces islets and beta cells to specifically up-regulate *Birc3* but not the other *Birc* genes. Some reports have shown that TNFα can also induce the other *Birc* members, *Xiap* and *Birc2*, in a number of cell types including endothelial cells (Stehlik et al., 1998; Wang et al., 1998). We therefore wondered whether this selective induction of *Birc3* is a phenomenon unique to pancreatic islets and beta cells. To test this, *Birc* family gene expression was measured in NIH3T3 mouse embryonic fibroblast cells and 2F2B endothelial cells, following TNFα stimulation. Like islets and beta cells, TNFα stimulated NIH3T3 and 2F2B cells also displayed selective induction of *Birc3* (5-fold; p≤0.0001; Figure 3.9A and 3-fold; p≤0.05; Figure 3.9B respectively). These results showed that selective regulation of TNFα-induced *Birc3* is not limited to islets and beta cells.

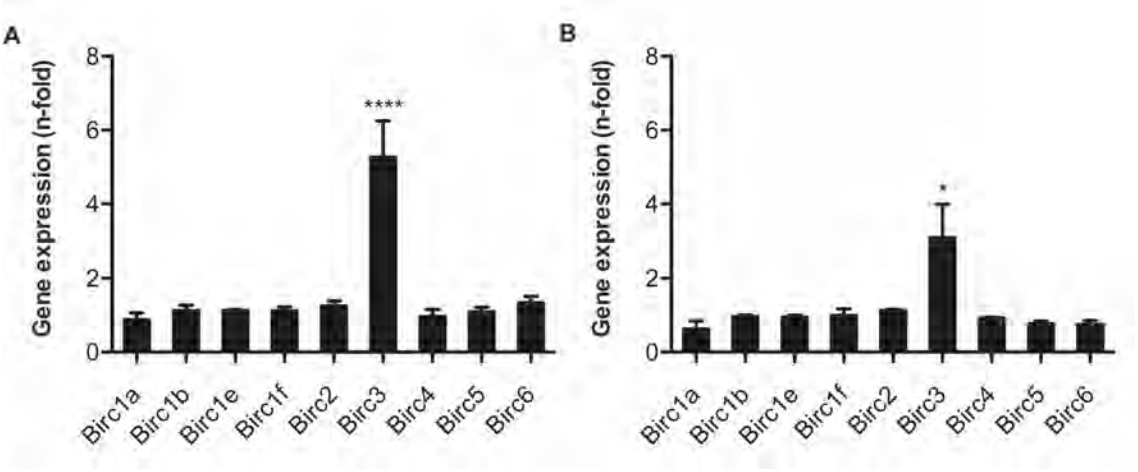


Figure 3.9 Expression of BIRC family genes in NIH-3T3 and 2F2B cells. Expression of *Birc1-6* in NIH-3T3 cells (A) or 2F2B cells (B) either left untreated or stimulated with TNFα (200 U/ml) for 4 h were measured by qRT-PCR. Data is expressed as mean ± SEM for 3 independent experiments. All differences are significant. Statistical comparisons were made by analysis of variance (ANOVA) with pair-wise multiple comparisons made using an unpaired t-test. (∗p<0.05 and ∗∗∗∗p<0.0001).

3.2.10 The role of HDACs in the regulation of BIRC family gene expression

Alterations in histone modifications can enhance or repress gene expression by modulating the accessibility of promoters to the transcription machinery (Berger, 2002; Cheung et al., 2000b; Eberhardy et al., 2000; Lomvardas and Thanos, 2002; Struhl, 2001). We questioned whether epigenetic changes may be contributing to the specific expression of BIRC family genes in beta cells, either by enhancing *Birc3* expression or by repressing the expression of the other *Birc* family genes.

A pilot experiment was carried out to test if HDACs modulate BIRC family gene expression in beta cells. BIRC family gene expression levels were analysed by qRT-PCR in MIN6 cells treated with the histone deacetylase inhibitor, trichostatin A (TSA), prior to stimulation with TNFα. Interestingly, TSA treatment resulted in 4-6-fold increase in basal and TNFα-induced *Birc1a* levels compared to untreated cells (Figure 3.10A). Like *Birc1a,* 25 ng/mL of TSA modestly increased basal and TNFα-induced *Birc1b* and *Birc4* (~2-folds) (Figure 3.10B and Figure 3.10D), while *Birc2*, *Birc5* and *Birc6* levels remained unaffected (Figure 3.10C, Figure 3.10E and Figure 3.10F). Thus HDACs may repress *Birc1a*, *Birc1b* and *Birc4* transcription in beta cells, by rendering the promoters of these genes inaccessible to the transcription machineries. Although more work is required to confirm this.

Additionally, TSA treatment also led to a marginal increase in basal *Birc3* expression but suppressed TNFα-induced *Birc3* expression by ≥50% (Figure 3.10G). These data suggested that HDACs may modulate *Birc3* expression in beta cells. As we have shown that TNFα-mediated *Birc3* is regulated by NF-κB signalling, we wondered whether HDACs play a role in the regulation of TNFα-induced NF-κB signalling. To test this, the effects of TSA on the expression of NF-κB-regulated genes, *Ccl2* and *A20,* were analysed by qRT-PCR. In support of our findings with *Birc3*, our trial experiment also showed that TNFα-mediated *Ccl2* and *A20* expression were suppressed by ≥50% and 25% respectively, with TSA-treatment (Figure 3.11A and Figure 3.11B). These findings collectively hinted that in addition to repressing *Birc1a, Birc1b* and *Birc4* expression, HDACs may also modulate NF-κB-regulated gene transcription in beta cells.

Indeed, it has been demonstrated that HDACs can modulate NF-κB signalling by interfering with the recruitment of RNA polymerase II (Furumai et al., 2011). Furthermore, it has been reported that NF-κB activation is also regulated by p65/RelA's interaction with HDAC corepressor proteins (Ashburner et al., 2001). A study found TSA treatment inhibits HDAC activity, induces increase in basal and TNFα-induced activation of a NF-κB-dependent reporter and the expression of the NF-κB-regulated gene *IL8* (Ashburner et al., 2001). Collectively, these findings supported the idea that HDACs may modulate NF-κB signalling in beta cells (Figure 3.10G and Figure 3.11A-B). This role of HDACs has been previously elucidated in beta cells. HDAC inhibition is associated with down-regulation of NF-κB transactivation, resulting in better beta cell survival and function in the presence of cytokine stimulation (Larsen et al., 2007b). Additionally, these findings also supported the idea that NF-κB is a key regulator of TNFα-mediated *Birc3* expression in beta cells.

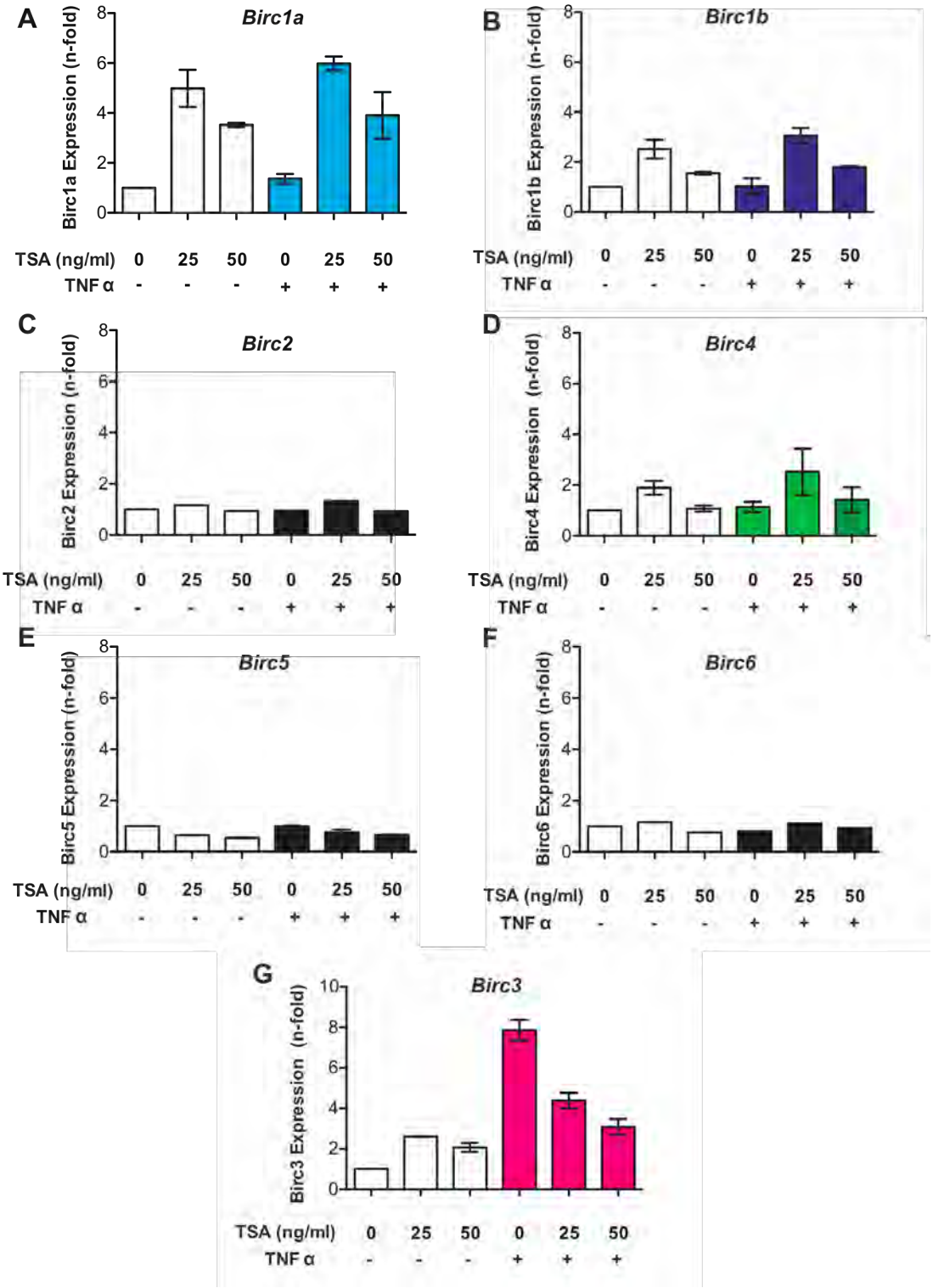


Figure 3.10 Effects of TSA on unstimulated and TNF-induced BIRC family gene expression in MIN6 cells. (A-G) The effects of 0-50 ng/mL of TSA on BIRC family gene expression in MIN6 with or without TNFα (200U/mL) stimulation for 4 h. Data represent mean ± SEM from two independent experiments.

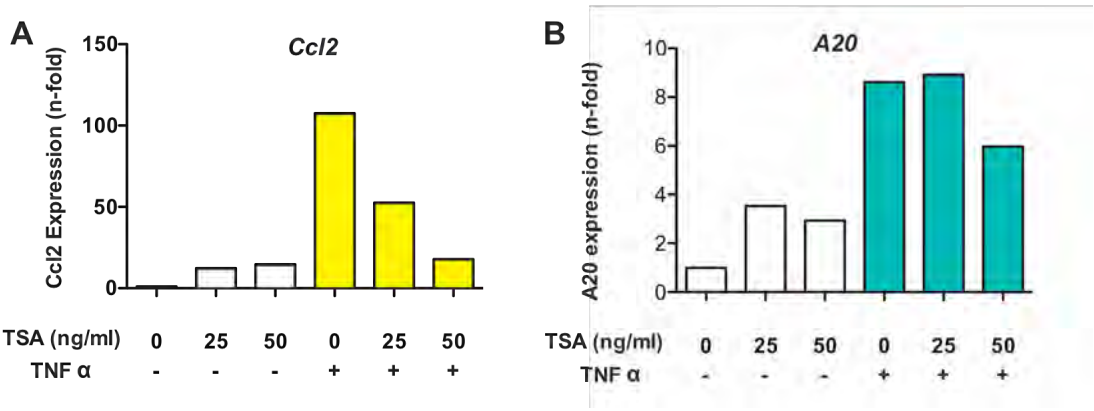


Figure 3.11. Effects of TSA on NF-κB-regulated gene expression in MIN6 cells. The effects of 0-50 ng/mL of TSA on *Ccl2* (A) and *A20* (B) in MIN6 with or without TNFα (200U/mL) stimulation for 4h. Data represent one experiment.

3.2.11 Induction of *Birc3* with TNFα-stimulated anti-apoptotic and inflammatory genes

The precise biological role of BIRC3 in pancreatic beta cells is not well defined. Accumulating evidences show that BIRC3 participates in diverse roles including regulation of apoptosis, as ubiquitin ligases, modulators of signal transduction, immune regulation, cell cycle regulation and embryogenesis. Of note, many of these processes involves the TNFα-mediated NF-κB signalling pathway (Bertrand et al., 2009; Bertrand et al., 2011; Bertrand et al., 2008; Chu et al., 1997; Chua et al., 2007; Gardam et al.; Gill et al., 2009; Hu and Yang, 2003; Huang et al., 2000; Jin and Lee, 2006; Mahoney et al., 2008; Park et al., 2004; Rothe et al., 1995; Shafey et al., 2006; Shu et al., 1996; Varfolomeev et al., 2007; Varfolomeev et al., 2008; Vince et al., 2007; Wang et al., 1998; Zhao et al., 2011a).

Beta cells are known to express a range of inflammatory and survival genes in response to cytokine stimulation. We examined the expression levels of a few known NF-κB-induced candidate genes in this study. *A20* represents a cytokine-induced early immediate response gene that has been previously shown to protect beta cells from cytokine-dependent apoptosis (Grey et al., 1999; Liuwantara et al., 2006). The inflammatory genes *Ccl2, Icam-1* and *Cxcl10* are examples of some of the most highly expressed cytokine-induced early immediate response genes in islet cells (unpublished data) that can contribute to beta cell inflammation and apoptosis (Bradley et al., 1999; Camacho et al., 2001; Campbell et al., 1989; Chen et al., 2001; Frigerio et al., 2002; Martin et al., 2008; Prieto et al., 1992; Roep et al.; Schulthess et al., 2009; Shigihara et al., 2006; Uno et al.).

To test this, mouse islets and MIN6 cells were pre-treated with the NF-κB inhibitor (PDTC) prior to stimulation with TNFα. Gene expression was measured by qRT-PCR. We found TNFα stimulation led to increase of *A20* (2.5-fold, $p \leq 0.001$), *Ccl2* (3-fold, $p \leq 0.001$), *Cxcl10* (4-fold, $p \leq 0.01$) and *Icam-1* (4-fold, $p \leq 0.0001$) in mouse islets (Figure 3.12A). Additionally, inhibition of NF-κB activation prevented induction of these genes by TNFα (Figure 3.12A). Thus, NF-κB signalling regulates the expression of TNFα-induced pro-survival and inflammatory genes in beta cells.

Similar to mouse islets, TNFα stimulation also resulted in the increase of *A20* (5-fold, p≤0.05), *Ccl2* (60-fold, p≤0.0001), *Cxcl10* (20-fold, p≤0.01) and *Icam-1* (60-fold, p≤0.0001) expression in MIN6 cells, at higher levels than mouse islets. Furthermore, TNFα induction of these genes was also suppressed by PDTC (Figure 3.12B). Collectively, our data implied a model whereby TNFα activated NF-κB, which subsequently induced increase expression of a range of survival and pro-inflammatory genes including *Birc3,* in islets and beta cells (Figure 3.13).

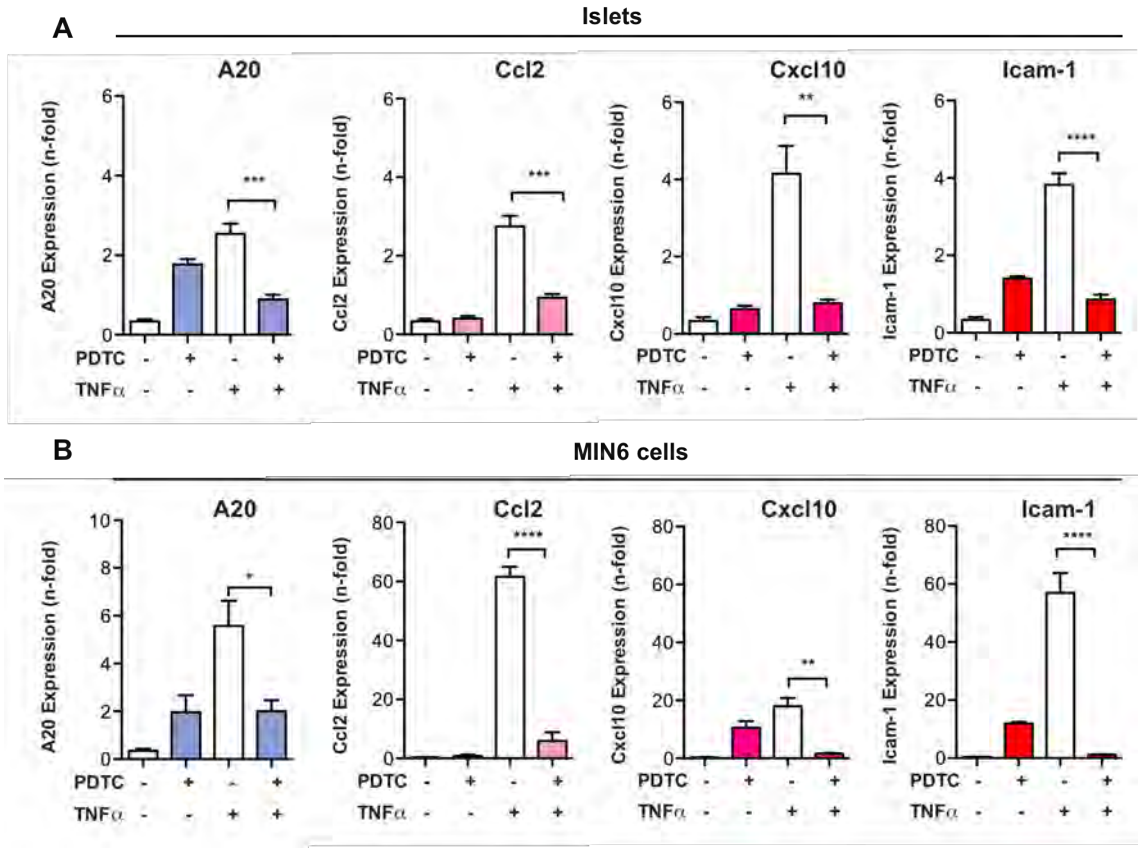


Figure 3.12. Induction of anti-apoptotic and inflammatory genes by TNFα in mouse islets and MIN6 cells. Expression of *A20, Ccl2, Cxcl10* and *Icam-1* in mouse islets (A) and MIN6 cells (B) either left untreated or pretreated with PDTC and stimulated with TNFα. Data represent mean ± SEM from three independent experiments. Statistical comparisons were made by single unpaired t-test between two groups. All differences are significant. (∗p≤0.05, ∗∗p≤0.01, ∗∗∗ p≤0.001 and ∗∗∗∗ p≤0.0001).

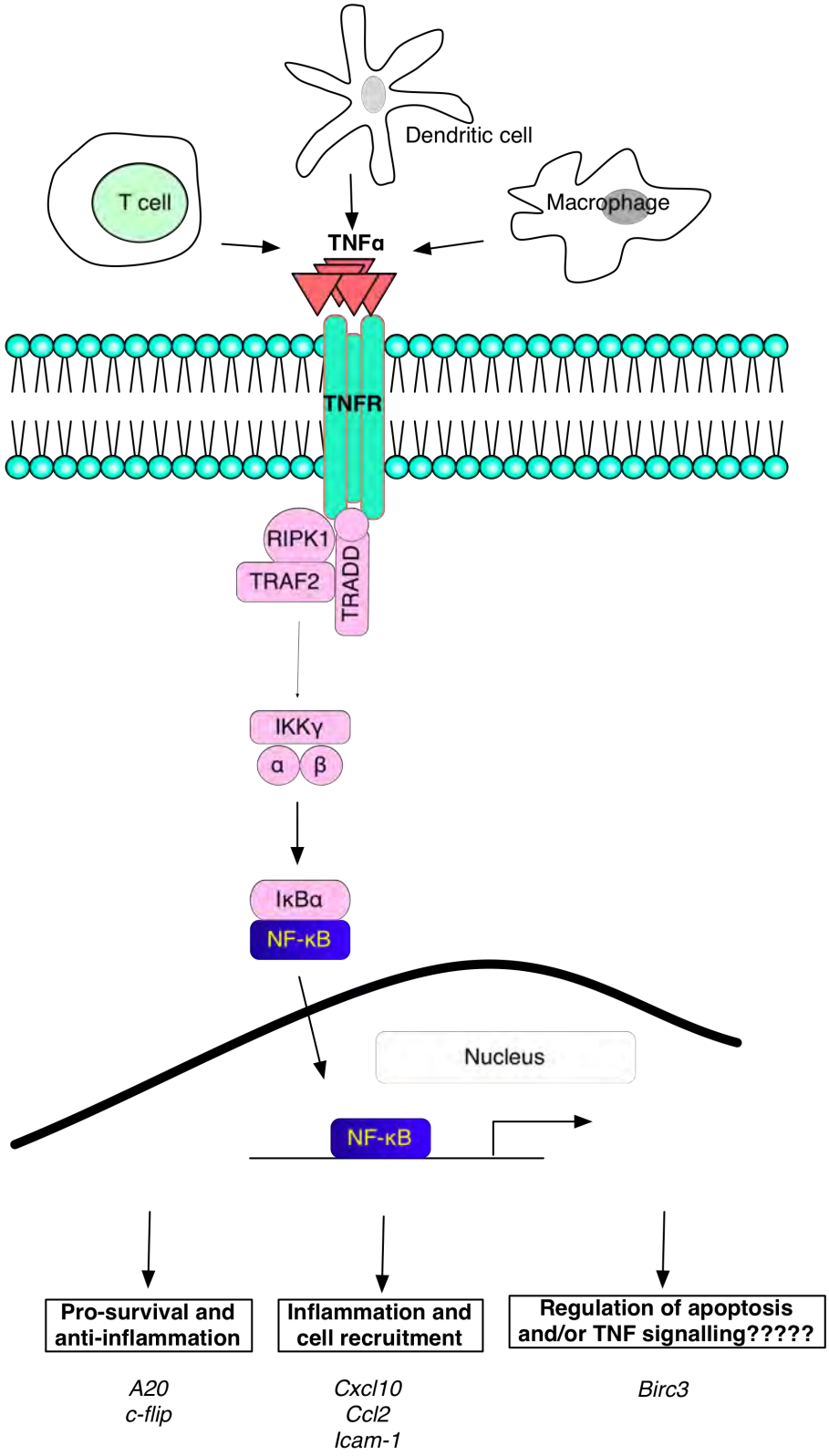


Figure 3.13. A proposed model of TNFα-mediated NF-κB signalling in pancreatic beta cells. TNFα secreted by T cells, dendritic cells and macrophages surrounding the islets stimulate TNFR signalling in beta cells within the islets. Activation of the TNF signalling cascade leads to NF-κB transcription, which is responsible for mediating the expression of genes involved in cell survival, inflammation, immune cell recruitment as well as *Birc3,* which role is not fully known in pancreatic beta cells.

3.2.12 Increased BIRC3 regulates NF-κB signalling in beta cells

Since BIRC3 is thought to play a major role in TNFα-mediated NF-κB signalling, we wondered if increased BIRC3 can feedback on TNFα-mediated NF-κB signalling in beta cells. To facilitate over-expression, the 1800 bp coding sequence of *Birc3* (NM_007464.3) was generated by gradient PCR using IL-1β-stimulated mouse islet cDNA as a template. The size of this product was confirmed on a 0.6% agarose gel (Figure 3.14A). To generate the *Birc3* expression vector, the resultant PCR product was subcloned into the pIRES2-eGFP vector and the sequence of *Birc3* was verified by gene sequencing. Subsequently, MIN6 cells were transfected with either the pIRES2-eGFP control vector (EV) or 0.3-0.6 μg of pIRES2-eGFP-*Birc3* (eGFP-*Birc3*). The transfection efficiency of this vector was determined via flow cytometry analysis. Our analysis showed no GFP expression was detected in non-transfected MIN6 cells while the EV and eGFP-*Birc3* plasmids were expressed at 10.6% and 6.5% of total transfected MIN6 cells, respectively (Figure 3.14B). At this level of transfection efficiency, eGFP-*Birc3*-transfected MIN6 expressed *Birc3* in a range similar to TNFα-stimulated MIN6 cells (~10 to 12-fold, $p \leq 0.05$ and $p \leq 0.001$) (Figure 3.14C).

To test the effects of increased BIRC3 on NF-κB signalling, MIN6 cells were transfected with a luciferase reporter containing two tandem NF-κB responsive elements (NF-κB.Luc) plus either 0.6 μg of EV or 0.1-0.6 μg of eGFP-*Birc3*. *Birc3*-transfected MIN6 cells showed dose-dependent increase in NF-κB activation. The NF-κB-dependent reporter was induced by 2.5-fold ($p \leq 0.05$) in the presence of 0.6 μg of eGFP-*Birc3* (Figure 3.14D). When combined with TNFα stimulation, 0.6 μg eGFP-*Birc3* showed further increase in NF-κB reporter activity, from 3.4-fold with TNFα alone to 7-fold with eGFP-*Birc3* in MIN6 cells (Figure 3.14D). Thus, increased BIRC3 alone can induce NF-κB signalling and further enhance NF-κB signalling in the presence of TNFα.

3.2.13 Increased BIRC3 also activates the native NF-κB-driven A20 promoter

To elucidate how BIRC3 would modulate a native NF-κB promoter, the activity of *Birc3* on the NF-κB-driven *A20*-reporter (Liuwantara et al., 2006) was examined. β-TC_3 cells were transfected with 2 μg of *A20*.Luc and with either 0.25 μg of EV or eGFP-*Birc3*. Experiments were performed on β-TC_3 cells here because transfection of *A20*-reporter was previously optimised in our laboratory on this cell line (Liuwantara et al., 2006). We found eGFP-*Birc3* increased the *A20* reporter by ~4-fold ($p \leq 0.01$) (Figure 3.14E). Additionally, the *A20*-reporter activity was increased from 7-fold ($p \leq 0.001$) for TNFα alone to 9.5-fold with TNFα plus eGFP-*Birc3* (Figure 3.14E), demonstrating that increased BIRC3 levels were sufficient to drive NF-κB activity and enhance TNFα-induced NF-κB signalling.

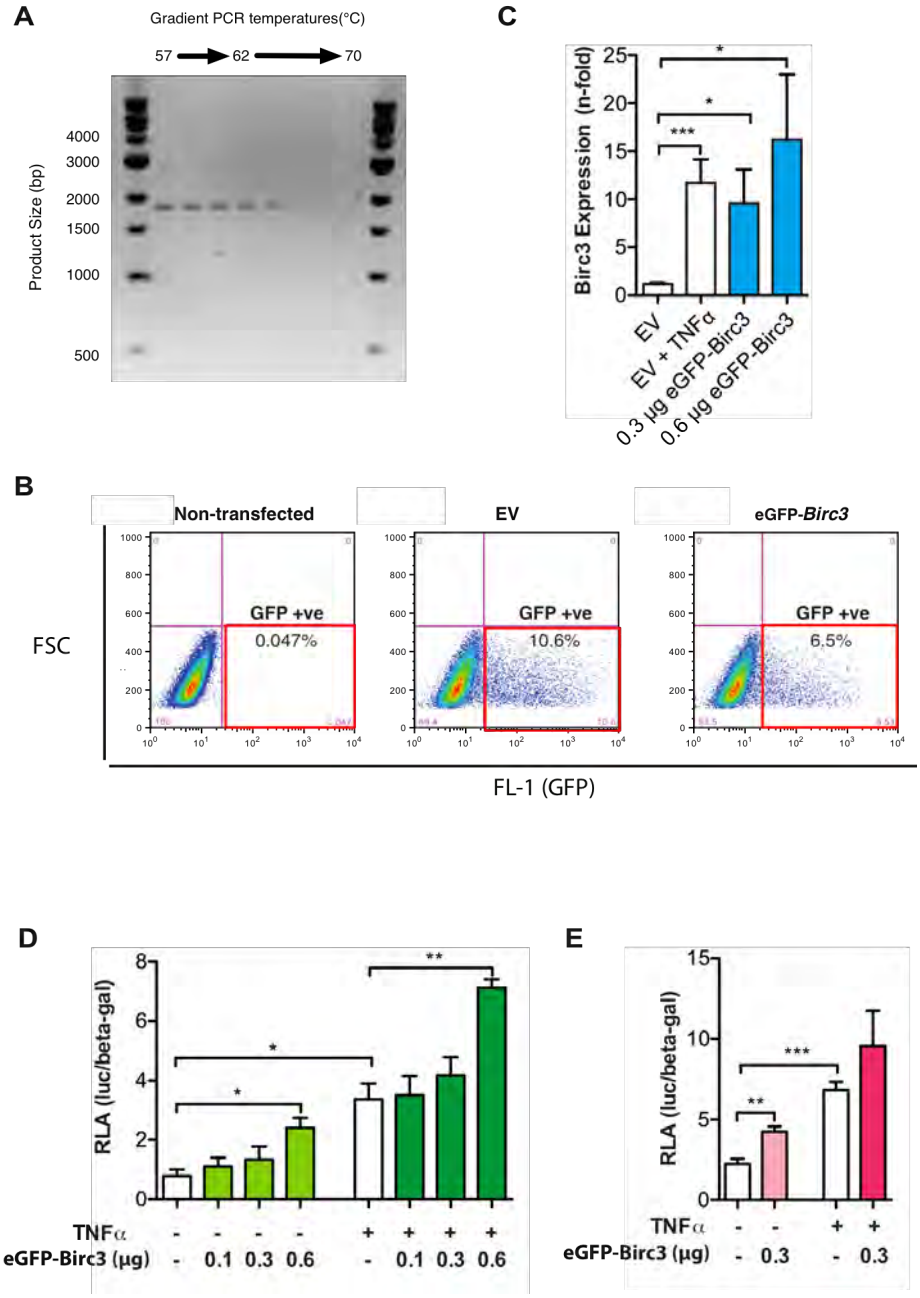


Figure 3.14. Increased BIRC3 regulates NF-κB signalling in MIN6 cells. (A) The 1800 bp PCR product of the coding sequence of mouse BIRC3. (B) Transfection efficiency analysed by flow cytometry was determined by the percentage of GFP in non-transfected MIN6 cells, or cells transfected with either 2 µg EV or eGFP-*Birc3* for 24 h. Representative plots of triplicate experiments show similar percentages. (C) Endogenous expression of *Birc3* for MIN6 cells transfected with 0.6 µg EV for 16 h in the presence or absence of TNFα (200 U/ml) for 4 h and MIN6 cells transfected with 0.3-0.6 µg eGFP-*Birc3* for 16 h. Data represent mean ± SEM from six independent experiments. (D) Induction of the NF-κB reporter in MIN6 cells transfected with either 0.1-0.6 µg of EV or eGFP-*Birc3* for 16 h, in the absence or presence of TNFα (200 U/ml) for 8 h. Data represent mean ± SEM from three independent experiments. (E) Induction of the *A20* reporter in β-TC_3 cells transfected with either 0.3 µg of EV or eGFP-*Birc3*, in the absence or presence of TNFα (200 U/ml). Data represent mean ± SEM from four independent experiments. Statistical comparisons were made by single unpaired t-test between two groups. All differences are significant. (*p≤0.05, **p≤0.01 and ***p≤0.001).

3.2.14 Increased BIRC3 also regulates NF-κB signalling in NIH-3T3 cells

Since *Birc3* expression is also increased in TNFα-stimulated NIH-3T3 cells, we examined whether increased BIRC3 also modulated NF-κB signalling in these cells. NIH-3T3 cells were transfected with either 1 μg of EV or eGFP-*Birc3* and stimulated with TNFα. We found eGFP-*Birc3* expression increased endogenous *Birc3* expression in NIH-3T3 cells by 46-fold ($p \leq 0.001$) (Figure 3.15A). The addition of TNFα further increased *Birc3* to 60-fold ($p \leq 0.01$) compared to EV-transfected cells (Figure 3.15A). Similar to MIN6 cells, BIRC3 alone also activated the NF-κB reporter by 3-fold ($p \leq 0.001$), demonstrating that BIRC3 was sufficient to activate NF-κB signalling (Figure 3.15B). In the presence of TNFα, the addition of BIRC3 slightly increased NF-κB reporter activity from 3.9-fold with TNFα alone to 4.7-fold in the presence of high levels of BIRC3 (Figure 3.15B). Collectively, these results indicated that BIRC3 positively increased TNFα-mediated NF-κB signalling in mouse embryonic fibroblast cells just as it did in pancreatic beta cells.

3.2.15 Increased BIRC3 can enhance expression of select NF-κB-target gene

We predicted that BIRC3 would trigger activation of NF-κB and induce the transcription of endogenous NF-κB-target genes. In MIN6 cells, NF-κB-target gene levels remained unaffected with elevated *Birc3,* which may be a result from the poor transfection efficiency of eGFP-*Birc3* in MIN6 cells (6.5%) (Figure 3.14B). Therefore, we tested this on NIH-3T3 cells where eGFP-*Birc3* resulted in higher expression of *Birc3*. Unlike MIN6 cells, increased BIRC3 resulted in a 2-fold ($p \leq 0.05$) increase in *Cxcl10* expression (Figure 3.15E) but did not affect resting levels of *A20*, *Ccl2* and *Icam-1* (Figure 3.15C, Figure 3.15D and Figure 3.15F respectively). These data implied that some NF-κB-regulated genes were more sensitive to increased BIRC3 levels than others in the absence of TNFα. In addition, we found that TNFα-induced NF-κB-regulated genes also tended to increase with high levels of BIRC3 (Figure 3.15C-E), with the exception of *Icam-1* (Figure 3.15E). TNFα-induced *A20* increased from 10-fold to 13-fold, *Ccl2* from 11-fold to 15-fold and *Cxcl10* from 30-fold to 44-fold with TNFα alone and TNFα with eGFP-*Birc3,* respectively. Therefore, increasing BIRC3 in beta cells may selectively enhance basal *Cxcl10* and TNFα-induced *A20*, *Ccl2 and* Cxcl10 expression.

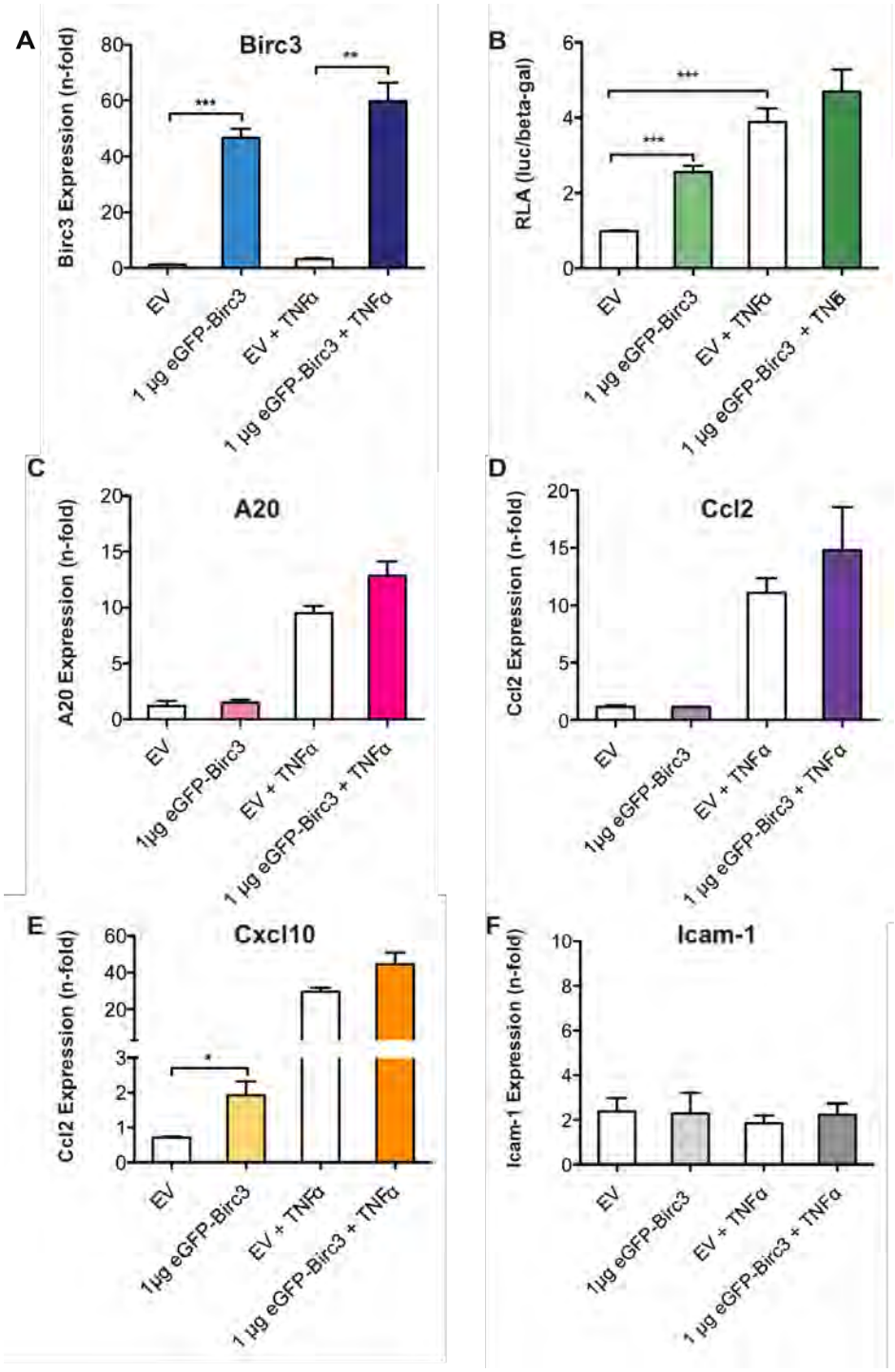


Figure 3.15 Increased BIRC3 regulated NF-κB signalling in NIH-3T3 cells. (A) Expression of *Birc3* by NIH-3T3 cells transfected with EV or 1 µg of eGFP-*Birc3* in the presence or absence of TNFα. Data represent mean ± SEM from three independent experiments. (B) Induction of the NF-κB reporter by NIH-3T3 cells transfected with either EV or eGFP-*Birc3*, in the absence or presence of TNFα. Data represent mean ± SEM from three independent experiments. (C-D) Induction of *A20*, *Ccl2*, *Cxcl10* and *Icam-1* in NIH-3T3 cells transfected with either EV or 1 µg of eGFP-*Birc3*, in the presence or absence of TNFα. Data represent mean ± SEM from three independent experiments. Statistical comparisons were made by single unpaired t-test between two groups. All differences are significant. ($*p \leq 0.05$, $**p \leq 0.01$ and $***p \leq 0.001$).

3.2.16 Increased TRAF2 can activate NF-κB signalling in MIN6 cells

As BIRC3 can associate and cooperate with the upstream TNFR signalling protein, TRAF2 (Rothe et al., 1995; Zheng et al., 2010), we proposed that increasing TRAF2 levels should also positively influence TNFα-mediated NF-κB signalling like BIRC3. Therefore, NF-κB reporter activation was studied in MIN6 cells transfected with either 0.9 μg of EV or *Traf2*. *Traf2*-transfected MIN6 cells displayed 10-fold ($p \leq 0.0001$) increase in NF-κB activation, thus agreeing with the idea that TRAF2 is sufficient to drive NF-κB signalling in beta cells (Figure 3.16). Further, high levels of TRAF2 also potentiated activation of the TNFα-mediated NF-κB signalling pathway from 2.4-fold with TNFα alone to 14-fold ($p \leq 0.001$) in the presence of high levels TRAF2 (Figure 3.16). These observations illustrated that similar to BIRC3, TRAF2 can also induce NF-κB activation and can further enhance TNFα-mediated NF-κB signalling in beta cells.

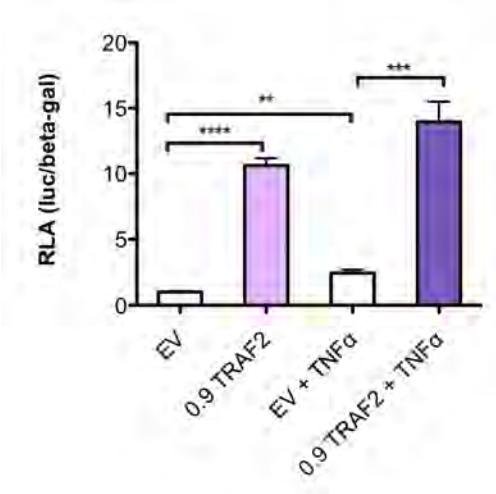


Figure 3.16. Increased TRAF2 regulated NF-κB signalling in MIN6 cells. Induction of the NF-κB reporter in MIN6 cells transfected with either EV or the TRAF2 expression vector for 16 h, in the absence or presence of TNFα for 8 h. Data represent mean ± SEM from three independent experiments. Statistical comparisons were made by single unpaired t-test between two groups. All differences are significant. (∗∗$p \leq 0.01$, ∗∗∗$p \leq 0.001$ and ∗∗∗∗ $p \leq 0.0001$).

3.2.17 TRAF2 enhanced the expression of select TNFα-induced NF-κB-target gene

So far, our data showed that in beta cells, BIRC3 can induce NF-κB activation and drive the expression of selected NF-κB-target genes. In addition, over expression of BIRC3 also enhanced TNFα-mediated NF-κB signalling and increased the expression of TNFα-induce NF-κB-target genes. This led us to question whether TRAF2 was also able to induce expression of NF-κB-target genes. NIH-3T3 cells were transfected with either 0.9 μg of EV or *Traf2* and the expression of downstream NF-κB-target genes were examined by qRT-PCR. We found increasing TRAF2 had no effect on the induction of basal NF-κB-target genes (Figure 3.17A-E). Similar to having high levels of BIRC3, TNFα-induced levels of *A20, Ccl2* and *Cxcl10* also showed trends to increase with more TRAF2 (Figure 3.17B-D). Different to BIRC3, TNFα-induced *Icam-1* levels were significantly higher than TNFα stimulated EV-transfected cells with *Traf2* (Figure 3.17E). These results indicated that although TRAF2 can activate NF-κB signalling, it is not sufficient to induce basal transcription of NF-κB-target genes by itself. In contrast, TRAF2 in the presence of TNFα can positively influence TNFα-induce gene expression such as *Icam-1*.

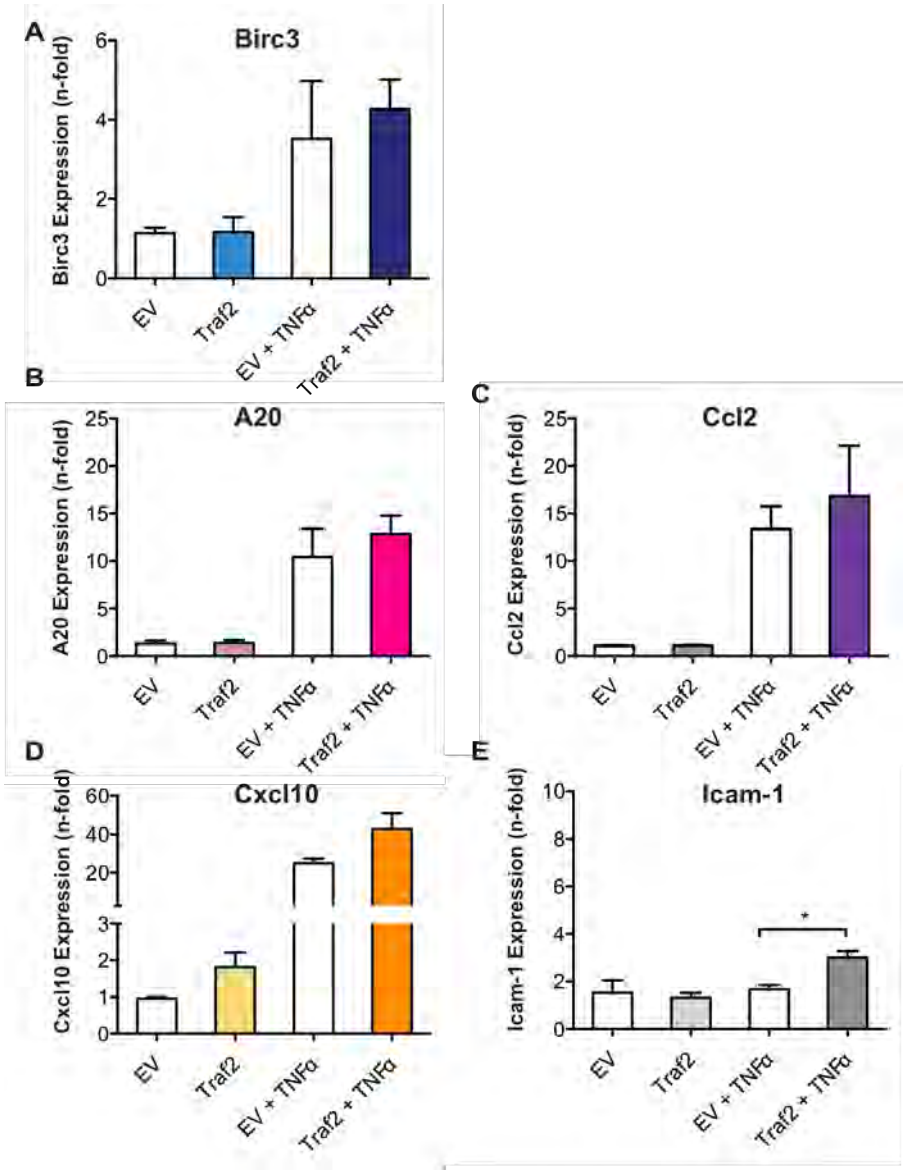


Figure 3.17 Increased TRAF2 on NF-κB-target gene expression in NIH-3T3 cells. (A-E) NIH-3T3 cells were transfected with either 0.9 μg of EV or *Traf2* and expression of NF-κB-target genes *Birc3*, *A20*, *Ccl2*, *Cxcl10* and *Icam-1* were analysed by qRT-PCR. Data represent mean ± SEM from three independent experiments. Statistical comparisons were made by single unpaired t-test between two groups. All differences are significant. ($*p \leq 0.05$)

3.3 Discussion

3.3.1 TNFα induces the expression of survival and inflammatory genes in beta cells

Apoptosis is a main form of beta cell death in T1D (Cnop et al., 2005; Mathis et al., 2001). The process of apoptosis is highly regulated and is determined by the balance between the expression and synthesis of apoptotic and anti-apoptotic genes and proteins (Figure 3.1) (Bach et al., 1997). Under conditions of stress, the beta cells react to their environment by expressing survival genes to counteract the detrimental effects that may cause cellular damage and apoptosis (Laybutt et al., 2002). Furthermore, cytokine-exposed beta cells are found to modify the expression of several genes involved in regulation of cell survival and apoptosis (Cardozo et al., 2001b; Cardozo et al., 2003; Eizirik et al., 2012). Indeed, this study found that TNFα-mediated NF-κB signalling in islets and beta cells regulates the expression of pro-survival gene (*A20*) as well as inflammatory genes (*Ccl2, Cxcl10* and *Icam-1*). Of interest, cytokine-stimulated islets and beta cells also specifically induce *Birc3* but not other members of its family. As BIRC3 has been implicated in apoptosis regulation and signal transduction pathways, which led to our interest to determine its function in this study.

3.3.2 Differential expression of BIRC members in response to TNFα

BIRC family genes show distinct patterns of expression amongst different cell types. Similar to our observations, human Jurkat T cells can induce *BIRC3* but not *BIRC2* following exposure to TNFα (Chu et al., 1997). Different to our data, fibroblast and endothelial cells such as human endothelial cell line (ECV 304), fibrosarcoma cells (HT1080), multiple myeloma and HeLa cells showed increase in both *BIRC2* and *BIRC3* with TNFα stimulation (Furusu et al., 2001; Furusu et al., 2007; Mitsiades et al., 2002; Wang et al., 1998). Furthermore, BIRC4 was also reported to increase transiently with TNFα stimulation in human eosinophillic acute myeloid leukaemia cells (AML14) (Qin et al., 2007), while primary cell cultures of human umbilical vein endothelial cells (HUVECs) and human skin

microvascular endothelial cells (HSMECs) showed simultaneous increase in *BIRC2*, *BIRC3* and *BIRC4* (Stehlik et al., 1998). In contrast, rat mesangial cells did not express *Birc2* and showed no changes in expression of *Birc2, Birc4* and *Birc5* with TNFα stimulation (Furusu et al., 2001). Our analysis of BIRC family genes in mouse embryonic fibroblast cells (NIH-3T3) and mouse endothelial cells (2F2B) suggested that this specificity of *Birc3* induction was not unique to beta cells. These data collectively show a diverse spread of BIRC family gene expression between different cell types and may reflect the different functions played by BIRC proteins within different tissues.

3.3.3 BIRC3 is regulated by NF-κB signalling

NF-κB-binding sites present in the *Birc3* promoter are responsible for up-regulation of *Birc3* transcripts in beta cells upon exposure to TNFα and IL-1β. Consistent with data from Jurkat T cells, the *Birc3* promoter is highly responsive to NF-κB activation in pancreatic beta cells (Chu et al., 1997; Mahoney et al., 2008). The *Birc3* proximal promoter contained a region that has three putative NF-κB binding sites and was responsive to cytokines including TNFα, IL-1β and a combination of cytokines consisting of TNFα + IL-1β + IFNγ. In MIN6 cells, the *Birc3* reporter was activated by p65 overexpression and was partially blocked by co-expression with the NF-κB inhibitor, IκBα. This demonstrated that NF-κB is sufficient and required for *Birc3* reporter activation in MIN6 cells. These data agreed with the findings by Sarkar and colleagues (Sarkar et al., 2009), although over-expression of IκBα did not inhibit cytokine-stimulated *Birc3* reporter activity as strongly as reported in their paper. It has been shown by electromobility shift assay (EMSA) that two of the NF-κB elements within the *Birc3* promoter (NF-κB#1 and NF-κB#3) can actively bind to the NF-κB p50/p65 heterodimer complexes in the presence of TNFα (Figure 3.5A) (Hong et al., 2000). As our cloned *Birc3*-reporter only contained NF-κB#1 and NF-κB#2, and NF-κB#1 is actively involved in *Birc3* transcription (Hong et al., 2000), this may explain the partial inhibition of the *Birc3* reporter by IκBα in our experiment (Figure 3.8B). In agreement with our promoter analysis, antagonizing NF-κB but not JNK or p38 pathways prevented TNFα-induced *Birc3* expression. Additionally, blockade of NF-κB activation by

overexpression of IκBα super-repressor also prevented TNFα-, IL-1β- and TNFα + IL-1β + IFNγ-induced *Birc3* expression. Together, these observations demonstrated that NF-κB is the master regulator of BIRC3 in beta cells at the promoter level.

3.3.4 The NF-κB pathway has dual role in regulating survival and death signals

The transcription factor NF-κB plays an important role in cytokine-dependent beta cell apoptosis. In some cases, blocking NF-κB signalling in primary islets and beta cell lines impedes cytokine-mediated apoptosis (Baker et al., 2001; Chang et al., 2003; Giannoukakis et al., 2000; Heimberg et al., 2001) and results in prolonged islet graft survival in transplantation (Ding et al., 2012). NF-κB mediates the expression of multiple genes that contribute to beta cell destruction. In addition, NF-κB can promote the invasion of mononuclear cells to the islets, generation of beta cell toxins, impairment of beta cell function and cause beta cell apoptosis (Amrani et al., 2000; Heimberg et al., 2001; Li and Mahato, 2008; Moriwaki et al., 1999; Suarez-Pinzon et al., 1999) through the induction of genes such as *Fas* (Darville and Eizirik, 2001), *iNos* (Kwon et al., 1995) and *Cox-2* (Sorli et al., 1998). Furthermore, our study confirmed that TNFα-induced inflammatory genes *Ccl2, Cxcl10* and *Icam-1* are regulated by NF-κB signalling. Thus, blocking NF-κB may be beneficial for beta cell survival in T1D.

Paradoxically, other NF-κB-regulated genes have been shown to inhibit apoptosis in beta cells through the expression of *c-Flip* (Cottet et al., 2002) and *A20* (Grey et al., 1999; Liuwantara et al., 2006). Some studies found that NF-κB activation is in fact required for islet cell survival (Chang et al., 2003; Sarkar et al., 2009; Thomas et al., 2006; Yang et al., 2000a). Hence, targeting NF-κB may prevent the expression of anti-apoptotic genes crucial for protecting beta cells from cytokine-mediated apoptosis. Collectively, these data suggested in beta cells, TNFα activates NF-κB, which plays a dual role in controlling the expression of anti-apoptotic and inflammatory genes alongside with *Birc3.* Thus, the survival of the beta cells in the presence of TNFα, may rely on the balance between anti-apoptotic and pro-apoptotic signals controlled by NF-κB. NF-κB-regulated BIRC3 has been reported

to protect glioma and multiple myeloma cells from TNFα-mediated apoptosis (Mitsiades et al., 2002; Zhao et al., 2011a). When NF-κB activation is inhibited, these cells succumb to TNFα-induced cell death because of the loss of *Birc3* expression. Therefore NF-κB-induced BIRC3 may defend beta cells from TNFα-mediated apoptosis. These data also suggests to us that examining the function of BIRC3 may reveal the full biological significance of NF-κB signalling in beta cells.

3.3.5 BIRC3 functions in the NF-κB positive feed-back loop in beta cells

This study presents a novel role for BIRC3 and the molecular mechanisms of TNF signalling in pancreatic beta cells. BIRC3 together with TRAF2, have been identified as key components of the TNFα-mediated NF-κB activation pathway (Rothe et al., 1995). Our data suggested that this is also true for beta cells since ectopic expression of BIRC3 is sufficient to activate NF-κB in beta cells (Chu et al., 1997). Further, increased BIRC3 can enhance NF-κB transcription in the presence of TNFα. This study also found that over-expression of BIRC3 could sufficiently induce the basal expression of the NF-κB-regulated gene, *Cxcl10*, and lead to subtle increase in TNFα-induced *A20, Cxcl10* and *Ccl2* levels. Consistent with the discoveries of BIRC3 as an anti-apoptotic gene (Chu et al., 1997; Sarkar et al., 2009; Wang et al., 1998; Zhao et al., 2011a), our study showed that BIRC3 may also provide subtle boosting of TNFα-mediated NF-κB signalling for added expression of anti-apoptotic genes such as *A20* to prevent islet inflammation and *c-flip* to protect cells from TNFα-induced apoptosis that may help tip the balance towards beta cell survival in the context of T1D.

However, BIRC3 may also promote beta cell inflammation by increasing levels of NF-κB-inflammatory genes. It is important to note that cells do possess added control on NF-κB signalling, by having inducible auto-regulatory pathways to negatively feedback on NF-κB signalling. For example NF-κB can induce the inhibitory IκB expression to shut down its own activation (Sun et al., 1993). In islets, the anti-apoptotic gene, *A20,* can also act to block NF-κB activation (Daniel et al., 2004; Grey et al., 1999; Jaattela et al., 1996; Liuwantara et al., 2006). Therefore, while BIRC3 may participate in a positive-feedback loop to provide added signal

for NF-κB under inflammatory stress, internal auto-regulatory networks are in place to regulate the level of NF-κB activation. In conclusion, BIRC3 may function as a positive regulator to fine-tune NF-κB activation in beta cells.

Furthermore, we found that increasing TRAF2 levels is sufficient to activate NF-κB signalling and potentiate TNFα-mediated NF-κB signalling in beta cells. However, over-expression of TRAF2 was insufficient to induce the expression of basal NF-κB genes, but could significantly enhance TNFα-induced *Icam-1* expression. A recent study suggests that TRAF2 must bind to BIRC3 for TNFα-mediated NF-κB signalling to proceed (Vince et al., 2009). In addition, biochemical analyses have shown that molecules of TRAF2 and BIRC3 bind in the ratio of 3:1 in solution (Zheng et al., 2010). These studies suggested that binding of BIRC3 to TRAF2 is required to appropriately drive the expression of downstream NF-κB signalling genes and that BIRC3 is a more potent inducer of TNFR signalling than TRAF2, since it is the limiting molecule in the TRAF2-BIRC3 complex. Therefore in beta cells, BIRC3 may bind to and work with TRAF2 to regulate TNFR signalling.

3.4 Conclusion

In conclusion, this study reports a novel role for BIRC3 and a model for TNF-induced NF-κB signalling in beta cells (Figure 3.18). In beta cells, TNFα induces NF-κB activation in response to inflammatory stress. The activated NF-κB up-regulates inflammatory genes including *Ccl2, Cxcl10* and *Icam-1*, and pro-survival genes like *A20* and *c-Flip,* to prevent activation of TNFα-mediated caspase-8 apoptosis and allow negative feedback on NF-κB activation to dampen inflammatory gene expression. In addition, NF-κB also induces *Birc* to fine-tune NF-κB signalling and boost NF-κB signalling downstream of TNFα. This is the first time such a role has been shown in beta cells. While BIRC3 alone is insufficient to induce expression of all the endogenous NF-κB-target genes, its role may be to associate with TRAF2 to allow NF-κB signalling to proceed and fine-tune TNFα-mediated NF-κB signalling in beta cells. Collectively, these findings suggested an important role for BIRC3 in T1D, where increased BIRC3 may contribute to enhanced NF-κB signalling, crucial for fine-tuning the balance between inflammatory and pro-survival genes that govern the beta cell's decision for life or death.

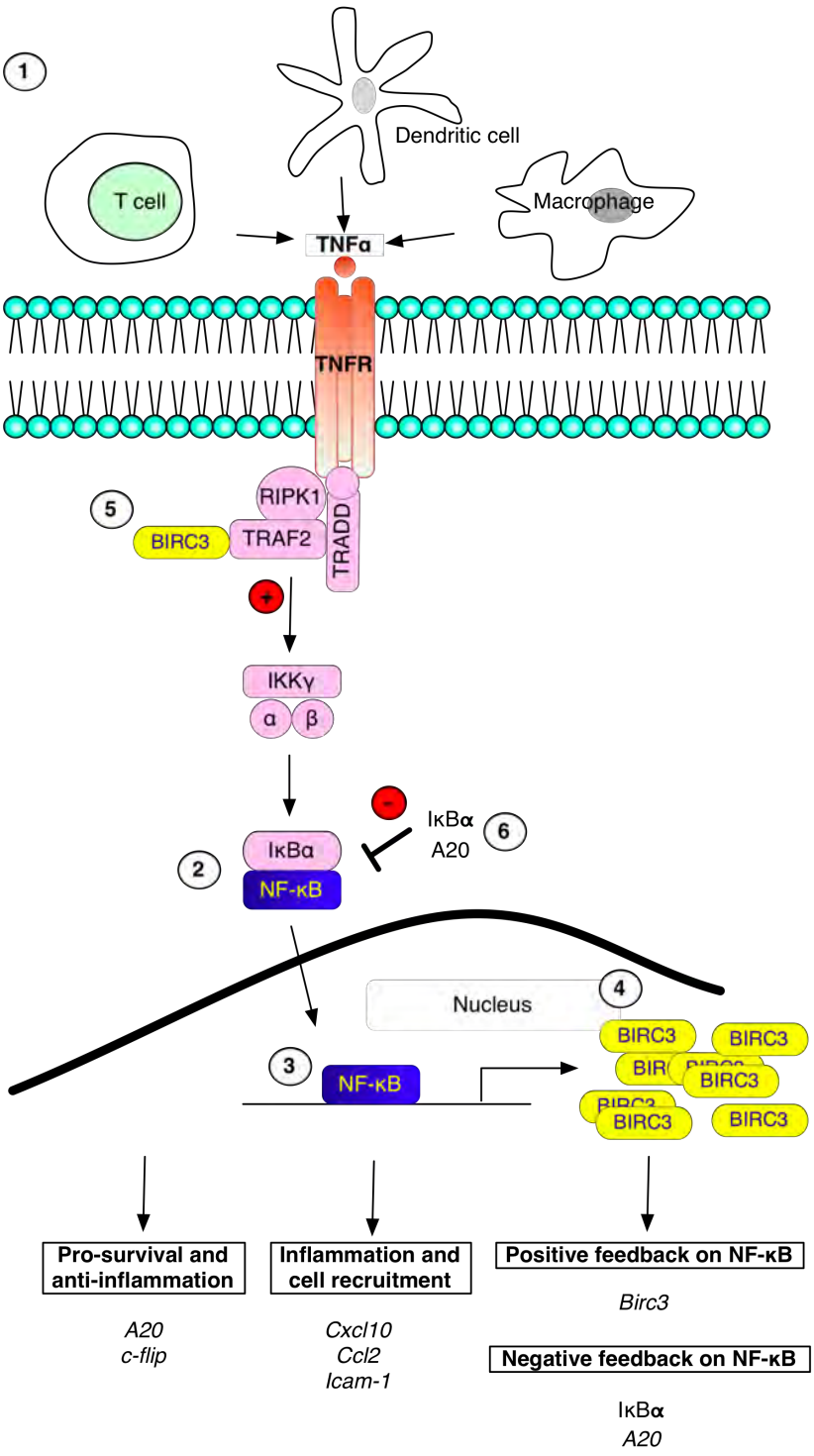


Figure 3.18 A proposed model of TNF signalling in pancreatic beta cells. (1) In T1D, lymphocytic infiltrates in the islets secrete TNFα. Beta cells respond to TNFα and activate complex signalling networks to modify gene expression to cope with inflammatory stress. (2) One such pathway is TNFα-mediated NF-κB activation. (3) NF-κB transcription leads to increase expression of genes that control survival and inflammation as well as (4) *Birc3*, which role is not well defined in beta cells. In this study we found that (5) elevated *Birc3* positively regulates TNFα-activated NF-κB signalling in beta cells. (6) Other internal self-regulatory networks are also in place to prevent over-activation of NF-κB signalling. NF-κB can induce the expression of *IκBα* and *A20* that can negatively feedback on NF-κB signalling.

4 Elucidating BIRC function in BIRC-knockout mouse islets

4.1 Introduction

In chapter 3 we established that TNFα induces NF-κB activation in pancreatic beta cells, which specifically drives the expression of the BIRC family gene, *Birc3*, but not the other members of the BIRC family. In our quest to understand why *Birc3* is up-regulated in beta cells, we discovered that ectopic expression of BIRC3 can induce NF-κB signalling and that BIRC3 actually participates in a feed-forward loop to further enhance NF-κB signalling. These data implied a novel role for BIRC3 as a positive regulator of TNFα-mediated NF-κB signalling in beta cells.

A flurry of work has implicated the importance of BIRC proteins as regulators of the NF-κB signalling pathway downstream of TNFR1 (Bertrand et al., 2008; Mahoney et al., 2008; Santoro et al., 2007; Varfolomeev et al., 2008). BIRC3 together with BIRC2 have been identified as key components of the TNF signalling pathway, through their association with TRAF2 (Rothe et al., 1995). TNFα mediates its actions through two distinct receptors TNFR1 and TNFR2, both of which are expressed on virtually all cell types (Brockhaus et al., 1990; Hohmann et al., 1989; Smith et al., 1990; Tartaglia et al., 1991). TNFR1 is predominantly responsible for TNF signalling in most cell types. It is mediated by the sequential formation of two complexes upon TNFR1 ligation (Micheau and Tschopp, 2003). Complex I forms with the recruitment of TRADD protein, which subsequently recruits TRAF2 and RIPK1 (Micheau and Tschopp, 2003). Within complex I, RIPK1 may be essential for activating the NF-κB pathway (Blonska et al., 2005; Devin et al., 2000; Kelliher et al., 1998; Li et al., 2006; Ting et al., 1996) while TRAF2 is required for activation of the JNK pathway (Habelhah et al., 2004; Lee et al., 1997; Nishitoh et al., 1998; Reinhard et al., 1997). Although an early report suggest that TRAF2 may also be responsible for regulating TNFα-mediated NF-κB and p38 activation (Carpentier et al., 1998). In addition, a secondary death complex,

referred to as complex II, comprising of FADD and caspase-8, triggers a pro-apoptotic cascade (Micheau and Tschopp, 2003). Normally, TNFα-stimulated cells are protected from the pro-apoptotic force of complex II by the expression of NF-κB-induced genes including *A20* and *c-FLIP* that are regulated by complex I. A20 and c-FLIP prevent activation of caspase-8 possibly at the level of complex II (Cottet et al., 2002; Daniel et al., 2004; Grey et al., 1999; Liuwantara et al., 2006). Thus, TNFα triggers a co-ordinated and complex cellular program regulating cell proliferation, inflammation and cell death.

BIRC proteins are thought to participate in TNFR signal transduction through ubiquitination and activation of the TNFR signalling cascade via molecules such as RIPK1 (Bertrand et al., 2011; Bertrand et al., 2008; Mahoney et al., 2008; Varfolomeev et al., 2008). Ubiquitination of RIPK1 allows the recruitment of downstream signalling complex proteins, which associates with and activates the kinase complex consisting of TAK1/TAB2/TAB3 (Besse et al., 2007; Blonska et al., 2005; Kanayama et al., 2004) and the IKK complex made up of NEMO/IKKα/IKKβ (Li et al., 1999; Solt et al., 2009; Zandi et al., 1997). IKKα and IKKβ then directly phosphorylate the NF-κB inhibitory protein IκB, which targets IκBα for degradation (Beg et al., 1993; DiDonato et al., 1995; Zandi et al., 1997). This allows the activation of TNFR1 and results in NF-κB-mediated expression of genes important for inflammation such as *Ccl2, Cxcl10, Icam-1* and cell survival such as *A20* and *c-Flip* as demonstrated in chapter 3. Hence BIRC proteins may play a significant role as regulators of TNFα-mediated NF-κB signalling and cell survival. This may be of relevance to pathological states such as in T1D, where NF-κB is important for regulating genes that modulate cell survival and inflammation. We therefore questioned whether BIRC3 is an important component for NF-κB signalling in beta cells. As this function of BIRC3 has never been examined in primary mouse beta cells, the aim for this chapter is to elucidate a function for BIRC proteins in pancreatic islets from BIRC-knockout mice and understand how TNFα signals in this cell type.

4.2 Results

4.2.1 BIRC3 is dispensable for gross beta cell function

Gain of BIRC3 expression positively regulated NF-κB signalling in beta cells. The effect of BIRC3 loss-of-function on the beta cells has never been examined. *Birc3*$^{-/-}$ mice were generated by targeting the Flippase Recognition Target (FRT) sites that flanked exon 2 and exon 3 of *Birc3* in the knock-in mice (WT) (Figure 4.1A) as previously described (Gardam et al.). Flp recombinase acts by deleting *Birc3* at the region flanked by FRT sites through site-specific recombination (Schlake and Bode, 1994; Zhu and Sadowski, 1995). Whole body suppression of BIRC3 was achieved by crossing WT mice with *ACTIN*-Flp transgenic mice (Figure 4.1B), leading to the removal of *Birc3*'s transcription start site in exon 2.

To verify gene deletion, we examined *Birc3* expression in *Birc3*$^{-/-}$ islets. TNFα-stimulated WT islets induced 9-fold increase of *Birc3* (Figure 4.1C) while *Birc3* mRNA was not detected in either non-stimulated or TNFα-stimulated *Birc3*$^{-/-}$ islets (Figure 4.1C). Having established that *Birc3* is not expressed in the islets of *Birc3*$^{-/-}$ mice, we next examined whether the loss of BIRC3 impacted on beta cell function.

Measurements of non-fasting blood glucose levels for 12-week old, male WT and *Birc3*$^{-/-}$ mice showed no significant differences. Both genotypes maintained non-fasting blood sugar levels at an average of 6.7 mmol/L (Figure 4.1D). Thus, the absence of BIRC3 in beta cells did not affect blood glucose homeostasis in mice. Further analysis of beta cell function was examined by IP-GTT. This was performed by IP-administration of 2 g/kg glucose on 12-week old, male mice, that were fasted for 16 h. IP-GTT analysis on WT mice showed normal glucose homeostasis. At 15 mins after glucose challenge, blood glucose levels were raised to 16 mmol/L and maintained at 30 min. By 60 min, blood glucose levels were decreased to 12 mmol/L and were further reduced to 6 mmol/L by 120 mins (Figure 4.1E). These data suggested that *Birc3*$^{-/-}$ mice did not reveal any abnormalities in beta cell

homeostasis under resting conditions. We interpreted this to mean that *Birc3* is not necessary for gross beta cell function.

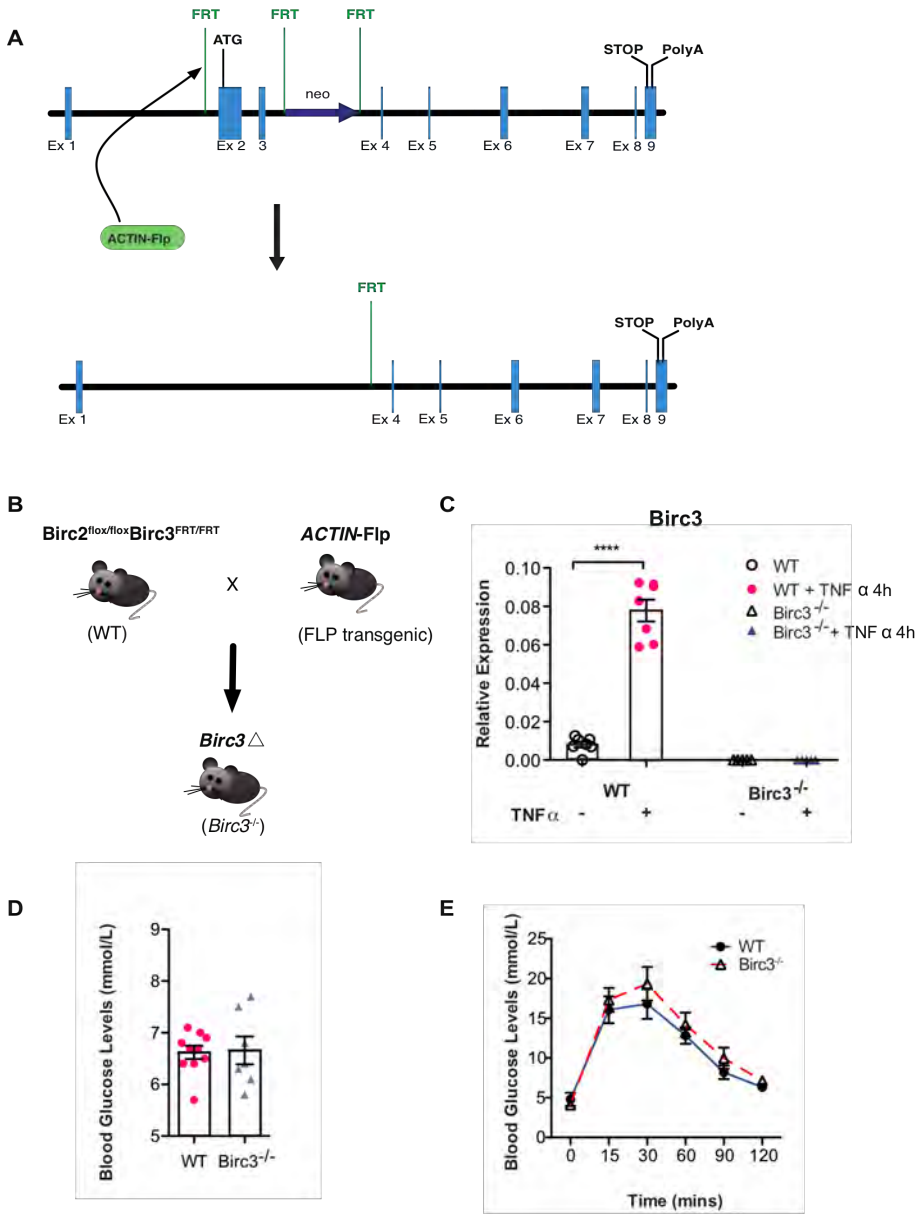


Figure 4.1. BIRC3 is dispensable for normal beta cell function. (A) The map of *Birc3* on mouse chromosome 9. Flp targets *Birc3* transcription start codon within exon 2 (ex 2) for gene deletion at the region flanked by FRT sites. (B) WT "knock-in" mice were bred with *ACTIN*-Flp mice to generate *Birc3*$^{-/-}$ mice. (C) Analysis of *Birc3* expression in WT and *Birc3*$^{-/-}$ mice in the absence and presence of TNFα stimulation for 4 h by qRT-PCR. Data represent mean ± SEM of *Birc3* expression compared to CPH from at least five independent experiments. (D) Blood glucose levels of non-fasted, 12-week old, male, WT and *Birc3*$^{-/-}$ mice. Data represent mean ± SEM from at least seven mice per group. (E) Blood glucose levels during IP-GTT of 12-week old, male WT and *Birc3*$^{-/-}$ mice fasted for 16 h. Data represent mean ± SEM from four mice per group. Statistical comparisons were made by unpaired t-test between two groups. All differences are significant. (****≤0.0001).

4.2.2 BIRC3 may be required for NF-κB signalling in islets

In chapter 3, we showed that BIRC3 positively regulates TNFα-mediated NF-κB signalling in beta cells. We next tested if the presence of BIRC3 is required for NF-κB signal transduction in beta cells. To do this, TNFα-mediated NF-κB signalling was examined in *Birc3*$^{-/-}$ islets. NF-κB activation is indicated by degradation of IκBα (Beg et al., 1993). TNFα-induced NF-κB signalling in WT or *Birc3*$^{-/-}$ islets were examined by Western blot analysis of IκBα levels. TNFα stimulation induced a rapid and transient degradation of IκBα in WT islets where IκBα was degraded at 15 mins and restored to baseline levels by 60 min (Figure 4.2A). In TNFα stimulated *Birc3*$^{-/-}$ islets IκBα degradation was delayed, but still functional (Figure 4.2A). These findings indicated that BIRC3 may be essential for the occurence of normal TNFα-mediated NF-κB signalling in islets.

4.2.3 BIRC3 fine-tunes NF-κB-regulated gene expression in islets

Next, the expression of NF-κB-regulated genes between WT and *Birc3*$^{-/-}$ islets were compared. To do this, the expression of *A20, Cc2, Cxcl10* and *Icam-1* were analysed in WT and *Birc3*$^{-/-}$ islets. These genes were previously established (Chapter3, Figure 3.12) as examples of NF-κB-regulated genes in pancreatic islets and beta cells. Compared to WT islets, TNFα-induced *A20* expression remained unchanged in the absence of BIRC3 (Figure 4.2B). However, the expression of TNFα-induced *Ccl2* and *Cxcl10* were significantly reduced by 27% and 63% respectively (Figure 4.2B). Conversely, *Birc3*$^{-/-}$ islets expressed 17% more TNFα-induced *Icam-1* than WT islets (Figure 4.2B). Therefore, while NF-κB signalling was apparent in the absence of BIRC3, the kinetics of NF-κB signal transduction and the expression of some NF-κB-regulated genes were affected. We interpreted these findings to suggest that BIRC3 may function to fine-tune TNF-induced gene expression in islets.

4.2.4 BIRC2 may compensate for the loss of BIRC3 in islets

A compensatory mechanism between BIRC proteins has been reported in splenocytes and thymocytes. A study found BIRC3 levels can increase in the absence of BIRC2 (Conze et al., 2005). In that study, BIRC2 is thought to modulate BIRC3 levels, by targeting BIRC3 for ubiquitin-mediated degradation. Therefore without BIRC2, BIRC3 levels were found to accumulate (Conze et al., 2005). This phenomenon is however not universal to all cell types and the converse might not be true. As an example, lung tissues from *Birc3*$^{-/-}$ mice were found to express normal levels of *Birc2* (Conte et al., 2006). These reports prompted the analysis of *Birc2* mRNA levels in *Birc3*$^{-/-}$ islets. We found *Birc3*$^{-/-}$ islets expressed normal endogenous levels of *Birc2*, thus suggesting that BIRC3 may not modulate BIRC2 levels in islet cells (Figure 4.2C). However, a difference was observed with TNFα stimulation. TNFα-stimulated *Birc3*$^{-/-}$ islets expressed ~50% more *Birc2* than WT islets (Figure 4.2C). These results indicated that BIRC2 might be compensating for loss of BIRC3, thus allowing TNFα-mediated NF-κB activation in *Birc3*$^{-/-}$ islets. Collectively, our analysis showed that BIRC3 is dispensable for NF-κB activation in islet cells, but may be required for fine-tuning the expression of some NF-κB-regulated genes. The occurrence of TNFα-mediated NF-κB signalling in *Birc3*$^{-/-}$ islets may be attributed to the elevated BIRC2 levels as a compensatory mechanism in islets.

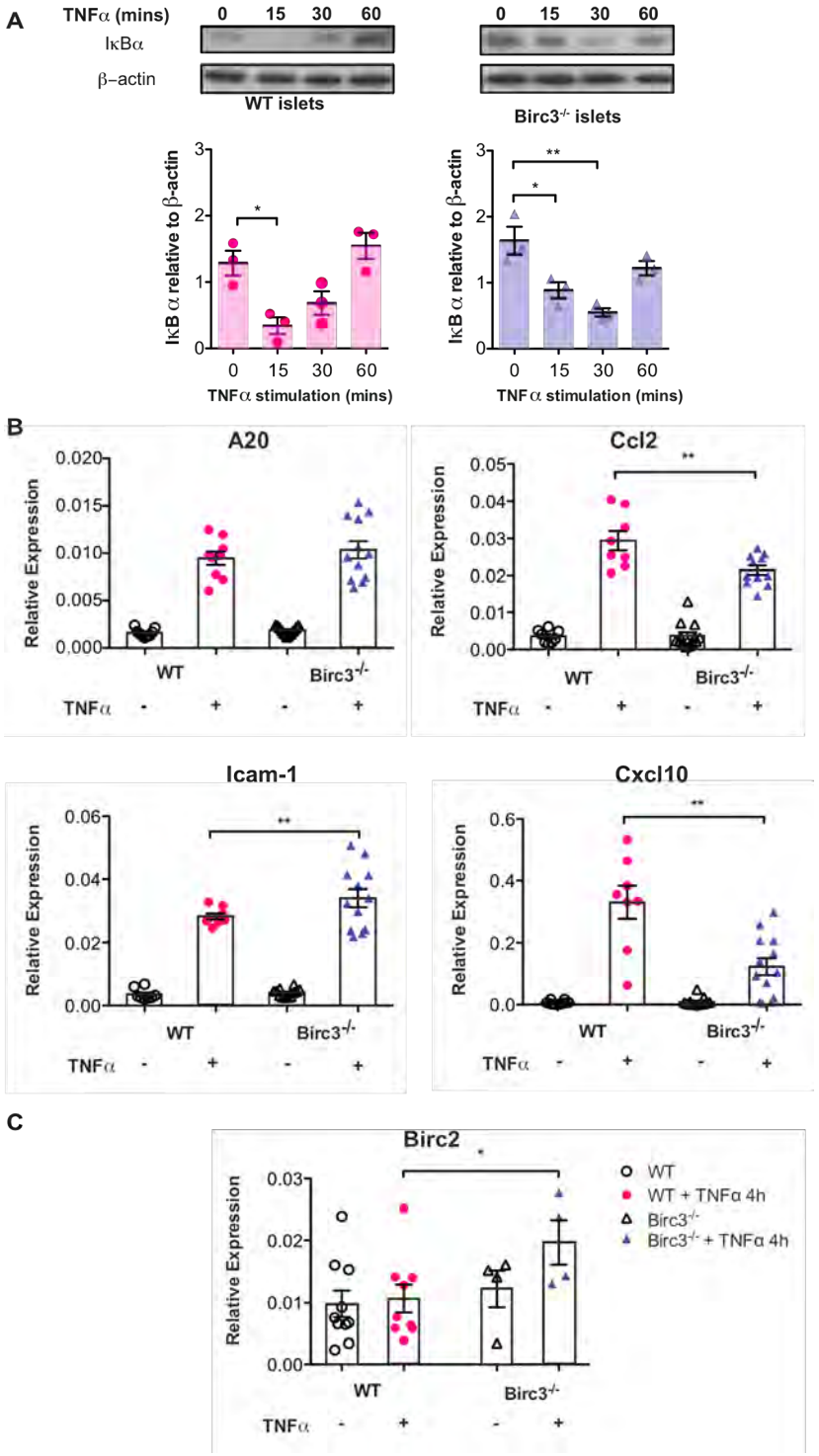


Figure 4.2. BIRC3 is redundant for NF-κB signalling in islet cells. (A) Western blot analysis of NF-κB activation measured by IκBα degradation in WT and *Birc3*$^{-/-}$ islets either left untreated or stimulated with TNFα at the indicated times. β-actin was used as a loading control. IκBα (39 kDa) was quantified by densitometry and representated as relative to β-actin. Data represent mean ± SEM from three independent experiments. (B-C) Analysis of NF-κB-regulated genes and *Birc2* expression in WT and *Birc3*$^{-/-}$ islets with or without TNFα for 4h, by qRT-PCR. Values from individual experiment are shown (n ≥ 4 per group). Bar represents mean ± SEM of gene expression relative to CPH. Statistical comparisons by single unpaired t-test between two groups. All differences are significant. (∗p≤0.05 and ∗∗p≤0.01).

4.2.5 Creating islet-specific deletion of BIRC2 and BIRC3 using HIP-*Cre* mice

NF-κB activation has been reported to remain functional in splenocytes, thymocytes, and embryonic fibroblasts (MEFs) derived from *Birc2*$^{-/-}$ mice, and in macrophages from *Birc3*$^{-/-}$ mice (Conte et al., 2006; Conze et al., 2005). Functional redundancy between BIRC2 and BIRC3 may explain the lack of NF-κB dysfunction in single deletion of BIRC2 or 3. Indeed, BIRC2 can compensate for the loss of BIRC3 and modulate intracellular signalling pathways in a number of cell types (Bertrand et al., 2009; Gardam et al.; Mahoney et al., 2008; Micheau and Tschopp, 2003; Vallabhapurapu et al., 2008; Varfolomeev et al., 2007; Varfolomeev et al., 2008; Vince et al., 2007; Zarnegar et al., 2008b). Hence, BIRC2 may be modulating TNFα-mediated NF-κB signalling in *Birc3*$^{-/-}$ islets.

To study the functional redundancy between BIRC2 and BIRC3, we generated double knockout *Birc3*$^{-/-}$*Birc2*Δβ-cell mice that are lacking of *Birc3* in their whole bodies and selectively lacking of *Birc2* in their beta cells (DKO). Analysis of TNFα-mediated NF-κB signalling in DKO islets may provide a better understanding of BIRC2 and BIRC3's function in TNF-signalling. DKO mice were generated using the *Birc3*$^{-/-}$ mice (showed in Figure 4.1) with lox-P sites within their *Birc2* sequence (Figure 4.3A). *Birc3*$^{-/-}$ mice were bred with HIP-*Cre* transgenic mice to obtain DKO-HIP mice (Figure 4.3B). Gene deletion by HIP-*Cre* examined by qRT-PCR showed that DKO-HIP islets expressed *Birc2* at 0-40% of WT islets (Figure 4.3C), while *Birc3* expression was not detected (Figure 4.3D). These indicated that HIP-*Cre* resulted in 60% reduction of *Birc2* expression in DKO-HIP islets.

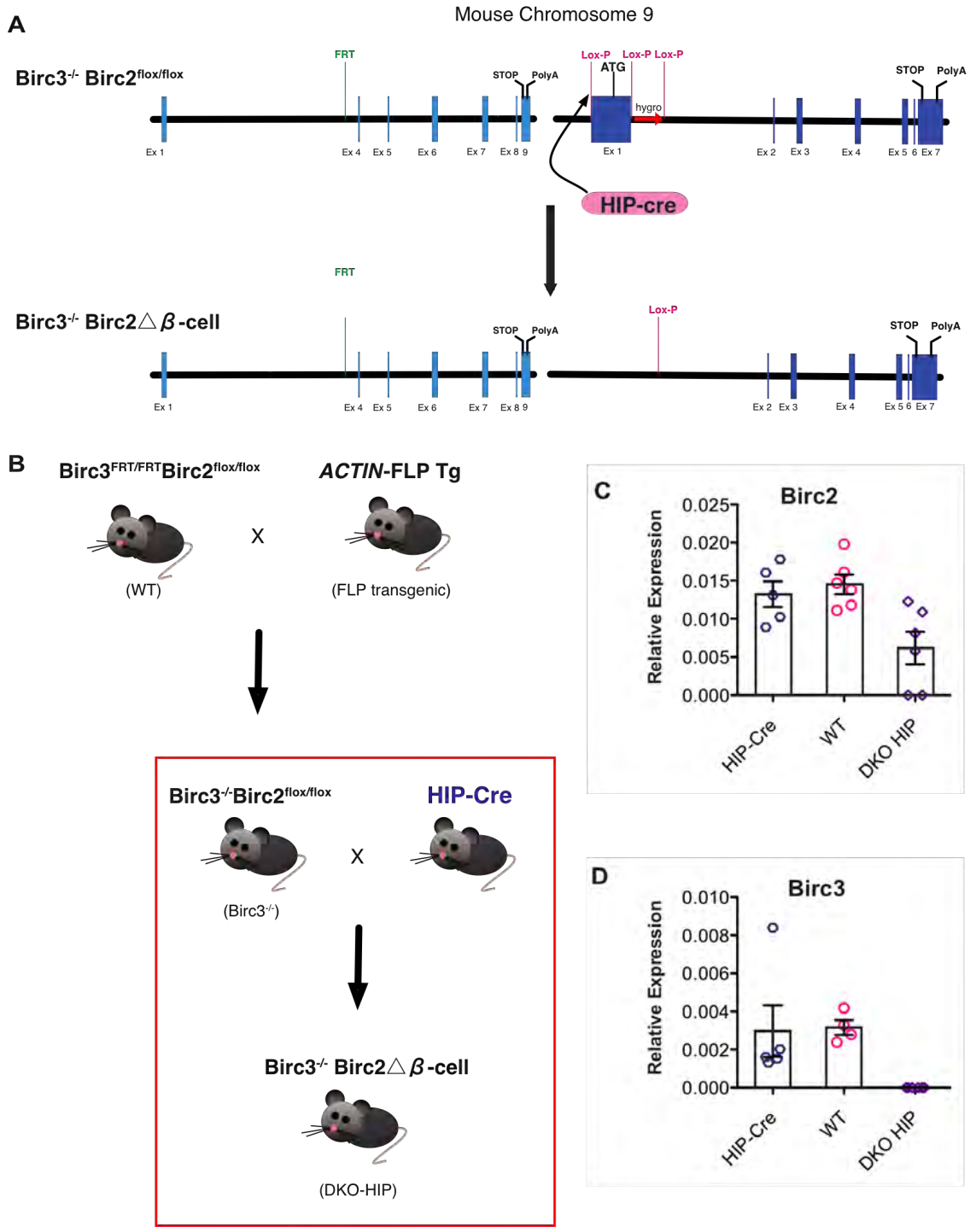


Figure 4.3. Conditional deletion of *Birc2* and *Birc3* in islets using HIP-*Cre* mice. (A) Map of *Birc2* and *Birc3* within chromosome 9 of *Birc3*$^{-/-}$ mouse. (B) Breeding strategy for the generation of *Birc3*$^{-/-}$*Birc2*Δβ-cell (DKO-HIP) mice. First, WT "knock-in" mice were bred with *ACTIN*-Flp. The resultant *Birc3*$^{-/-}$*Birc2*$^{flox/flox}$ (*Birc3*$^{-/-}$) mice were then bred with HIP-*Cre* mice. (C and D) Analysis of *Birc2* and *Birc3* expression in resting HIP-*Cre*, WT and DKO-HIP islets by qRT-PCR. Values from individual experiment are shown (n ≥ 5 per group). Bar represents mean ± SEM of gene expression relative to CPH.

4.2.6 The effects of Hip-*Cre* on beta cell function

Reports have shown that some strains of RIP-*Cre* mice have reduced beta cell mass, were less glucose tolerant and have impaired insulin secretion function compared to WT mice (Pomplun et al., 2007; Zhang and Kim, 1995). This prompted us to compare beta cell responses between HIP-*Cre* mice (*Cre*$^{+}$) and their *Cre*-deficient WT littermates (*Cre*-). Analyses of beta cell function were performed by IP-GTT using 2 g/kg of glucose on 12-week old male mice, which were fasted for 16 h. *Cre*- mice showed normal glucose homeostasis. At 15 mins after glucose challenge, plasma glucose levels of *Cre*- mice increased to 17.9 mmol/L (Figure 4.4A). Plasma glucose levels had decreased by 30 mins to 15.9 mmol/L and were further reduced to 8.7 mmol/L by 120 mins (Figure 4.4A). The glucose response of *Cre+* mice completely matched that of *Cre*- mice, suggesting that HIP-*Cre* mice have good glucose uptake response and beta cell function. We interpreted this to mean that our HIP-*Cre* mice exhibited normal beta cell homeostasis.

4.2.7 BIRC2 and BIRC3 are not required for beta cell function in DKO-HIP mice

Earlier in this chapter, we showed that the absence of BIRC3 did not impact on beta cell function in mice. The normal beta cell function in *Birc3*$^{-/-}$ mice may in fact be contributed by the presence of BIRC2. To test if BIRC2 was responsible for maintaining beta cell function in *Birc3*$^{-/-}$ mice, IP-GTT analysis was performed on DKO-HIP and compared to that of *Cre+* and WT mice. Our analyses showed that under resting conditions, DKO-HIP mice showed similar glucose response when compared to *Cre+* and WT (Figure 4.4B and Figure 4.4C respectively). Therefore, these findings indicated that BIRC2 and BIRC3 are not essential for normal beta cell function.

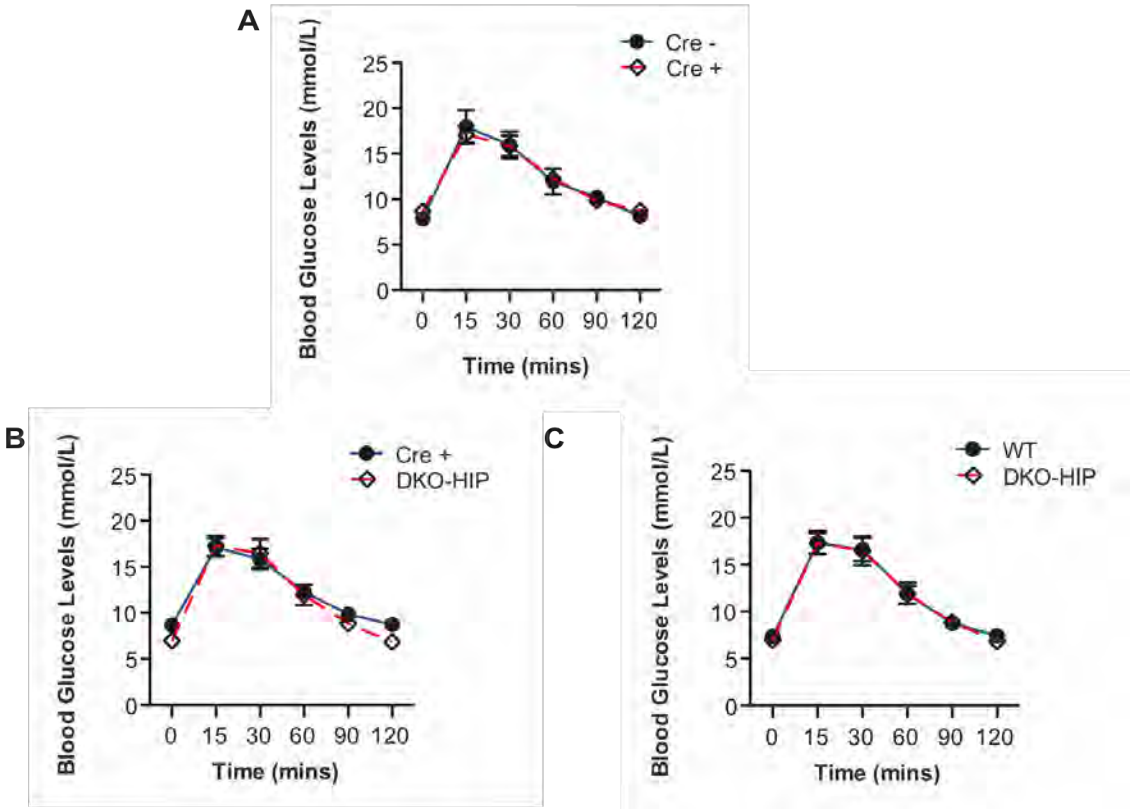


Figure 4.4 BIRC2 and BIRC3 are not required for beta cell function in DKO-HIP islets. (A) Blood glucose levels during IP-GTT for 12-week old, male *Cre*- (black circle, blue solid line) and *Cre*+ (white diamond, red dotted line) mice after fasting for 16 h. Data represent mean ± SEM from at least six mice per group. (B) Blood glucose levels during IP-GTT for 12-week old, male *Cre*+ (black circle, blue solid line) and DKO-HIP (white diamond, red dotted line) mice after fasting for 16 h. Data represent mean ± SEM from at least six mice per group. (C) Blood glucose levels during IP-GTT for 12-week old, male WT (black circle, blue solid line) and DKO-HIP (white diamond, red dotted line) mice, after fasting for 16 h. Data represent mean ± SEM from at least six mice per group.

4.2.8 BIRC2 and BIRC3 may function to maintain NF-κB signalling kinetics in islets

The requirement for BIRC2 and BIRC3 in TNFα signalling was next studied. This was examined by Western blot analysis of IκBα degradation in DKO-HIP islets. TNFα induced a rapid and transient degradation of IκBα in WT islets. Following TNFα stimulation, IκBα reaches maximum degradation at 15 min and was restored to baseline levels by 60 min (Figure 4.5A). Interestingly, DKO-HIP islets showed different kinetics of IκBα degradation compared to WT and *Birc3*$^{-/-}$ islets. Following TNFα stimulation, IκBα levels of DKO-HIP islets seemed to be reduced at 15 min. However, this may be due to lesser loading of the cell lysate at this time point, as less β-actin was observed in the 15 min lane compared to the others (Figure 4.5A). Therefore it is difficult to conclude if IκBα is degraded at 15 min by TNFα in DKO-HIP islets. Despite this, TNFα stimulation did not degrade IκBα at 30 mins in DKO-HIP islets, thus suggesting the kinetics of IκBα degradation may be delayed for DKO-HIP compared to WT islets. It was only at 45 min that TNFα-stimulated DKO-HIP islets started to show IκBα degradation, which did not rebound at 60 min. These findings suggested that DKO-HIP islets have delayed and prolonged kinetics of NF-κB activation compared to WT islets. We interpreted this to mean that in the absence of BIRC2 and BIRC3, islets have impaired NF-κB response to TNFα.

4.2.9 BIRC2 and BIRC3 fine-tune NF-κB-regulated gene expression in beta cells

The expression of downstream NF-κB-regulated genes was next analysed. Despite the impaired NF-κB activation kinetics in DKO-HIP islets at the protein level, TNFα-induced NF-κB transcription could still occur. Our results showed that DKO-HIP islets expressed TNFα-induced *A20*, *Ccl2* and *Cxcl10* levels to the same levels as WT islets (Figure 4.5B). We interpreted this to indicate that BIRC2 and BIRC3 may be dispensable for NF-κB signalling in islets. This finding was unexpected because TNFα-induced *Ccl2* and *Cxcl10* were reduced in *Birc3*$^{-/-}$ islets (Figure 4.2B). The differences in gene expression exhibited by *Birc3*$^{-/-}$ islets compared to DKO-

HIP islets indicated that BIRC2 and BIRC3 may play slightly different roles in fine-tuning NF-κB-target gene expression. However, like *Birc3*$^{-/-}$ islets, DKO-HIP islets showed 44% increased in TNFα-induced *Icam-1* levels compared to WT islets (Figure 4.5B), which indicated that BIRC2 and BIRC3 may play distinct and overlapping roles in controlling NF-κB-regulated gene expression in islet cells.

4.2.10 DKO-HIP islets show dysregulated stimulus independent gene expression

To our surprise, DKO-HIP islets also showed unexpected basal NF-κB-regulated gene expression. Resting DKO-HIP islets exhibited significant increase in basal *A20* (53%), *Ccl2* (61%), *Icam-1* (82%) and *Cxcl10* (85%) levels compared to WT islets (Figure 4.5B). These findings suggested that in addition to fine-tuning TNFα-induced gene expression; BIRC2 and BIRC3 may be negative regulators of basal gene expression in islets. Collectively, our data from DKO-HIP mice showed that BIRC2 and BIRC3 may contribute to fine-tuning the TNF signalling responses in islet cells. Such that in their absence, TNFα-mediated NF-κB signal transduction kinetics is delayed and expression of some NF-κB genes are dysregulated.

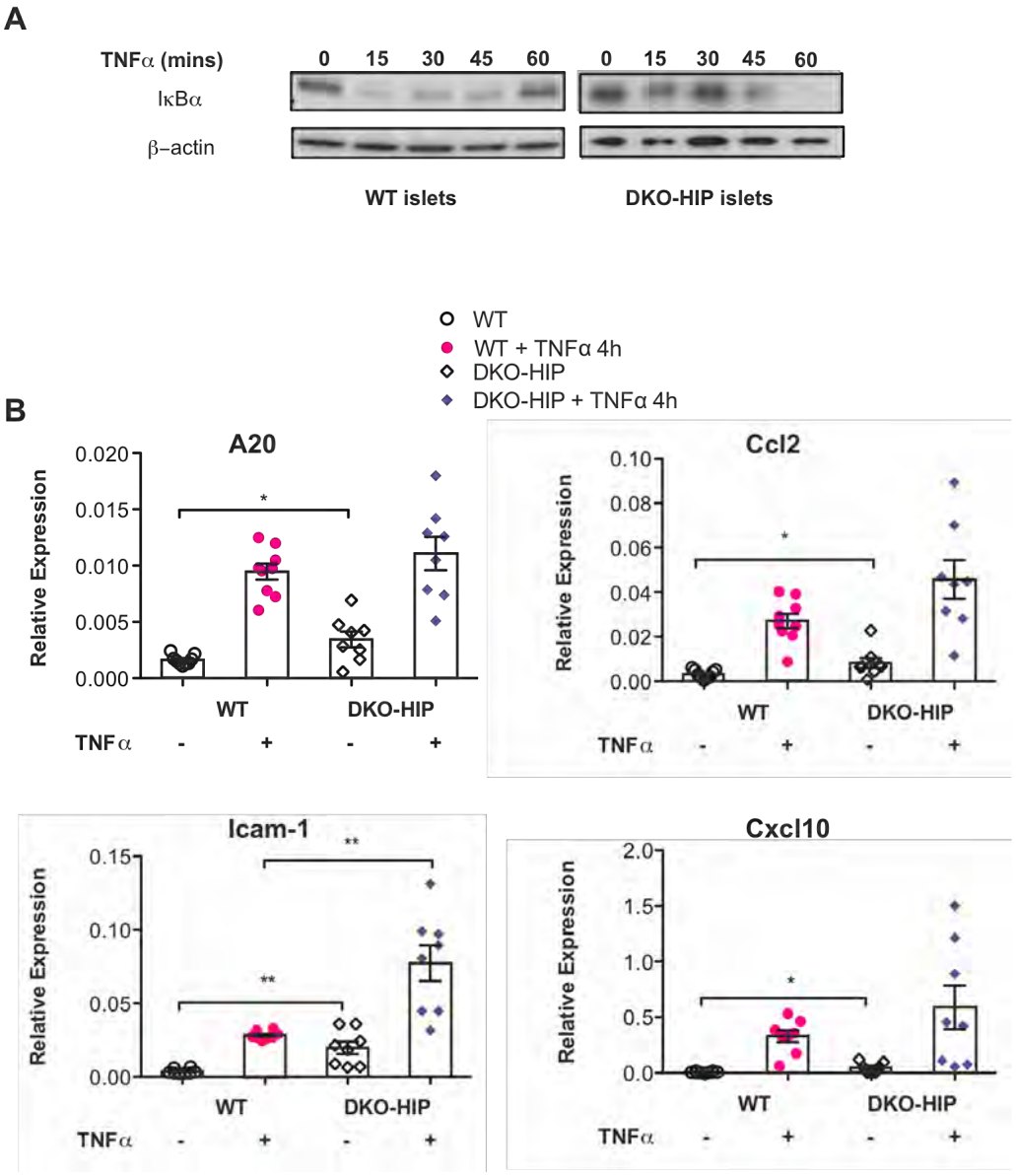


Figure 4.5. BIRC2 and BIRC3 are redundant for NF-κB signalling. (A) Western blot analysis of NF-κB activation measured by IκBα degradation in WT and DKO-HIP- islets either left untreated or stimulated with TNFα at the indicated times. β-actin was used as a loading control. Representative blots are shown. Duplicate experiments in each case generated similar results. (B) Analysis of NF-κB-regulated gene expression in WT and DKO-HIP islets in the absence or presence of TNFα for 4h by qRT-PCR. Values from individual experiment are shown (n ≥ 6 per group). Bar represents mean ± SEM of gene expression relative to CPH. Statistical comparisons were made by single unpaired t-test between two groups. All differences are significant. (*p≤0.05 and **p≤0.01).

4.2.11 Generation of beta cell specific deletion of *Birc2* and *Birc3* using RIP-*Cre* mice

Using DKO-HIP mice, our data showed that in contrast to the roles of BIRC2 and BIRC3 in other cell types, NF-κB signal transduction could proceed in the absence of BIRC3 and reduced BIRC2 in islets. Our analysis showed that DKO-HIP islets still expressed ~40% of *Birc2* compared to WT islets (Figure 4.3C). As BIRC2 and BIRC3 have been shown to be functionally redundant and BIRC2 has been shown to compensate in intracellular signalling pathways for the loss of BIRC3. Hence it is possible that the residual BIRC2 may have contributed to the regulation of TNFα-mediated NF-κB gene transcription in DKO-HIP islets, where DKO-HIP islets were found to expressed normal levels of TNFα-induced *A20, Ccl2* and *Cxcl10*. As *Birc2* expression was not efficiently suppressed in beta cells by HIP-*Cre*, DKO-HIP mice may not have provided us with the right conditions to study functional redundancy between BIRC2 and BIRC3 in islet cells.

Therefore, to fully appreciate the functional redundancy of BIRC2 and BIRC3 in TNF signalling, a stronger deletion of *Birc2* in beta cells was required. To achieve this, we utilised a mouse line where *Cre* expression is driven by the rat insulin II promoter (RIP) (Postic et al., 1999). It has been demonstrated by immunohistochemistry that RIP-*Cre* resulted in 82% of beta cells expressing Cre recombinase protein (Postic et al., 1999). Further, RIP-*Cre* mice have been shown to generate up to 95% gene deletion in the beta cells (Cantley et al., 2009). To do this, *Birc3*$^{-/-}$*Birc2*$^{flox/flox}$ mice were bred with RIP-*Cre* to generate DKO-RIP mice, that are lacking of *Birc3* in their whole bodies and selectively lacking of *Birc2* in their beta cells (*Birc3*$^{-/-}$*Birc2*Δβ-cell) as well as *Birc2* deletion in a small population of hypothalamic neurons (Choudhury et al., 2005) (Figure 4.6A). Our analysis found that RIP-*Cre* transgenic mice resulted in better deletion of *Birc2* compared to HIP-*Cre* mice (Figure 4.3C). In our hands, the DKO-RIP islets showed low levels of *Birc2* with RIP-*Cre* deletion, e.g. 0-20% of WT levels compared to 0-40% of WT with HIP-*Cre* mice; while *Birc3* mRNA was undetected (Figure 4.6B).

4.2.12 BIRC2 and BIRC3 are redundant for beta cell function in DKO-RIP mice

The requirements for BIRC2 and BIRC3 for glucose homeostasis in beta cells were examined in DKO-RIP mice. Comparison of blood glucose levels of non-fasting 12-week old, male WT and DKO-RIP mice showed no differences between the two genotypes (Figure 4.6C). Like DKO-HIP mice, DKO-RIP mice also showed normal glucose homeostasis under resting conditions, indicating their dispensability for physiological beta cell function (Figure 4.6D). Thus BIRC2 and BIRC3 are not required for beta cell function.

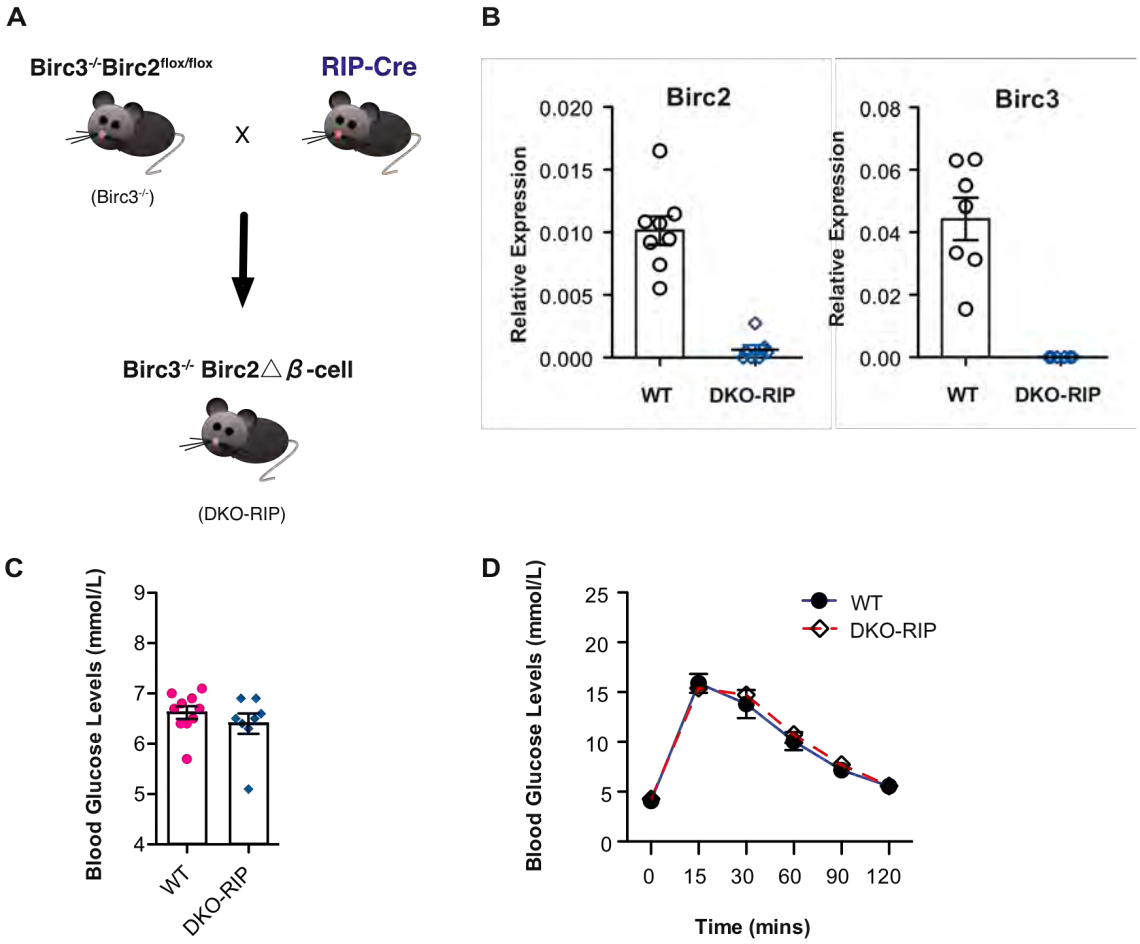


Figure 4.6. Beta cell specific deletion of *Birc2* and *Birc3* using RIP-*Cre* mice. (A) Breeding strategy to generate *Birc3*$^{-/-}$*Birc3*Δβ-cell (DKO-RIP) mice. *Birc3*$^{-/-}$*Birc2*$^{flox/flox}$ (*Birc3*$^{-/-}$) were bred with RIP-*Cre* mice to generate DKO-RIP mice. (B) Analysis of *Birc2* and *Birc3* expression in resting WT and DKO-RIP islets by qRT-PCR. Values from individual experiment are shown (n ≥ 7 per group). Bar represents mean ± SEM of gene expression relative to CPH. (C) Blood glucose levels for non-fasted 12-week old, male WT and DKO-HIP mice. Data represent mean ± SEM from at least eight mice per group. (D) Blood glucose levels during IP-GTT for 12-week old, male WT (black circle, blue solid line) and DKO-RIP (white diamond, red dotted line) mice. Data represent mean ± SEM from at least seven mice per group.

4.2.13 BIRC2 and BIRC3 fine-tune TNFα-mediated NF-κB signalling in beta cells

Our analysis of NF-κB activation in WT islets found IκBα levels were rapidly degraded by 15 mins of TNFα stimulation and restored to unstimulated levels by 60 mins (Figure 4.7A). Like DKO-HIP islets, TNFα-induced DKO-RIP islets exhibited impaired kinetics of IκBα degradation. Within the time points we used for TNFα stimulation, NF-κB signalling kinetics was significantly delayed in the absence of both BIRC2 and BIRC3 (Figure 4.7A). We interpreted this to mean that BIRC2 and BIRC3 are required to maintain the rapid and transient TNFα-induced NF-κB activation in beta cells.

4.2.14 BIRC2 and BIRC3 are required to fine-tune NF-κB-regulated gene expression

NF-κB-regulated gene expression was compared between WT and DKO-RIP islets. Loss of BIRC2 and BIRC3 in DKO-RIP islets led to significant disturbances in the regulation of TNF-target genes, a phenotype that was not seen in DKO-HIP islets. Analysis of DKO islets showed significantly higher expression of TNFα-induced *A20* (62%), *Ccl2* (71%) and *Icam-1* (95%) compared to WT islets, but *Cxcl10* induction was not more significant than WT (Figure 4.7B). These trends indicated that BIRC2 and BIRC3 may function to fine-tune TNF-induced genes in beta cells, by negatively regulating their expression.

4.2.15 Dysregulated gene expression in the absence of BIRC2 and BIRC3

In addition to the higher TNFα-stimulated gene expression, DKO-RIP islets also exhibited increased stimulus-independent expression of NF-κB-regulated genes. Like DKO-HIP islets, resting DKO-RIP islets showed significantly higher basal expression of *A20* (73%), *Ccl2* (87%), *Icam-1* (95%) and *Cxcl10* (94%) than WT islets (Figure 4.7B). These findings highlight a role for BIRC2 and BIRC3 in the regulation of NF-κB-driven genes even in the absence of TNFα stimulation. Our data from DKO-RIP mice reinforced the idea that BIRC2 and BIRC3 are important modulators that fine-tune the expression of NF-κB-regulated genes in beta cells

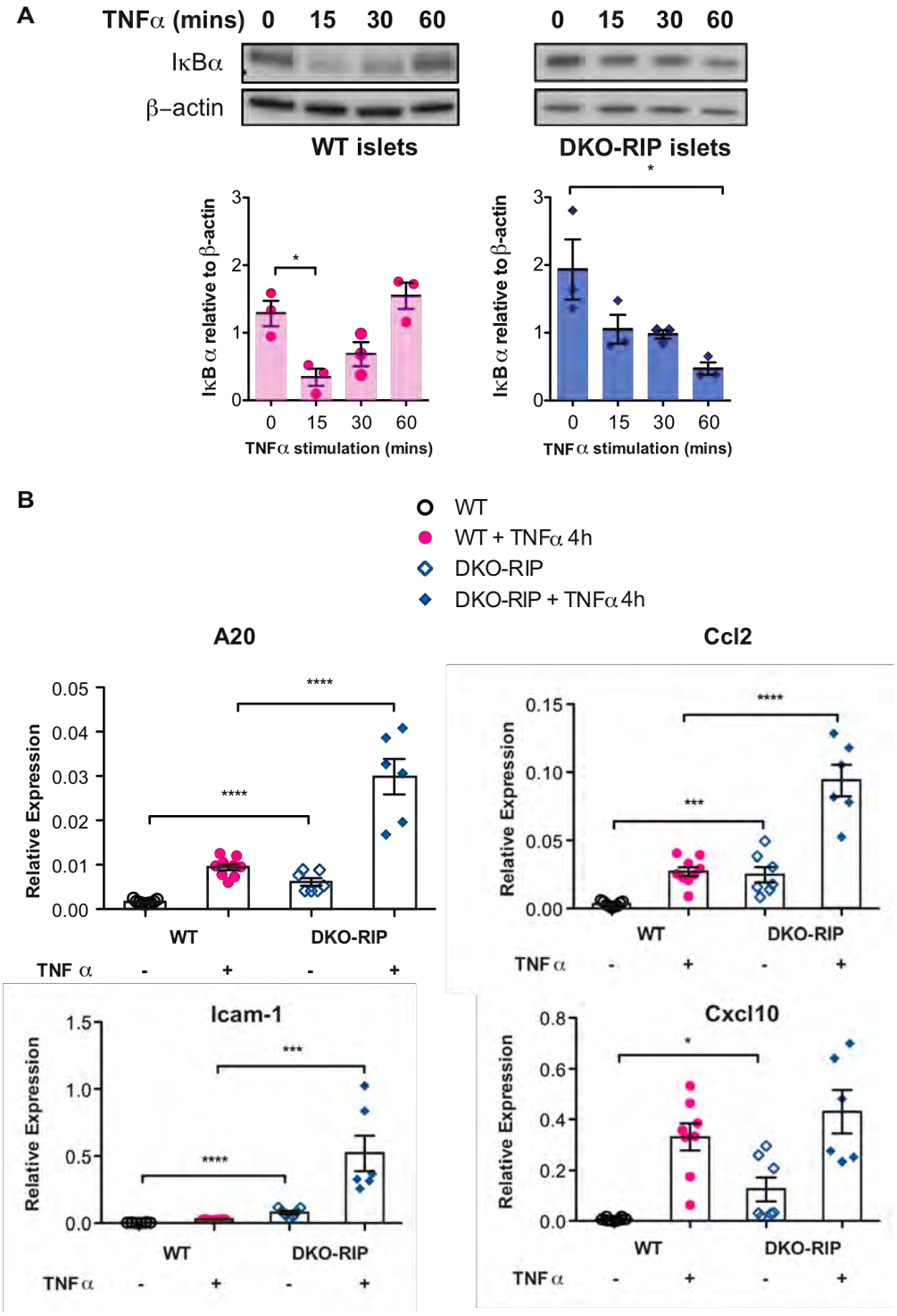


Figure 4.7. BIRC2 and BIRC3 fine-tune TNFα-mediated NF-κB signalling in beta cells. (A) Western blot analysis of NF-κB activation measured by IκBα degradation in WT and DKO-RIP islets either left untreated or stimulated with TNFα at the indicated times. β-actin was used as a loading control. IκBα (39 kDa) was quantified by densitometry and expressed as relative to β-actin. Data represent mean ± SEM from three independent experiments. (B) Analysis of NF-κB-regulated gene expression in WT and DKO-RIP islets with or without TNFα for 4h by qRT-PCR. Values from individual experiment are shown (n ≥ 6 per group). Bar represents mean ± SEM of gene expression relative to CPH. Statistical comparisons by single unpaired t-test between two groups. All differences are significant. (*p≤0.05, ***p≤0.001 and **** p≤0.0001).

4.3 Discussion

4.3.1 BIRC3 fine-tunes the expression of selected TNF-induced genes

Evidence for the participation of BIRC3 in TNFα-mediated NF-κB activation originated from studies demonstrating that BIRC3 can associate with TRAF2 and bind to TNFR (Rothe et al., 1995). BIRC2 and BIRC3 are thought to be requisite for RIPK1 ubiquitination, for TNFα-induced NF-κB signalling to proceed and for inhibition of TNFα-mediated apoptosis (Bertrand et al., 2011; Bertrand et al., 2008; Mahoney et al., 2008; O'Donnell et al., 2007; Schoemaker et al., 2002; Zhao et al., 2011a). This function stems from the RING domain of BIRC3, which acts as an E3 ubiquitin ligase to help maintain constitutive ubiquitination of RIPK1 (Bertrand et al., 2011; Bertrand et al., 2008). Our results from chapter 3 implied that this function of BIRC3 may be true for primary islets, as ectopic expression of *Birc3* is sufficient to activate NF-κB in beta cells. In this chapter, the requirement for BIRC3 in TNFα-mediated NF-κB signalling was analysed. Our study found that global suppression of BIRC3 did not affect gross beta cell function in mice. To our surprise, TNFα-induced NF-κB activation appeared to proceed with a mildly delayed kinetic in the absence of BIRC3. Thus, BIRC3 may be required for maintaining the kinetics of TNF-induced NF-κB activation in islet cells.

Furthermore, the expression of downstream TNFα-induced gene expression appeared to be deregulated in the absence of BIRC3. Our results revealed the complexities of the function of BIRC3 in beta cells. Compared to WT islets, *Birc3*$^{-/-}$ islets appeared to express less *Ccl2* and *Cxcl10* with TNFα stimulation but *Icam-1* levels were increased in the absence of BIRC3. The lowered expression of TNFα-induced *Ccl2* and *Cxcl10*, agreed with the idea from chapter 3 that BIRC3 served as a positive enhancer for TNFα-induced NF-κB signalling required for TNFα-induced gene transcription to reach its full potential. However the trend of increased TNFα-induced *Icam-1* levels in the absence of BIRC3 showed that it may also function as a negative regulator for some TNF-induced genes. Thus, in beta cells BIRC3 functions to allow immediate activation of NF-κB with TNFα and differentially fine-tune the expression of TNF-induced genes.

4.3.2 Evidence of functional redundancy between BIRC2 and BIRC3

BIRC2 and BIRC3 can perform redundant functions for TNFα-mediated NF-κB signalling in some cell types (Bertrand et al., 2009; Gardam et al.; Mahoney et al., 2008; Micheau and Tschopp, 2003; Vallabhapurapu et al., 2008; Varfolomeev et al., 2007; Varfolomeev et al., 2008; Vince et al., 2007; Zarnegar et al., 2008b). It has been reported that both primary muscle and C2C12 skeletal myoblasts cells are naturally occurring *Birc3*-deficient cells. This makes them a good model for the study of functional redundancy between BIRC2 and BIRC3. Expectedly, suppression of BIRC2 in these BIRC3-deficient muscle cells completely ablated TNFα-mediated NF-κB signalling (Mahoney et al., 2008). Further to this, BIRC2 deletion in C2C12 cells also prevented the expression of the NF-κB-regulated gene, *c-Flip* (Chang et al., 2006; Micheau et al., 2001). The attenuated NF-κB signalling in C2C12-*Birc2*$^{-/-}$ cells was however restored by ectopic expression of *Birc3* (Mahoney et al., 2008). Hence, these experiments demonstrated the functional redundancy of BIRC2 and BIRC3 in TNF signalling. Without BIRC3, BIRC2 is essential for TNFα-mediated NF-κB signalling to occur in muscle cells.

For other cell types such as primary hepatocytes and embryonic fibroblast (MEF) cells that express both BIRC2 and BIRC3, NF-κB activation remained impervious to single *Birc2*- or *Birc3*- siRNA treatments (Mahoney et al., 2008). Nonetheless, co-treatment of these cells with both *Birc2*- and *Birc3*-siRNA completely abolished TNFα-stimulated NF-κB signalling and RIPK1 ubiquitination. These findings further supported the idea that BIRC2 is required for TNFα-mediated NF-κB signalling, but BIRC3 can compensate for the loss of BIRC2. For beta cells, TNFα-induced NF-κB activation kinetics was slightly delayed but still apparent in the absence of BIRC3. We predicted that the residual NF-κB activation in *Birc3*$^{-/-}$ islets is probably contributed by BIRC2. Particularly, since *Birc3*$^{-/-}$ islets expressed twice more TNFα-induced *Birc2* than WT islets, implicating that BIRC2 levels may increase to compensate for the loss of BIRC3.

4.3.3 BIRC3 may function as a negative regulator of BIRC2

Our data showed that in the absence of BIRC3, TNFα stimulated BIRC2 levels are significantly increased, thus suggesting that BIRC3 may be a negative regulator of BIRC2. In some cell types, BIRC2 is found to control the levels of the RING-bearing BIRC proteins, BIRC3, BIRC4 and BIRC7, by targeting them for proteosome degradation (Cheung et al., 2008). Therefore, it is likely that in beta cells, BIRC3 may control BIRC2 levels by targeting it for proteosomal degradation. However, this conclusion cannot be made until further testing is performed. It has also been demonstrated that the promoter region of BIRC2 is occupied by the NF-κB p65/p50 homodimers. Additionally, blockade of NF-κB signalling with the inhibitor, bortezemib, prevented BIRC2 expression in cutaneous T-cell lymphoma Hut-78 cells (Juvekar et al., 2011), thus indicating that BIRC2 is an NF-κB-regulated gene. Therefore, these findings also demonstrated the point that BIRC3 plays a role to fine-tuning NF-κB-regulated gene expression.

4.3.4 The challenge of studying functional redundancy *in vivo*

Few studies explored the functional redundancy of BIRC proteins *in vivo*. This is because BIRC2 and BIRC3 are essential for the development and survival of mouse embryos. Concurrent global suppression of *Birc2* and *Birc3* in mice is embryonically lethal. The embryonic lethality of *Birc2*$^{-/-}$*Birc3*$^{-/-}$ mice can be rescued to birth with the deletion of TNFR1 while the deletion of RIPK1 afforded longer survival of embryos (Moulin et al., 2012). Therefore, the study shows BIRC2 and BIRC3 contribute to embryonic development and survival by modulating the TNFR1 pathway and RIPK1 activity, thus agreeing with the notion that BIRC2 and BIRC3 are important for TNF signalling and perform redundant functions. Further to the issue of embryonic lethality, generation of *Birc2*- and *Birc3*-double knockout mice is deemed an intricate task, as the coding sequences of *Birc2* and *Birc3* lie in close proximity in mouse chromosome 9 (Green and Flavell, 1999). Hence, it is not possible to generate double knockout mice by simply breeding single knockout mice of each genotypes together (Gardam et al.). Fortunately, Professor David Vaux and colleagues (Cell death and differentiation division, Walter and Eliza Hall Institute, VIC, Australia) created mice that permit either independent or

simultaneous suppression of *Birc2* and *Birc3*. These mice possessed lox-P and FRT sites "knocked-in" to the region flanking the transcription start sites of the *Birc2* and *Birc3* sequence respectively. The generation of these mice enabled the use *Cre* and flippase recombinase enzyme (Flp) to enable tissue specific deletion of *Birc2* and *Birc3* respectively, by site-directed recombination. Using this system, we were able to generate *Birc2*- and *Birc3*- beta cell specific knockout mice.

4.3.5 The challenge of achieving efficient gene deletion by *Cre* recombinase

Another challenge in this study, was the use of *Cre* recombinase to target deletion of *Birc2* in the beta cells of our *Birc3* global knockout mice. The *Birc3* knockout mice had lox-P sites "knock-in" to it which can be targeted by *Cre* for site specific recombination as described above. We found that deletion of *Birc2* in beta cells generated by HIP-*Cre* mice only led to ~60% knockdown of the gene. Although, the 60% deletion of *Birc2* in the beta cells resulted in dysregulated basal gene expression and may have impaired TNFα-induced NF-κB activation, the remaining 40% *Birc2* was still able to compensate and allowed normal expression of some TNFα-induced genes. Thus, this model was not ideal for us to study the functional redundancy of BIRC2 and BIRC3 in beta cells. The use of RIP-*Cre* mice, however, provided with efficient deletion of *Birc2* (~80% deletion) and allowed us to better understand the roles played by these BIRC proteins in primary beta cells. One may wonder why the RIP-*Cre* mice were not utilised in the first place. This was because while the RIP-*Cre* showed high expression of *Cre* specific for beta cells (Gannon et al., 2000; Postic et al., 1999), low levels of *Cre* were also expressed in a small population of hypothalamic neurons (Choudhury et al., 2005). Furthermore, some strains of RIP-*Cre* were glucose intolerant (Lee et al., 2006; Pomplun et al., 2007; Zhang and Kim, 1995), which led to our first choice of using the HIP-*Cre* line as a *Birc2*-deletor. Despite controversial reports, the strain of RIP-*Cre* mice used in this study (also known as RIP2-*Cre* or B6.Cg-Tg(Ins2-cre)25Mgn/J) showed normal beta cell function as demonstrated by our DKO-RIP mice. In support of our findings, Fex *et al.* have shown that glucose intolerance in RIP-*Cre* can be avoided through rigorous backcrossing onto the C57BL/6J background (Fex et al., 2007).

RIP-*Cre* mice expressed bacterial *Cre* recombinase with a nuclear localization sequence and a 2.1 kb fragment from the human growth hormone gene driven by a 668 bp fragment of the rat insulin II promoter (RIP2) (Jax mice, Bar Harbour, ME, USA). On the other hand, HIP-*Cre* was made by joining the 1.9-kb 5′ region of the human insulin promoter (HIP) to the 1.2-kb *Cre*-*Mlu*I fragment excised from the plasmid pMC-*Cre* (Gu et al., 1993; Hamilton-Williams et al., 2003; Sarvetnick et al., 1988). Another reason for the less efficient gene deletion by HIP-*Cre* compared to RIP-*Cre* could be due to the difference in their promoter elements. Not surprising, Hay and Docherty showed that insulin gene promoters of rodents are significantly different to that of human (Hay and Docherty, 2006). In particular, the human insulin promoter lacked the CCAAT box, COUP-TFII binding element and regulatory elements HNF-4α and STAT that are present in rodent promoters (Hay and Docherty, 2006). Although these elements are yet to be associated with the regulation of insulin expression, these findings point out the fundamental differences between human and rodent promoters, which may explain why *Cre* expressed by the human promoter in mouse is weaker than that expressed by the rat promoter in mouse.

Another reason that may explain the incomplete phenotype in DKO-HIP islets compared to DKO-RIP islets may be due to the loss of *Cre*-recombinase expression in transgenic mouse strains over time. It has been reported that Cre recombinase expression can be reduced with increasing number of breeding generations, leading to eventual loss of *Cre* expression in the inserted *Cre* cDNA (Schulz et al., 2007). When this happens, loss of *Cre* expression led to partial or complete loss of disruption of the protein of interest. In this situation, *Cre* expression could still be detected at the genomic level by tail-snips genotyping, misleading researchers into thinking that the protein of interest is being suppressed. Therefore, this study highlights the importance of screening for *Cre* expression within the beta cells. Although this phenomenon has not been tested in our DKO-HIP mice, it may explain the partial phenotype seen in our DKO-HIP islets.

4.3.6 BIRC2 or BIRC3 are required for TNFα-induced NF-κB activation

Despite the challenges, the use of *Birc3*$^{-/-}$, DKO-HIP and DKO-RIP mice enabled us to fully appreciate the function of BIRC2 and BIRC3 in TNF-signalling in primary beta cells. Furthermore, some phenotypes that were observed in DKO-HIP islets were duplicated in DKO-RIP islets, thus providing a robust method of studying the roles of BIRC2 and BIRC3 in beta cells. Our study found in primary beta cells, NF-κB activation by TNFα was mildly delayed but functional in the absence of BIRC3. However, when coupled with the absence of BIRC2, DKO-RIP islets showed markedly delayed TNFα-induced NF-κB activation, indicating that BIRC2 can compensate for the loss of BIRC3 in this pathway. Despite this, the presence of BIRC3 is still required for the fast response of TNFα-induced NF-κB activation seen in WT islets. Thus, the presence of either BIRC2 or BIRC3 is essential for TNFα-mediated NF-κB activation to proceed in beta cells. The effects of single *Birc2* deficiency on beta cells will be presented in the next chapter.

4.3.7 The contribution of p38 and JNK pathways in TNFα-induced gene transcription

Unexpectedly, downstream gene transcription was still able to proceed despite the severely delayed NF-κB activation in TNFα-stimulated *Birc2*$^{-/-}$*Birc3*$^{-/-}$ islets. In fact, TNFα-induced NF-κB-regulated inflammatory genes were increased with both BIRC2- and BIRC3- loss. This phenotype may have arisen from complex counter-regulation of other TNFα-induced pathways. In addition to NF-κB activation, TNFα also signals through the p38 and JNK pathways (Reinhard et al., 1997; Song et al., 1997; Thiefes et al., 2005; Thompson and Van Eldik, 2009). Moreover, p38 and JNK pathways have been reported to play a role in the regulation of some NF-κB-induced genes. For example, the NF-κB, JNK and p38 pathways are found to regulate TNFα-induced *Ccl2* expression (Chen et al., 2004; Ho et al., 2008). Particularly, c-Jun binding sites within the *Ccl2*-promoter are required for its gene induction in the presence of IL-1β (Wolter et al., 2008). Some data showed that c-Jun regulates *Ccl2* expression by participating in histone modifications and the recruitment of HDACs, NF-κB subunits and RNA polymerase II (Wolter et al., 2008).

These findings implicate a role for JNK and p38 signalling in regulating *Ccl2* expression in beta cells.

In addition, JNK was also reported to regulate *Cxcl10* expression (Alrashdan et al., 2012). In airway smooth muscle cells, both JNK and NF-κB are required to regulate cytokine-mediated *Cxcl10* expression (Alrashdan et al., 2012), implicating a role for JNK in modulating *Cxcl10* expression in beta cells. While NF-κB seems to play a dominant role in TNFα-induced *Icam-1* (Chen and Manning, 1995; Rajan et al., 2008), there is evidence suggesting a role for p38 and JNK in regulation of TNFα-induced *Icam-1* expression (Yang et al.). Together, these studies implicate a role for JNK and p38 signalling pathways in the regulation of the TNF-induced inflammatory genes that we have screened in this study. Hence, in the absence of BIRC2 and BIRC3, hyper-expression of *Ccl2, Cxcl10* and *Icam-1* may have occurred via the p38 and/or the JNK pathways. The roles of p38 and JNK in the regulation of these genes will be further explored in the next chapter.

4.3.8 BIRC2 and BIRC3 negatively fine-tune basal gene transcription

Further to the delayed TNFα-mediated NF-κB activation and accumulation of downstream TNF-induced genes, another major phenotype we discovered is the loss of fine-tuning of basal gene expression in beta cells without BIRC2 and BIRC3. Our analysis showed DKO islets exhibited dysregulated basal NF-κB-target gene expression. In line with our findings, a study found inhibition of BIRC2 and BIRC3 by BIRC antagonist resulted in increased basal levels of NF-κB-target genes including *TNFα, Il-8* and *Ccl2* (Varfolomeev et al., 2007). Further, it was reported that *Birc2* degradation induced by BIRC-antagonist in MEF cells also resulted in increased RIPK1 binding to TNFR1 and constitutive activation of canonical NF-κB signalling in the absence of a stimulus (Vince et al., 2007). Thus, these studies implied that loss of BIRC2 and BIRC3 could result in increased basal activation of NF-κB signalling in beta cells, manifested as increased basal NF-κB-target genes expressed by DKO-RIP islets. Overall, these findings supported the idea that BIRC2 and BIRC3 may be negative regulators that fine-tune basal NF-κB-regulated gene levels in beta cells.

4.4 Conclusion

We showed for the first time the complex roles played by BIRC proteins in TNF-signalling in islets and beta cells. Firstly, we found BIRC3 was not essential for TNFα-mediated NF-κB signalling in islet cells, as NF-κB signalling in *Birc3*$^{-/-}$ islets can be compensated by BIRC2, due to functional redundancy between the two proteins. Although, our study showed that BIRC3 is needed by primary beta cells to maintain normal tempo of TNFα-induced NF-κB activation and to fine-tune the expression of some TNFα-induced genes. Secondly, co-absence of BIRC2 and BIRC3 resulted in impaired TNFα-activated NF-κB kinetics suggesting that either BIRC2 or BIRC3 is required to regulate the kinetics of NF-κB activation. However, the impaired NF-κB response did not prevent TNF-induced gene transcription. In fact, basal NF-κB-target gene transcripts were accumulated in resting DKO islets and TNF-inducible genes were also increased. These data suggest that BIRC2 and BIRC3 function to fine-tune basal and TNFα-induced gene expression in beta cells.

Overall, our study shows a model whereby under resting conditions, BIRC2 and BIRC3 act to prevent spontaneous basal induction of some NF-κB-regulated genes. Under inflammatory conditions, beta cells require BIRC2 and BIRC3 for immediate/transient NF-κB activation in response to TNFα. Together, BIRC proteins function to fine-tune the expression of NF-κB-regulated genes and balance the signals of cellular survival and apoptosis in beta cells. Thus, BIRC proteins may be crucial in regulating the beta cell's fate under conditions of inflammatory stress.

5 Examining the mechanism of dysregulated gene expression in BIRC-knockout islets

5.1 Introduction

BIRC proteins are thought to be essential players for TNF signalling (Bertrand et al., 2008; Mahoney et al., 2008; Santoro et al., 2007; Varfolomeev et al., 2008). BIRC2 and BIRC3 can contribute to TNF signalling by ubiquitinating and activating RIPK1 to allow TNFα-mediated NF-κB signalling to proceed. Studies on other cell types suggest that BIRC2 and BIRC3 can function redundantly in TNFα-mediated NF-κB signalling (Mahoney et al., 2008; Varfolomeev et al., 2008). It has been reported that global suppression of BIRC2 or BIRC3 displayed no overt changes in TNF signalling. *Birc2*- and *Birc3*-null mice were asymptomatic, indicating that BIRC2 can compensate and allow NF-κB signalling to still occur in the absence of BIRC3 and *vice versa* (Mahoney et al., 2008; Varfolomeev et al., 2008).

In chapter 4, analysis of DKO islets showed unexpected function of BIRC2 and BIRC3. NF-κB activation kinetics determined by IκBα degradation was impaired in the absence of BIRC2 and BIRC3. To our surprise, despite the impaired NF-κB activation, the basal and TNFα-induced gene transcription in DKO islets were highly upregulated. These findings indicated that BIRC2 and BIRC3 may function as negative regulators of gene expression in primary beta cells. Therefore, one of the aims for this chapter is to study the contribution of BIRC2 in TNFα-mediated NF-κB signalling in beta cells with the use of *Birc2*$^{-/-}$ mice. As TNFα is known to signal through the NF-κB, JNK and p38 pathways (Reinhard et al., 1997; Song et al., 1997; Thiefes et al., 2005; Thompson and Van Eldik, 2009), we aimed to determine whether these pathways contribute to the regulation of basal and TNF-induced *A20, Ccl2, Icam-1* and *Cxcl10* expression. The result of this study will give us an idea of the pathways that BIRC proteins modulate to fine-tune the expression of these genes in beta cells.

5.2 Results

5.2.1 BIRC2 is not required for normal beta cell function

It has been shown in some cell types that BIRC2 can compensate for the loss of BIRC3 to modulate intracellular signalling pathways. We have shown in chapter 4 that this redundancy between BIRC2 and BIRC3 also applies to islets in the context of TNFα-mediated NF-κB signalling. In chapter 4, we showed that removing BIRC3 did not significantly impact on NF-κB signalling, while BIRC2/3-DKO islets presented with impaired kinetics of TNFα-mediated NF-κB signalling, where NF-κB activation was delayed and prolonged compared to WT islets. Based on these findings, we wondered whether BIRC2 alone is sufficient to compensate for BIRC3-loss in TNFα-induced NF-κB activation in islets. In addition, we also wanted to test whether dysregulated NF-κB signalling in DKO-RIP islets was a result of combined absence of BIRC2 and BIRC3 or of a single BIRC2-deficiency. To answer these questions, NF-κB signalling was examined in islets isolated from *Birc2*$^{-/-}$ mice.

Birc2$^{-/-}$ mice were generated by Cre deletion of *Birc2* via site-specific recombination. The Cre recombinase targets the start codon of *Birc2* within exon 1 of WT "knock-in" mice, in the region flanked by lox-P sites (Orban et al., 1992; Sauer, 1987; Sauer and Henderson, 1988) (Figure 5.1A). To do this, *ACTIN*-Cre transgenic mice were bred with WT "knock-in" mice to obtain *Birc2*$^{-/-}$ mice (Figure 5.1B). To verify gene deletion, *Birc2*$^{-/-}$ islets were screened for *Birc2* expression by qRT-PCR. Untreated and TNFα-stimulated WT islets expressed same levels of *Birc2,* while no *Birc2* was detected from TNFα-stimulated and unstimulated *Birc2*$^{-/-}$ islets (Figure 5.1C).

Next, *Birc2*$^{-/-}$ mice were analysed for beta cell function. We found no significant differences between the non-fasting, random blood glucose levels of 12-week old, male WT and *Birc2*$^{-/-}$ mice. Both strains of mice maintained non-fasting blood glucose levels at an average of 6.7 mmol/L (Figure 5.1D). We interpreted this to mean that BIRC2 does not have a role in blood glucose homeostasis in mice. Analysis of beta cell function by IP-GTT found WT mice showed normal glucose

homeostasis. Following glucose challenge, plasma glucose levels were raised to 15.4 mmol/L at 15 mins and continued to rise to 16.8 mmol/L at 30 mins before decreasing to 12.8 mmol/L at 60min (Figure 5.1E). At 120 min, blood glucose levels were at 6.3 mmol/L (Figure 5.1E). Although *Birc2*$^{-/-}$ mice showed slightly reduced blood glucose levels at 30 mins compared to WT, there was no significant difference between the general glucose response between WT and *Birc2*$^{-/-}$ mice (Figure 5.1E). Thus, *Birc2*$^{-/-}$ mice have normal glucose homeostasis, indicating that BIRC2 is dispensable for gross beta cell function.

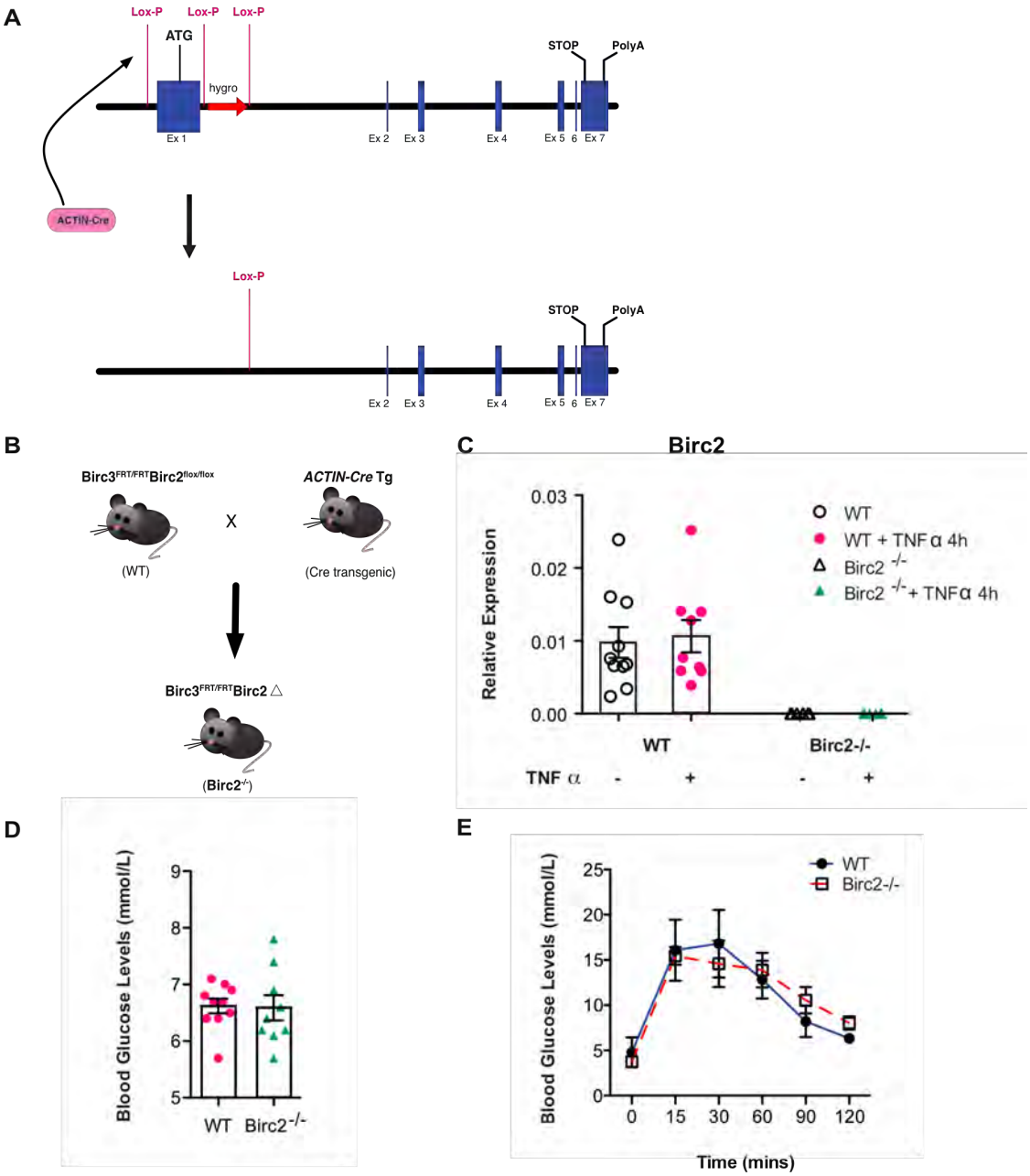


Figure 5.1. BIRC2 is not required for normal beta cell function. (A) The map of *Birc2* from mouse chromosome 9. The enzyme Cre targets the transcription start codon of *Birc2* at the region denoted "ATG" within the exon (Ex) 1 region. (B) WT "knock-in" mice were bred with *ACTIN*-Cre mice to generate *Birc2*$^{-/-}$. (C) The expression of *Birc2* in WT and *Birc2*$^{-/-}$ islets in the absence and presence of TNFα stimulation determined by qRT-PCR. Data represent mean ± SEM from three independent experiments. Gene expression are shown relative to housekeeping gene, CPH. (D) Random blood glucose levels for non-fasted 12-week old, male WT and *Birc2*$^{-/-}$ mice. Data represent mean ± SEM from at least nine mice per group. (E) Blood glucose levels during IP-GTT for WT (black circle, blue solid line) and *Birc2*$^{-/-}$ (white square, dotted red line) mice. Data represent mean ± SEM from four mice per group. Statistical comparisons were made by single unpaired t-test between two groups.

5.2.2 BIRC2 is redundant for NF-κB signalling in beta cells

To determine if BIRC2 is necessary for TNFα-mediated NF-κB signalling in islets, we compared the TNFα-induced NF-κB activation kinetics of WT and *Birc2*$^{-/-}$ islets. Regarding NF-κB signalling, IκBα was degraded following TNFα stimulation for 15 min, was replenished at 30 min and restored to baseline levels by 60 min in WT islets (Figure 5.2A) (Beg et al., 1993). Similar to *Birc3*$^{-/-}$ islets, *Birc2*$^{-/-}$ islets showed delayed kinetics of TNFα stimulated IκBα degradation but was restored to baseline levels at 60 min (Figure 5.2A). Therefore, NF-κB signalling was still functional in the absence of BIRC2, suggesting that BIRC2 is redundant for TNFα-mediated NF-κB signalling in primary beta cells. Furthermore, these results also suggested that BIRC3 may be contributing to TNFα-mediated NF-κB signalling in the absence of BIRC2.

5.2.3 BIRC2 negatively fine-tunes TNF-induced genes.

The role of BIRC2 in the modulation of NF-κB-target genes was next examined. To do this, TNFα-induced NF-κB-regulated genes expressed by WT and *Birc2*$^{-/-}$ islets were compared. Different to *Birc3*$^{-/-}$ islets, *Birc2*$^{-/-}$ islets expressed normal TNFα-induced *A20* and *Cxcl10* but showed significantly higher levels of TNFα-induced *Ccl2* (by 70%) and *Icam-1* (by 64%) compared to WT (Figure 5.2B). Therefore, BIRC2 may be required to dampen the expression of some TNFα-induced NF-κB-driven genes in primary beta cells.

5.2.4 BIRC2 also functions to fine-tune basal gene expression

Noticeably, the basal levels of *Ccl2* and *Cxcl10* were increased by ≥86% in non-stimulated *Birc2*$^{-/-}$ compared to WT islets (Figure 5.2B). These data suggested that the function of BIRC2 expands beyond the negative regulation of TNFα-mediated NF-κB transcription. BIRC2 may be required as a negative regulator of basal NF-κB-regulated gene expression in resting islets.

5.2.5 *Birc3* may compensate for loss of *Birc2* in beta cells

As mentioned in chapter 4, some cell types exhibit compensatory mechanism between BIRC2 and BIRC3, where BIRC3 levels were found to increase in the absence of BIRC2 (Conze et al., 2005). In these cells, BIRC2 was thought to control the levels of BIRC3 by targeting it for degradation. Hence, BIRC3 proteins accumulate in the absence of BIRC2 (Conze et al., 2005). These findings prompted the analysis of *Birc3* levels in *Birc2*$^{-/-}$ islets. Contrary to expectations, basal *Birc3* levels remained unaffected in unstimulated *Birc2*$^{-/-}$ islets (Figure 5.2C). Interestingly, in the presence of TNFα, *Birc3* levels were significantly reduced by 50% in the absence of BIRC2. These findings indicated that BIRC2 does not regulate BIRC3 levels in islet cells but may be needed for full expression of TNFα-induced BIRC3. Furthermore, BIRC3 expressed only at 50% of WT levels was still sufficient for TNFα-mediated NF-κB activation in *Birc2*$^{-/-}$ islets.

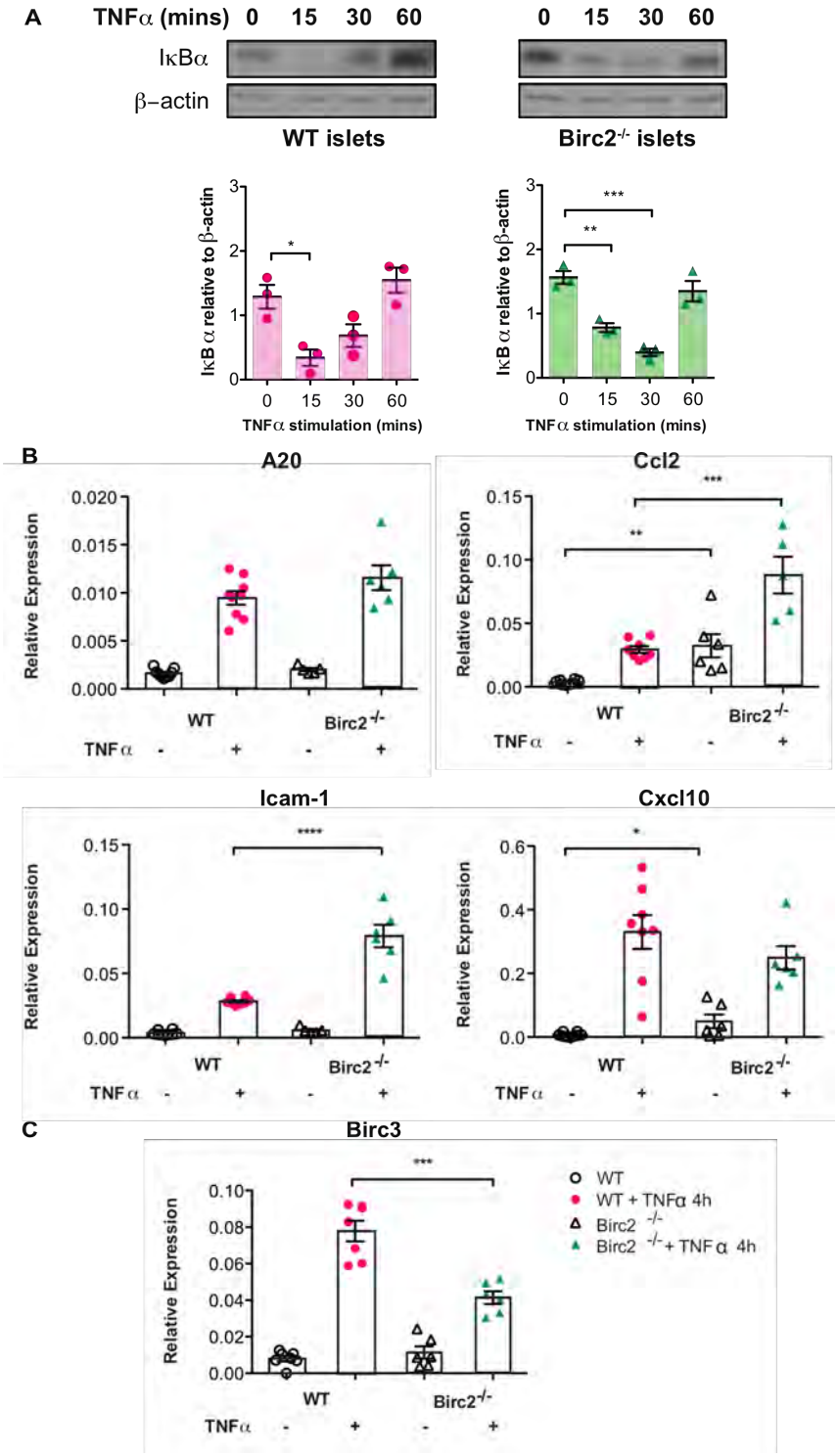


Figure 5.2. BIRC2 provides negative control over TNF pathways in beta cells. (A) Western blot analysis of NF-κB activation in WT and *Birc2*$^{-/-}$ islets with or without TNFα at times indicated. β-actin was used as a loading control. IκBα (39 kDa) was quantified by densitometry and expressed as relative to β-actin. Data represent mean ± SEM from three independent experiments. (B and C) Analysis of NF-κB-regulated genes and *Birc3* expression in WT and *Birc2*$^{-/-}$ islets with or without TNFα for 4h, determined by qRT-PCR. Gene expression shown are relative to housekeeping gene, CPH. Values from individual experiment are shown (n ≥ 5 per group). Bar represents mean value. Statistical comparisons were made by single unpaired t-test between two groups. All differences are significant. (*p≤0.05, **p≤0.01, ***p≤0.001 and ****p≤0.0001).

5.2.6 DKO-RIP islets expressed higher basal gene expression than DKO-HIP islets

Overall, our *Birc2*$^{-/-}$, *Birc3*$^{-/-}$ and DKO islets showed different patterns of NF-κB-target gene expression. To determine the relative contribution of the BIRC genes in stimulus-independent gene expression, the basal levels of NF-κB-regulated gene expressed by *Birc2*$^{-/-}$, *Birc3*$^{-/-}$, DKO-HIP and DKO-RIP islets were compared. Overall, DKO-RIP islets exhibited the highest level of basal *A20*, *Ccl2*, *Icam-1* and *Cxcl10* amongst the four strains of mice (Figure 5.3). From our analysis, it was apparent that DKO-RIP islets exhibited stronger phenotype of gene expression than DKO-HIP islets. DKO-RIP islets expressed higher basal *A20* (44%), *Ccl2* (67%), *Icam-1* (76%) and *Cxcl10* (62%) compared to DKO-HIP islets. The differences resulted from RIP-Cre being a more efficient deletor of *Birc2* compared to HIP-Cre mice.

5.2.7 BIRC2 and BIRC3 differentially fine-tune basal gene expression

The relative contributions of BIRC2 and BIRC3 to the phenotypes observed for DKO islets were also examined. In the absence of stimulus, both *A20* and *Icam-1* levels were increased for DKO-HIP and DKO-RIP, but not for *Birc2*$^{-/-}$ or *Birc3*$^{-/-}$ islets. These indicated that either BIRC2 or BIRC3 could singly provide negative control over basal *A20* and *Icam-1* expression in primary beta cells (Figure 5.3). In contrast, BIRC2 strictly controlled the expression of basal *Ccl2*, as the phenotype of *Birc2*$^{-/-}$ islets was identical to DKO-RIP islets (Figure 5.3). In addition, our data suggested a dose-dependent effect of BIRC2 on *Ccl2* expression. At 40% expression, *Birc2* could still provide control over basal *Ccl2* levels but at 20%, *Birc2* could no longer suppress basal *Ccl2* expression. At 20% *Birc2* expression, the *Ccl2* levels of DKO-RIP islets were increased to levels similar to *Birc2*$^{-/-}$ islets, where no *Birc2* was detected. Like *Ccl2,* the control of *Cxcl10* appeared to be more strongly suppressed by *Birc2*, but *Birc3* also seemed to contribute to the negative regulation of *Cxcl10*; as combined absence of *Birc2* and *Birc3* further increased *Cxcl10* expression. The increased *Cxcl10* of DKO-RIP islets surpassed the levels expressed by *Birc2*$^{-/-}$ and DKO-HIP islets (Figure 5.3). Together, these results demonstrated that BIRC proteins play complex and differential roles in the fine-tuning of TNF-target gene expression in primary beta cells.

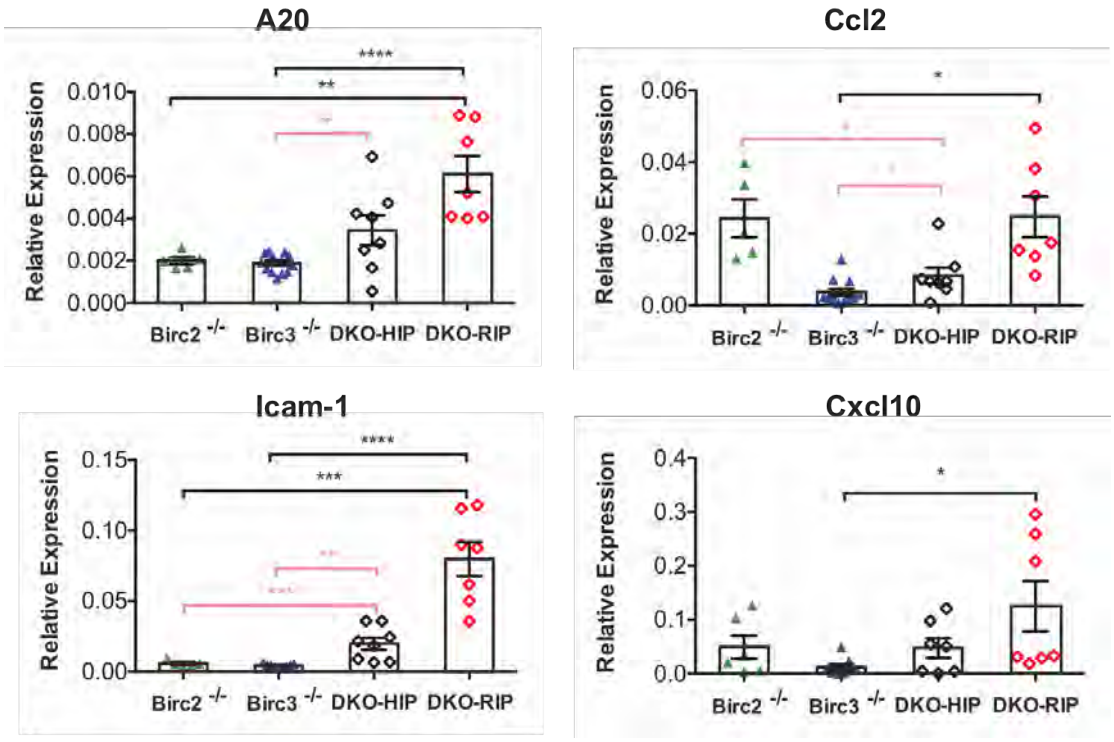


Figure 5.3. BIRC2 and BIRC3 differentially fine-tune inflammatory gene expression in islet cells. Expression of NF-κB-target genes in resting unstimulated *Birc2*$^{-/-}$, *Birc3*$^{-/-}$ DKO-HIP and DKO-RIP islets (n ≥ 5 per group) determined by qRT-PCR. Gene expression shown are relative to housekeeping gene, CPH. Bar represents mean value. Statistical comparisons were made by single unpaired t-test between two groups. Black lines represent significant differences between *Birc2*$^{-/-}$ or *Birc3*$^{-/-}$ with DKO-RIP and pink lines represent significant differences between *Birc2*$^{-/-}$ or *Birc3*$^{-/-}$ with DKO-HIP. All differences are significant. (∗p≤0.05 and ∗∗p≤0.01).

5.2.8 BIRC2 and BIRC3 also differentially regulate TNFα-stimulated genes

As we have discovered that BIRC2 and BIRC3 contributed differently to the regulation of basal gene expression in beta cells, we wondered if the same rules were applied on the regulation of TNFα-stimulated genes. To do this, the relative contributions of BIRC2 and BIRC3 on TNFα-mediated genes were compared between *Birc2*$^{-/-}$, *Birc3*$^{-/-}$, DKO-HIP and DKO-RIP islets. Due to the stronger *Birc2* deletion by RIP-Cre, we found that with the exception of *Cxcl10*, DKO-RIP islets expressed higher TNFα-stimulated *A20* (63%), *Ccl2* (51%) and *Icam-1* (85%) than DKO-HIP islets (Figure 5.4).

Overall, our analyses found BIRC2 and BIRC3 applied the same rules of basal gene regulation on TNFα-induced gene expression in beta cells. BIRC2 or BIRC3 alone was sufficient for the control of TNFα-induced *A20* and *Icam-1* expression (Figure 5.4). Further, BIRC2 was solely responsible for modulating TNFα-induced *Ccl2* expression, while BIRC2 and BIRC3 are both required for TNFα-induced *Cxcl10* expression (Figure 5.4). Collectively, our results indicated that BIRC2 and BIRC3 negatively regulate basal and TNFα-induced gene expression in beta cells.

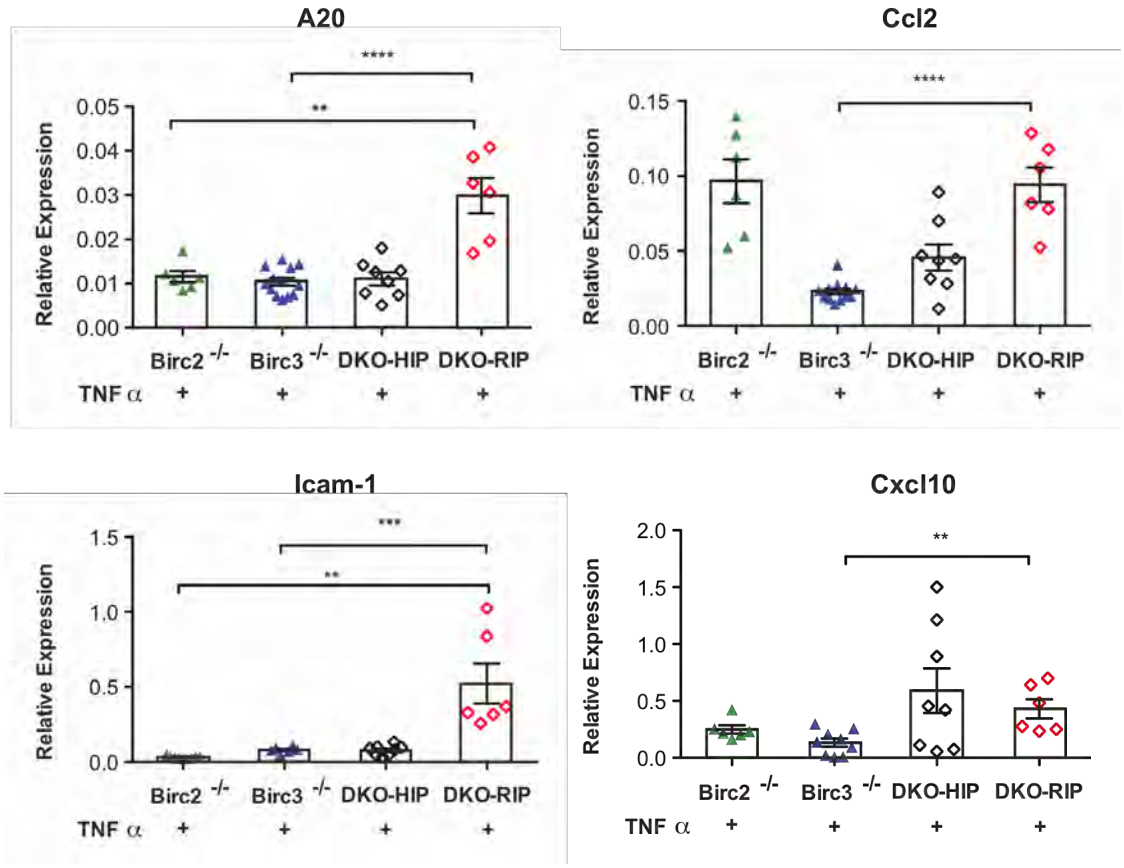


Figure 5.4. BIRC2 and BIRC3 also differentially regulate TNFα-stimulated genes. Expression of NF-κB-target genes in *Birc2*$^{-/-}$, *Birc3*$^{-/-}$ DKO-HIP and DKO-RIP islets stimulated with TNFα for 4 h (n ≥ 5 per group) determined by qRT-PCR. Gene expression shown are relative to housekeeping gene, CPH. Bar represents mean value. Statistical comparisons were made by single unpaired t-test between two groups. All differences are significant. (**p≤0.01, ***p≤0.001 and **** p≤0.0001).

5.2.9 Transcriptional control of TNFα-induced gene expression in WT islets

Increased gene expression could result from alterations in mRNA stability or increased *de novo* transcription. TNFα can regulate gene expression via transcription or through posttranscriptional modifications (Lentz et al., 1991). We tested whether TNFα induction of *A20*, *Cxcl10*, *Ccl2* and *Icam-1* expression were under transcriptional control. To do this, WT islets were pre-treated with the transcriptional inhibitor, AcD, prior to TNFα stimulation for 4 h. In untreated WT islets, TNFα led to the induction of *A20* expression. However blockade of transcription inhibited TNFα-induced *A20* by 90% (Figure 5.5). Similarly, TNFα-induced *Ccl2, Cxcl10* and *Icam-1* were inhibited by 64%, 92% and 83% respectively by AcD treatment (Figure 5.5). These data implicated that TNFα induction of *A20, Ccl2, Cxcl10* and *Icam-1* were regulated by *de novo* gene transcription.

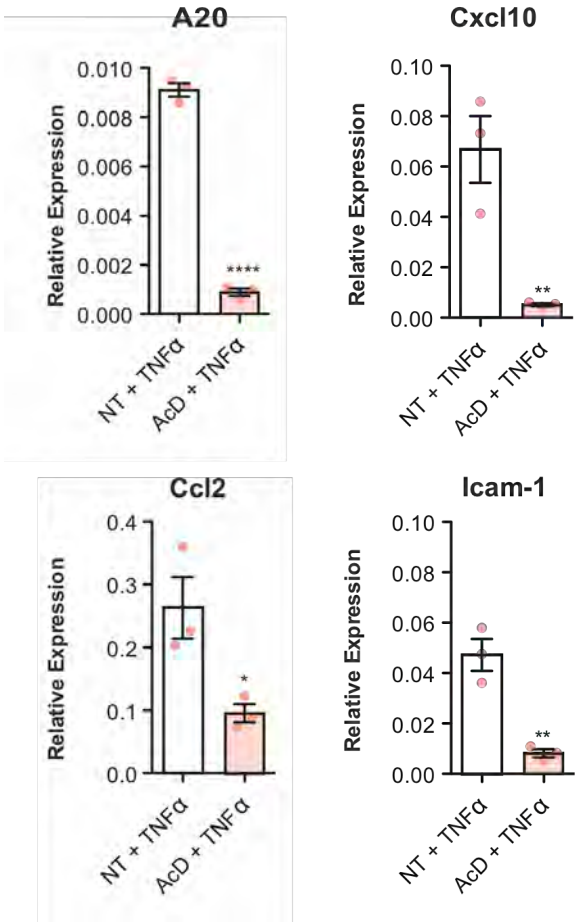


Figure 5.5. Transcriptional control of TNFα-induced genes in WT islets. Expression of *A20, Cxcl10, Ccl2* and *Icam-1* mRNA in WT islets either left untreated or treated with Actinomycin D (AcD) for an hour, prior to TNFα stimulation for 4 h (n=3 per group). Gene expression shown are relative to housekeeping gene, CPH. Bar represents mean value. Statistical comparisons were made by single unpaired t-test between two groups. All differences are significant. (*p≤0.05, **p≤0.01 and ****p≤0.0001).

5.2.10 BIRC-dependent control over the beta cell transcriptional responses

Having established that TNFα-induced *A20*, *Cxcl10*, *Ccl2* and *Icam-1* were under the transcriptional control; we next determined if stimulus-independent basal gene expression was also regulated by gene transcription in DKO-RIP islets. For this experiment, we tested DKO-RIP islets because they were found to express the highest level of basal gene expression as demonstrated in Figure 5.3. DKO-RIP islets treated with AcD prevented stimulus-independent basal expression of *A20* and *Cxcl10* by 90%, but did not affect basal *Ccl2* or *Icam-1* levels (Figure 5.6). These indicated that basal *A20* and *Cxcl10* mRNA levels were regulated by active gene transcription in DKO-RIP islets. We interpreted these data to mean that without BIRC2 and BIRC3, the stimulus-independent transcription rates of some TNF-regulated genes are altered in primary beta cells.

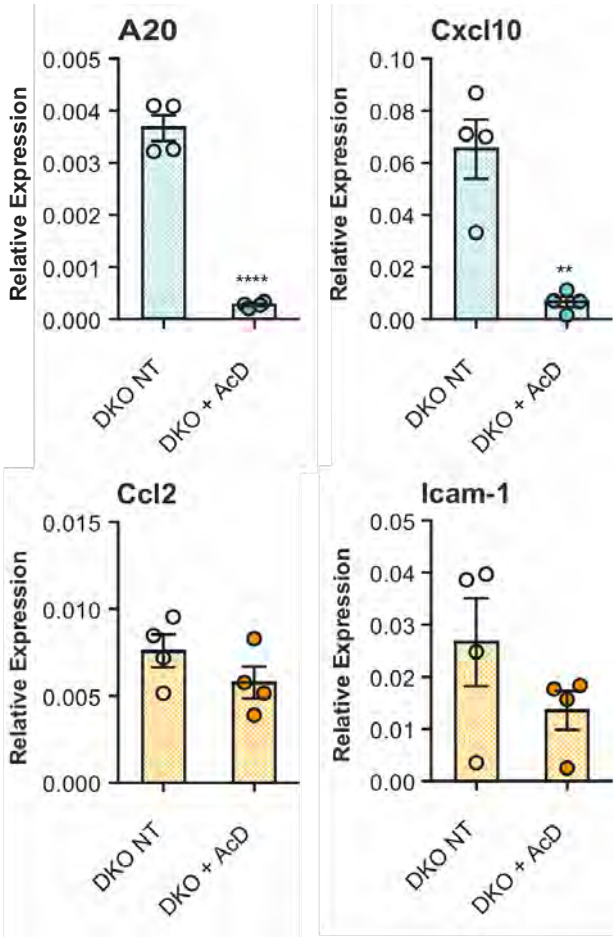


Figure 5.6. BIRC-dependent control over the beta cell's transcriptional response. Expression of *A20, Ccl2, Icam-1* and *Cxcl10* mRNA in DKO-RIP islets treated with 1 μM Actinomycin D (AcD) for 8 h (n=4 per group). Gene expression shown are relative to housekeeping gene, CPH. Bar represents mean value. Statistical comparisons were made by single unpaired t-test between two groups. All differences are significant. (**p≤0.01).

5.2.11 Regulation of TNFα-induced genes by NF-κB, JNK and p38 pathways

Beta cells respond to TNFα and activate the NF-κB, JNK and p38 pathways (Carpentier et al., 1998; Liu et al., 1996b; Reinhard et al., 1997; Song et al., 1997). There has been evidence that suggests that JNK and p38 can also contribute to the regulation of TNFα-induced *Ccl2* (Chen et al., 2004; Gao et al., 2009; Wolter et al., 2008)*, Cxcl10* (Clarke et al.; Shen et al., 2006; Yeruva et al., 2008) and *Icam*-1 (Chen and Manning, 1995; Rajan et al., 2008; Yang et al.). To examine if JNK and p38 contributed to the regulation of TNFα-stimulated *A20*, *Cxcl10*, *Ccl2* and *Icam-1,* MIN6 cells that we had previously treated with PDTC, SP600125 or SB203580 to inhibit NF-κB, JNK or p38 respectively (in chapter 3) were screened for *A20*, *Ccl2*, *Cxcl10* and *Icam-1* levels.

We found PDTC treatment resulted in 67% inhibition of TNFα-induced *A20* expression, but were unaffected with SP600125 or SB203580 treatment (Figure 5.7). These results indicated that TNFα-induced *A20* was specifically regulated by NF-κB activation. Further, our analysis found TNFα-induced *Ccl2* expression was prevented by 98% and 82% by PDTC and SP600125 treatments respectively, but not by SB203580 (Figure 5.7). These suggested that TNFα-induced *Ccl2* is regulated by the NF-κB and JNK pathways in beta cells. Similar to *Ccl2,* PDTC and SP600125 treatment resulted in ≥75% blockade in TNFα-stimulated *Icam-1* and *Cxcl10* but not with SB203580. Therefore in beta cells, TNFα-stimulated *Icam-1* and *Cxcl10* were also co-regulated by NF-κB and JNK activation (Figure 5.7).

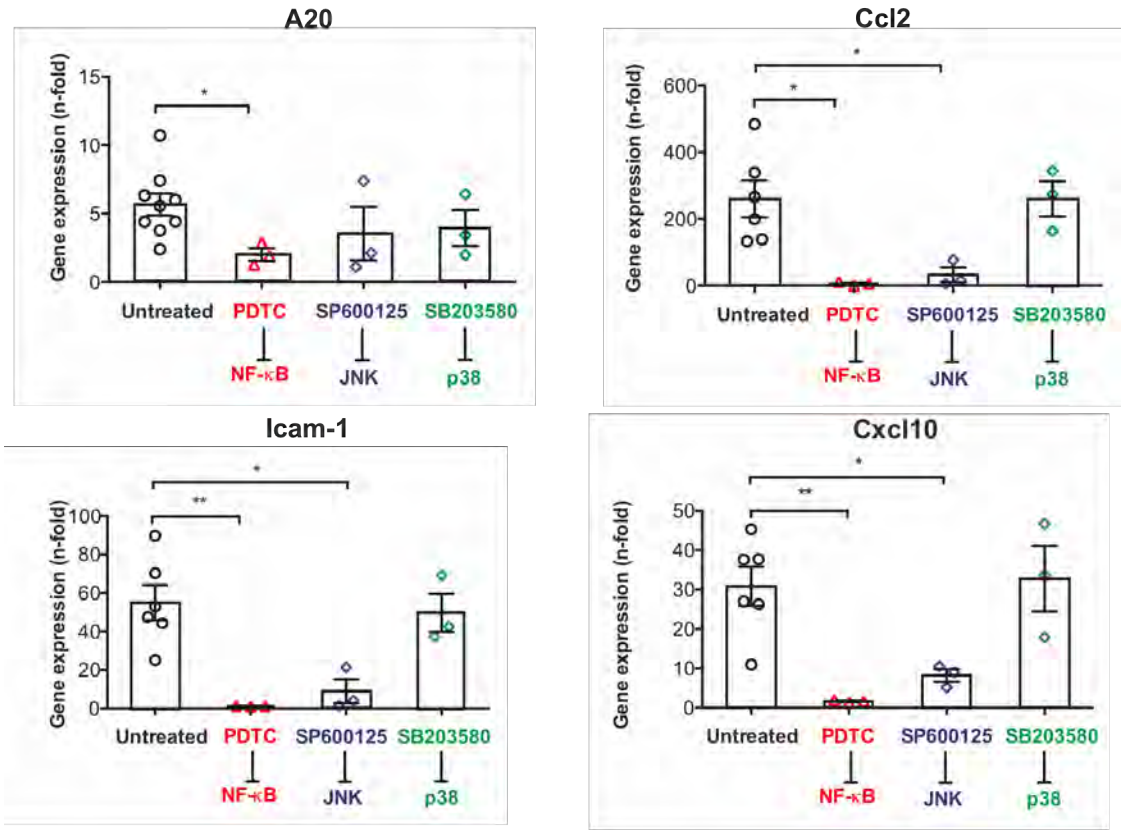


Figure 5.7. The regulation of TNFα-induced genes by the NF-κB, JNK and p38 pathways. Expression of *A20, Ccl2, Icam-1* and *Cxcl10* mRNA in MIN6 cells either left untreated or treated with PDTC, SP600125 or SB203580 for 1 h prior to stimulation with TNFα for 4h, measured by qRT-PCR. (n ≥ 3per group). Gene expression shown are relative to housekeeping gene, CPH. Bar represents mean value. Statistical comparisons were made by single unpaired t-test between two groups. All differences are significant. (∗p≤0.05 and ∗∗ p≤0.01).

5.2.12 JNK inhibition reigned in the DKO-RIP phenotype

We next tested the idea that increased basal gene expression in DKO-RIP islets might be due to hyper-activation of the same pathways involved in the regulation of normal TNFα-induced gene expression. To test this, DKO-RIP islets were treated with PDTC, SP600125 or SB203580 to inhibit NF-κB, JNK or p38 respectively, and gene expression was analysed by qRT-PCR. These analyses revealed that the basal expression of *Ccl2* (50%), *Icam-1* (67%) and *Cxcl10* (56%) were sensitive to SP600125, but not to SB203580 or PDTC treatment (Figure 5.8A). These findings indicated that loss of both BIRC2 and BIRC3 disrupted negative control over the JNK pathway. As a result, unstimulated *Birc2/3*-DKO beta cells spontaneously up-regulated JNK-signalling. Importantly, examination of JNK activation by analysis of c-Jun phosphorylation showed hyper-phosphorylation of c-Jun in DKO-RIP versus WT islets (Figure 5.8B). We interpret these findings to indicate that loss of both BIRC2 and BIRC3 impairs fine-control over the JNK pathway, such that beta cells are hypersensitive to triggering JNK-signalling.

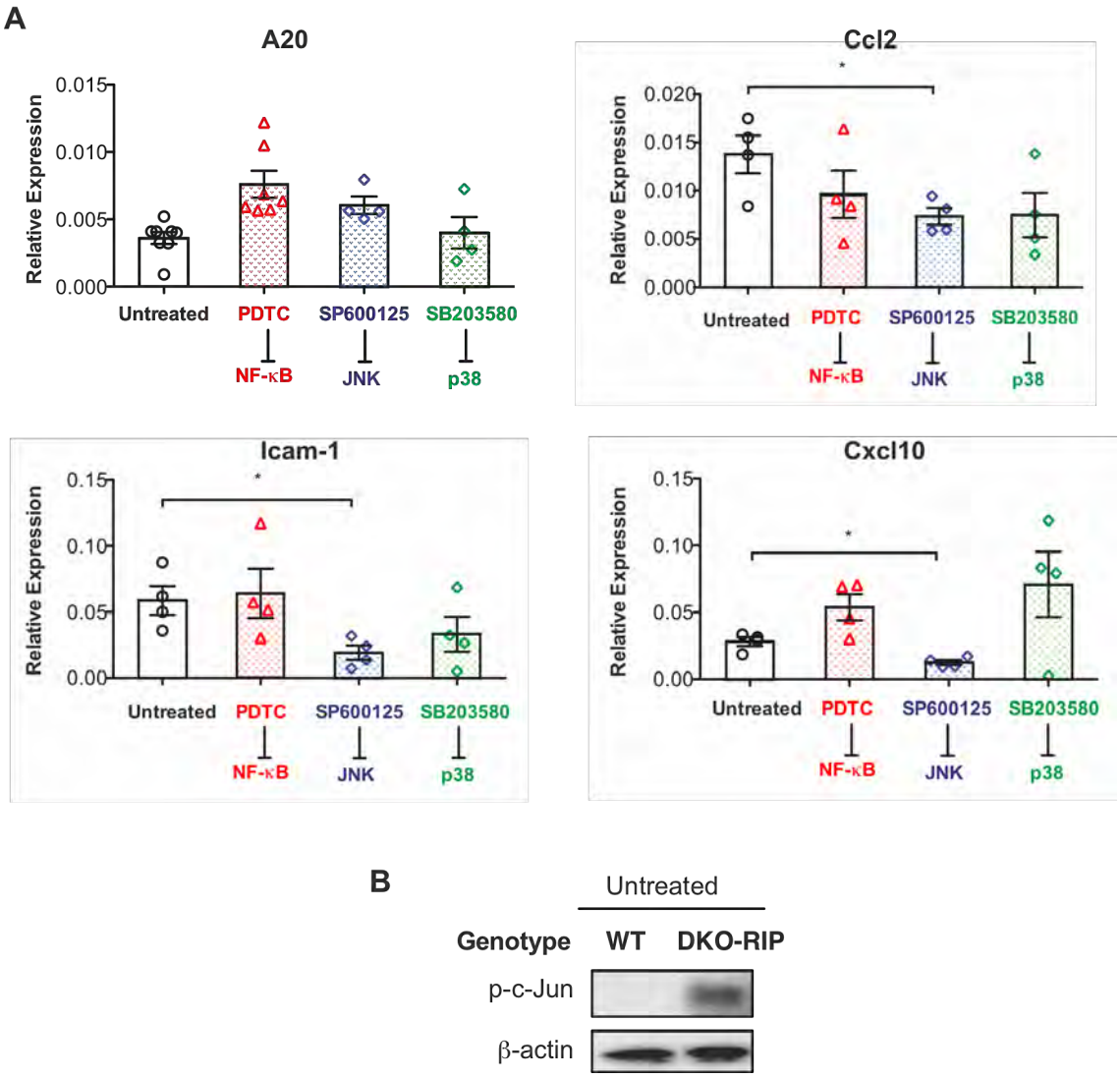


Figure 5.8. JNK inhibition reigned in the DKO-RIP phenotype. (A) Expression of *A20, Ccl2, Icam-1* and *Cxcl10* mRNA in DKO-RIP islets either left untreated or treated with PDTC, SP600125 or SB203580 for 8 h (n ≥ 4 per group), determined by qRT-PCR. Gene expression shown are relative to housekeeping gene, CPH. Bar represents mean value. (B) Western blot analysis of JNK activation by c-Jun phosphorylation (48 kDa) in WT and DKO islets left untreated. β-actin was used as a loading control. Representative blots are shown. Triplicate experiments in each case generated similar results. Statistical comparisons were made by single unpaired t-test between two groups. All differences are significant. (*p≤0.05).

5.2.13 Proteosome inhibition mimicked the DKO beta cell phenotype

BIRC proteins function as ubiquitin modifying enzymes that target proteins for proteosomal degradation (Hu and Yang, 2003; Huang et al., 2000; Li et al., 2002). We tested the idea that in the absence of BIRC proteins, accumulation of signalling components normally targeted for degradation, perhaps regulatory-kinases, could be triggering activation of signalling pathways without appropriate external inputs. To do this, we examined whether loss of proteosome activity could replicate the DKO islet phenotype. MIN6 cells were treated with the proteosome inhibitor, MG-132. Compared to control MIN6 cells, MG-132-treated MIN6 cells exhibited increased expression of *Ccl2* (50%), *Icam-1* (50%) and *Cxcl10* expression (80%) but not *A20* (Figure 5.9A). Further, analysis of active c-Jun phosphorylation showed MG132-treated MIN6 cells exhibited increased JNK pathway activation (Figure 5.9B). Thus blocking the proteosome resulted in dysregulated JNK pathway activation in a stimulus-independent manner, with hyper-expression of *Ccl2, Icam-1* and *Cxcl10* inflammatory genes. This phenotype is reminiscent of primary beta cells that lack both BIRC2 and BIRC3.

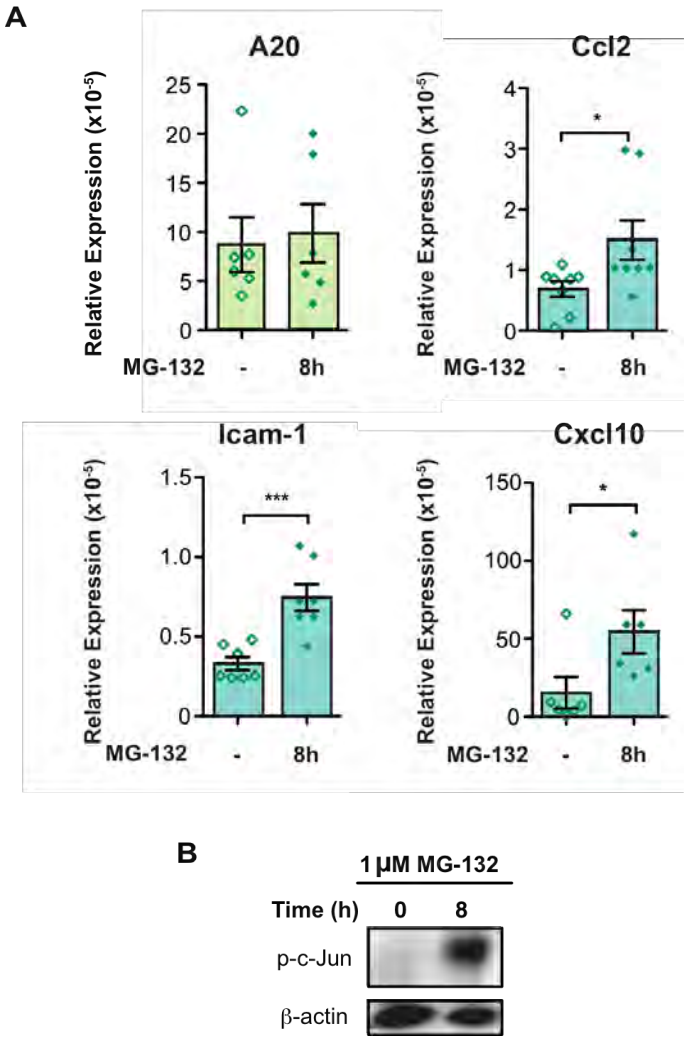


Figure 5.9. BIRC2 and BIRC3 fine-tune the TNF-signalling in islet cells. (A) Expression of *A20, Ccl2*, *Icam-1* and *Cxcl10* in MIN6 either left untreated or treated with MG-132 for 8 h, measured by qRT-PCR. Gene expression shown are relative to housekeeping gene, CPH. Values from individual experiment are shown (n ≥ 5 per group). Bar represent mean value. (B) Western blot analysis of JNK activation by c-Jun phosphorylation (48 kDa) in MIN6 with or without MG-132 treatment. β-actin was used as a loading control. Representative blots are shown. Triplicate experiments in each case generated similar results. Statistical comparisons were made by single unpaired t-test between two groups. All differences are significant. (∗p≤0.05 and ∗∗∗p≤0.001).

5.2.14 BIRC2 and BIRC3 are crucial for islet graft survival

During the process of isolation, JNK is acutely activated in islets (Fornoni et al., 2008; Fukuda et al., 2008; Varona-Santos et al., 2008). Specifically, antagonizing JNK signalling can improve islet survival post isolation (Noguchi et al., 2005). Thus, we predicted that DKO islets are less tolerant to the stress of isolation, and exhibited hyper-JNK signalling and increased expression of TNFα-induced inflammatory genes. Further, we also wondered whether the increased levels of inflammatory genes expressed by DKO-RIP islets were sufficient to affect physiological responses of the islets. To test the role of BIRC2 and BIRC3 in an inflammatory setting, WT or DKO-RIP islets were transplanted into diabetic, allogeneic recipients. We hypothesised that DKO-RIP islets would fair worse *in vivo* following transplantation. For this model, donor islets obtained from WT or DKO-RIP mice of C57BL/6 background, which carried the MHC haplotype H-2^d, were transplanted under the kidney capsule of CBA recipient mice of MHC haplotype H-2^k. These strain combinations represent a MHC-mismatched model. A ratio of three-donor pancreata to one recipient was used and the recipient mice were administered with 300 mg/kg of streptozotocin to induce diabetes.

Analysis of blood glucose levels showed that both WT and DKO-RIP islets provided good metabolic control immediately after transplantation (Figure 5.10A). As early graft function can be affected by loss of islet mass to cell death (Biarnes et al., 2002; Grey et al., 2003), these data suggested that DKO-RIP islets were not hypersensitive to death stimuli directly after isolation and transplantation procedures. However, by post-operative day (POD) 6, DKO-RIP islets struggled to restore euglycaemia, which exacerbated with time, while WT islets showed good metabolic control till at least POD 18 (Figure 5.10A). Kaplan-Meier survival analysis showed that mice with WT islets rejected their grafts with a mean survival time (MST) of 26 days, while recipients of DKO-RIP islets rejected their grafts at a faster rate of MST 17 days (Figure 5.10B). We interpreted the earlier loss of DKO-RIP islet function to reflect loss of control of inflammatory signalling.

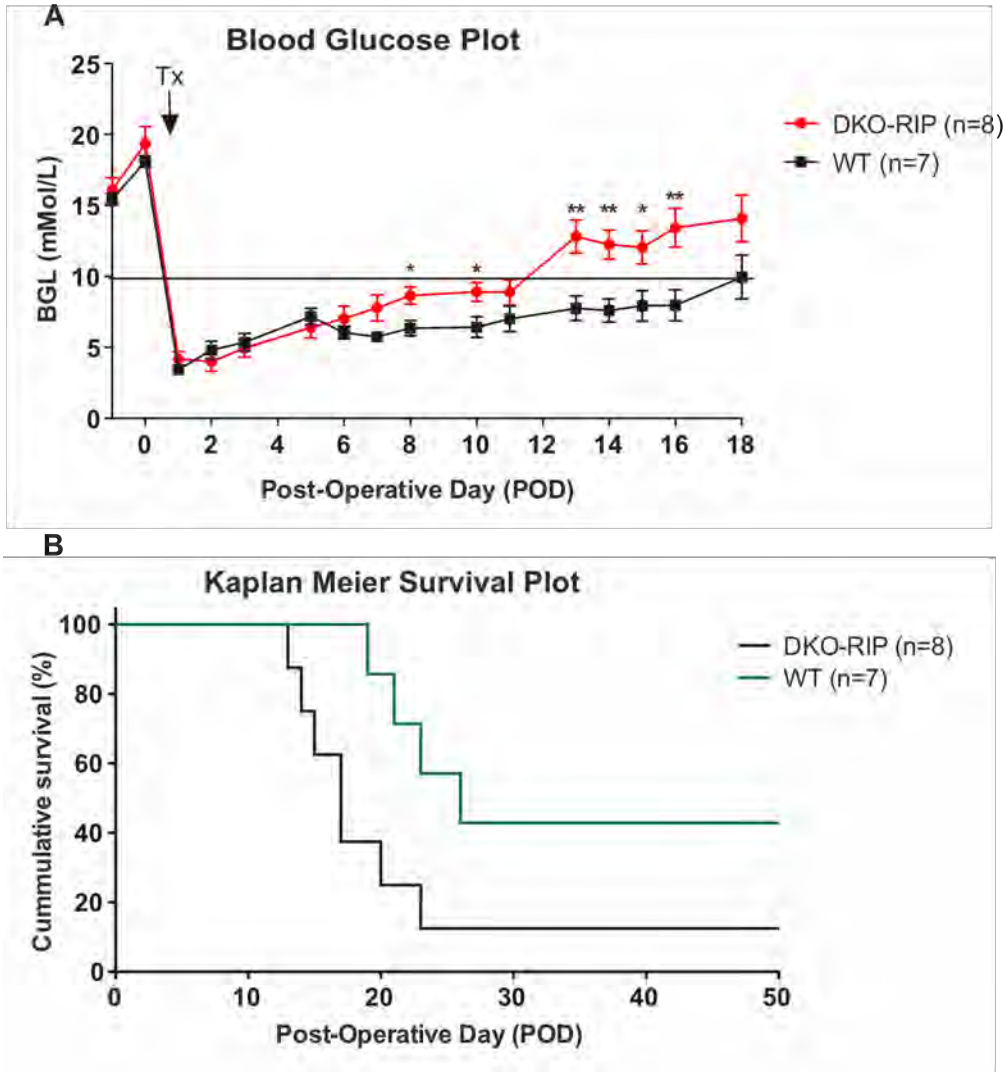


Figure 5.10. BIRC2 and BIRC3 are crucial for islet graft survival. (A) Graphical illustration of blood glucose values over time from diabetic CBA mice receiving allogeneic WT (black line) or DKO-RIP islets (red line). (B) Kaplan-Meier analysis showing islet allograft survival. Islets were isolated from either WT (black line, MST 26 days; n=7) or DKO-RIP (green line, MST 17 days, n=8) mice. (Tx = Transplantation; *p≤0.05 and **p≤0.01)

5.3 Discussion

5.3.1 BIRC2 and BIRC3 maintain the kinetics of TNFα-mediated NF-κB signalling.

Our present data from this chapter provided further findings on the role of BIRC2 and BIRC3 in TNF signalling in primary beta cells. As previously suggested, BIRC2 and BIRC3 were reported to function redundantly in various intracellular signalling pathways (Bertrand et al., 2009; Gardam et al.; Mahoney et al., 2008; Micheau and Tschopp, 2003; Vallabhapurapu et al., 2008; Varfolomeev et al., 2007; Varfolomeev et al., 2008; Vince et al., 2007; Zarnegar et al., 2008b). In this chapter, we showed that like *Birc3*-deficient islets, TNFα-induced NF-κB activation was delayed but still functional in *Birc2*$^{-/-}$ islets. We considered that residual NF-κB signalling in the absence of BIRC2 was contributed by BIRC3. In chapter 4, we showed that *Birc2*$^{-/-}$*Birc3*$^{-/-}$ islets were significantly delayed in TNFα-induced NF-κB activation kinetics. Together, these data supported the idea that in primary beta cells, BIRC3 can compensate and allow TNFα-induced NF-κB activation to occur in the absence of BIRC2. However, both BIRC proteins are required to maintain normal NF-κB activation kinetics in response to TNFα stimulation, such that in their absence, NF-κB activation kinetics is markedly impaired.

BIRC2 has been reported to regulate BIRC3 levels by targeting the protein for degradation. In the absence of BIRC2, BIRC3 levels are found to increase in some cell types (Conze et al., 2005). Our analysis found BIRC2 and BIRC3 levels remained unaffected in untreated islets. Therefore, contrary to this report, BIRC2 does not play a role in controlling the levels of BIRC3 in beta cells. Of note, *Birc2*-deficient islets expressed 50% less TNFα-induced *Birc3* compared to WT islets, indicating that BIRC2 may contribute to the regulation of *Birc3* expression. Previously, we showed in chapter 3 that NF-κB is responsible for the regulation of TNFα-induced *Birc3* expression. We therefore interpreted these findings to mean that BIRC2 may participate in TNFα-mediated NF-κB signalling that is required to control full expression of TNFα-induced BIRC3 in beta cells. Together, these findings implicated a model whereby BIRC2 participates in TNFα activated NF-κB

signalling, which induced an increase in *Birc3* expression. BIRC3 then functions to fine-tune TNFα-mediated NF-κB signalling to control proper expression of NF-κB-target genes.

5.3.2 Increased JNK activation may arise from dysregulated NF-κB activation

Analysis of the increased basal gene expression in DKO-RIP islets with the use of specific pathway inhibitors showed that the increased gene expression was controlled by the JNK but not NF-κB or p38 pathway. Notably, DKO-RIP islets showed c-Jun hyper-phosphorylation, indicating that BIRC2 and BIRC3 function to suppress basal JNK activation. Transient and modest JNK activation by TNFα has been associated with cell survival and proliferation while prolonged JNK activation is associated with TNFα-mediated cell death (Chen et al., 1996; Guo et al., 1998; Tang et al., 2002). For example, transient activation of JNK has been reported to induce genes required for T cell proliferation. However, prolonged JNK activation results in aberrant gene expression and T cell apoptosis (Chen et al., 1996). Thus, the function of BIRC2 and BIRC3 in islets may be to inhibit hyper-JNK phosphorylation to prevent JNK-mediated beta cell apoptosis under conditions of inflammatory stress.

Additionally, it has been demonstrated that inhibition of NF-κB activation can result in extended JNK activation and eventually JNK-induced cell death (Liu et al., 2002; Tang et al., 2002; Zhang et al., 2004). Conversely, cell death induced by blocking NF-κB signalling, can be reversed by inhibiting JNK activation (Zhang et al., 2004), suggesting that NF-κB signalling can protect cells from JNK-mediated apoptosis. This cytoprotective role of NF-κB is supported by reports showing that it can suppress the secondary prolonged phase of JNK activation by inducing JNK-inhibitory genes and by inhibiting ROS production (Bubici et al., 2006; Sakon et al., 2003; Tang et al., 2001). Collectively, these reports suggested that in primary beta cells, BIRC2 and BIRC3 are required to provide proper NF-κB activation kinetics, allowing NF-κB to control the duration of JNK activation and prevent JNK-induced cytotoxicity in beta cells.

5.3.3 DKO islets are more susceptible to external stress

Using the islet transplantation model, we showed that DKO-RIP islets were less able to tolerate the inflammatory stress of an MHC mismatched transplant conditions and rejected at a faster rate than WT islets. Therefore, consistent with previous findings that implicated BIRC2 and BIRC3 as anti-apoptotic proteins, in this setting, BIRC2 and BIRC3 seem to provide pro-survival support to islet cells through their participation as regulators of the TNF- signalling pathway (Bertrand et al., 2008; Csomos et al., 2009; Schoemaker et al., 2002). Our results showed that BIRC2 and BIRC3 are required to protect islets from conditions of stress including islet isolation stress, inflammatory stress during islet transplantation and T1D, by dampening basal JNK and inflammatory responses.

5.3.4 Up-regulation of inflammatory genes impair beta cell function and viability

Uncontrolled expression of inflammatory genes such as *Ccl2, Cxcl10* and *Icam-1* has adverse effects on islets. CXCL10 is implicated in the pathogenesis of both type 1 and type 2 diabetes. Elevated *CXCL10* levels were found to impair beta cell function and viability (Schulthess et al., 2009), while RIP-*Cxcl10* mice showed spontaneous islet infiltration by immune cells and impaired beta cell function, although these mice do not proceed to diabetes (Rhode et al., 2005). The islets of type 2 diabetic patients were reported to secrete 33.5-folds more *CXCL10* than normal islets (Schulthess et al., 2009), while serum CXCL10 levels were found to heighten in NOD mice just before or at the onset of diabetes (Shigihara et al., 2006). Conversely, *in vivo* treatment with CXCL10 DNA vaccine (pCAGGS-CXCL10) in young NOD mice to induced anti-CXCL10 antibody production, alleviated disease progression. Although anti-CXCL10 treatment did not prevent immune infiltration and insulitis, it was able to suppress the incidence of spontaneous diabetes in treated mice (Shigihara et al., 2005). Like CXCL10, the inflammatory genes *Ccl2* and *Icam-1* are also cytotoxic to beta cells. Low levels of *Ccl2* expression were sufficient to impair beta cell function (Martin et al., 2008). Transgenic mice expressing high levels of intra-islet *Ccl2* showed increased macrophage infiltration into the islets and developed diabetes spontaneously (Martin et al., 2008). Like

Ccl2 and *Cxcl10*, increased *Icam-1* expression was also linked to impaired beta cell viability. *Icam-1* was found to promote beta cell destruction by cytotoxic T cells in NOD mice (Yagi et al., 1995), while *Icam-1*$^{-/-}$ NOD mice are protected from diabetes (Martin et al., 2001). These findings suggested the importance of BIRC2 and BIRC3 in islet cells, where BIRC proteins function to prevent spontaneous expression of inflammatory genes that can be detrimental to beta cell viability and function.

5.4 Conclusion

In conclusion, a number of new functions of BIRC2 and BIRC3 in pancreatic beta cells were presented in this chapter. Firstly, we confirmed that BIRC2 and BIRC3 are redundant for TNFα-mediated NF-κB signalling but are both required to maintain the immediate early kinetics of NF-κB activation in beta cells. BIRC2 and BIRC3 also provide distinct and overlapping functions in fine-tuning the expression of basal and TNFα-induced genes. In addition, the role of BIRC2 and BIRC3 extends beyond the regulation of NF-κB activation. This study found that BIRC2 and BIRC3 are also required to negatively regulate JNK activation in the absence of stimulus. The regulation of BIRC proteins on JNK signalling could be via an unknown direct method, or indirectly through their participation in NF-κB signalling. In their absence, islets may be more susceptible to external stress such as inflammatory stress in a MHC-mismatched immune environment. Without these BIRC proteins, islets exhibit increased inflammatory gene expression, which can contribute to the failure and destruction of beta cells. Hence, our findings in this chapter reinforced the idea that BIRC2 and BIRC3 are pro-survival genes, which are crucial for controlling NF-κB activation, fine-tuning of basal and TNF-induced gene expression and dampening the activation of JNK pathway in pancreatic islets. The balance of these pathways may be crucial in the survival of islets under inflammatory conditions such as T1D.

6 Discussion and implications for future work

6.1.1 BIRC2 and BIRC3 fine-tune NF-κB responses in islets

It is now established that T1D is a consequence of lymphocytic invasion to the pancreatic islets, resulting in eventual beta cell failure and destruction (Amano and Yoon, 1990; Hanenberg et al., 1989; Jansen et al., 1994; Voorbij et al., 1989). Final beta cell destruction involves the participation of cytokines and T lymphocytes (Nerup et al., 1988). Of interest, the pro-inflammatory cytokine TNFα, secreted by invading T cells, macrophages and dendritic cells into the islets during insulitis (Arnush et al., 1998; Cantor and Haskins, 2005; Dahlen et al., 1998; Uno et al., 2007), has been found to directly impair beta cell function (Zhang and Kim, 1995) and cause beta cell death (Liuwantara et al., 2006; Stephens et al., 1999). TNFα also contributes to insulin resistance and T2D pathology by impairing lipid metabolism (Dandona et al., 1998; Hotamisligil, 1999). Despite the importance of TNFα in the pathogenesis of diabetes, its effect on beta cells is not fully understood. Previous data from our laboratory and others have shown that *Birc3* is upregulated in beta cells following cytokine stimulation (Liuwantara et al., 2006; Sarkar et al., 2009). Of interest, BIRC3 plays a role in TNF signalling and regulation of apoptosis and its function has not been elucidated in beta cells. Furthermore, the role of its binding partner, BIRC2, has never been examined in beta cells. This inspired us to examine the role of BIRC proteins in beta cells in this thesis.

Our study have provided better understanding of TNFα signalling in primary beta cells and beta cell lines by examining the role of BIRC proteins in this pathway. Firstly, beta cells specifically induced *Birc3* but not other BIRC family members with TNFα stimulation. Further to this, we found *Birc3* was also induced in the presence of other cytokines such as IL-1β and IFNγ that also contribute to the pathology of T1D (Baquerizo and Rabinovitch, 1990; Mori et al., 2008; Thomas et al., 2004; Wachlin et al., 2003). When added together, the cytokines TNFα + IL-1β + IFNγ synergized and significantly induced high levels of *Birc3*.

This prompted us to study the regulation of BIRC3 and determine the function of BIRC3 in pancreatic beta cells. We identified a number of putative transcription

factor binding sites within the proximal promoter of *Birc3* and that the *Birc3*-proximal promoter was inducible by the same cytokines and combination that induced the *Birc3* transcript. It has been reported that TNFα-induced *BIRC3* is modulated by p38 but not by NF-κB signalling in ECV304 endothelial cells (Furusu et al., 2007), while TNFα-stimulated BIRC3 was regulated primarily by NF-κB in Jurkat and primary T cells (Chu et al., 1997; Hong et al., 2000). Our study found that TNFα-induced *Birc3* was regulated by NF-κB but not by p38 or JNK signalling, which correspond to the phenotype observed in T cells but not that of ECV304 cells. Exactly why beta cells choose to up-regulate only BIRC3 in the presence of TNFα is not known. Our preliminary findings implied that HDACs may play a role in inhibiting the up-regulation of *Birc1* and *Birc4* but further experimentation is required to confirm this.

Noticeably, our study found an important function of BIRC3 in beta cells. Overexpression of BIRC3 positively enhanced NF-κB signalling and led to subtle up-regulation of the NF-κB-regulated inflammatory genes, *Cxcl10* and *Ccl2.* This function of BIRC3 has been deemed important in the context of oncogenesis. Mucosa-associated lymphoid tissue (MALT) lymphoma is an inflammation-induced cancer of the mucosa (primarily in gut and lung) (Zhou et al., 2005). Occurrence of MALT lymphoma is thought to occur from reciprocal translocation of *BIRC3* and *MALT1* resulting in a chimeric protein, BIRC3-MALT1, that is made up of the three BIRs and one UBA domains of BIRC3, as well as the paracaspase domain of MALT1 (Zhou et al., 2005). BIRC3-MALT1 positively feeds back on NF-κB signalling, resulting in further expression of BIRC3-MALT1. The UBA domain of BIRC3-MALT1 is responsible for stimulating constitutive NF-κB activation by binding to NEMO, which increases cell survival and the oncogenic potential of MALT lymphoma (Gyrd-Hansen et al., 2008). Thus, increased BIRC3-dependent NF-κB activation is associated with increased cell survival, which may be important for protecting beta cells from cytokine-induced apoptosis in T1D. However, this function of BIRC3 remains to be examined. The BIRC members, BIRC3, Survivin, ML-IAP and XIAP are found to be highly upregulated in pancreatic cancer and BIRC3, in particular, is associated with its resistance to chemotherapeutic agents

(Lopes et al., 2007). Hence, furthering our understanding of BIRC3's function in beta cells may also provide treatment for pancreatic cancer.

To determine whether BIRC3 is essential for beta cell function, BIRC3 loss-of-function was studied in islets. Without BIRC3, TNFα-mediated NF-κB signalling was delayed, but still functional. Further, BIRC3 was required to fine-tune the expression of some TNF-regulated genes. The loss of BIRC3 was compensated by BIRC2 and this redundancy has been reported in diverse cell types (Bertrand et al., 2008; Mahoney et al., 2008; Varfolomeev et al., 2008). Like BIRC3, loss of BIRC2 also led to delayed but apparent NF-κB signalling. Interestingly, in addition to maintaining the normal expression of TNF-induced genes, BIRC2 was also required to negatively regulate basal expression of TNF-induced genes. Furthermore, the loss of fine-tuning of basal gene expression in the absence of BIRC2 was further exacerbated when combined with the loss of BIRC3.

The kinetics of NF-κB signalling was impaired in the absence of BIRC2 and BIRC3. This demonstrated their importance in maintaining the kinetics of TNFα-induced NF-κB activation in beta cells. The function of BIRC2 and BIRC3 in beta cells reflected their roles described in the literature where the combined absence of BIRC2 plus BIRC3 strongly impaired the activation of TNFα-mediated NF-κB signalling (Bertrand et al., 2008; Mahoney et al., 2008; Varfolomeev et al., 2008). During conditions of inflammatory stress, such as in T1D, BIRC2- and BIRC3-deficient beta cells may fail to respond to cytokines quickly. This may have detrimental effects on the beta cells, as they may not be able to activate NF-κB to drive the expression of pro-survival genes such as *c-Flip* and *A20* that are known to exert protective functions under conditions of external stress (Cottet et al., 2002; Daniel et al., 2004; Grey et al., 1999; Liuwantara et al., 2006; Micheau et al., 2001). However, our cells did not show reduction in *A20* expression. Instead *A20* levels were increased in the absence of BIRC2 and BIRC3, and despite the delayed kinetics of NF-κB activation, IκBα levels remained phosphorylated at 60 mins with TNFα, which suggests a prolonged NF-κB activation. The extended duration of NF-κB activation may explain the accumulation of TNFα-induced gene expression.

6.1.2 A role for BIRC2 and BIRC3 in non-canonical NF-κB signalling

In addition to the canonical NF-κB pathway, emerging data implicate a role for BIRC2 and BIRC3 in the non-canonical NF-κB pathway (Vallabhapurapu et al., 2008; Varfolomeev et al., 2007; Vince et al., 2007). *Birc2*$^{-/-}$ MEFs and *Birc2*$^{-/-}$*Birc3*$^{-/-}$ cancer cells accumulate NIK to high levels. Subsequently, NIK accumulation results in IKKα phosphorylation and induces activation of the non-canonical NF-κB pathway (Varfolomeev et al., 2007; Vince et al., 2007). BIRC2 and BIRC3 are implicated as negative regulators of the non-canonical NF-κB pathway. Moreover, both the canonical and non-canonical NF-κB pathways have been reported to modulate *ICAM-1* expression in HUVEC cells stimulated with LIGHT and LTα1β2 (Madge et al., 2008). These findings suggested that the increased basal *Icam-1* that was observed in *Birc2*$^{-/-}$*Birc3*$^{-/-}$ islets may relate to dysregulated non-canonical NF-κB signalling in islet cells. The non-canonical NF-κB subunit, RelB, is thought to resolve inflammation in fibroblasts by regulating chemokine expression. Our study found that TNFα-induced chemokine expression in *Birc2*$^{-/-}$*Birc3*$^{-/-}$ islets resembled that of *RelB*$^{-/-}$ fibroblasts where TNFα-induced *Cxcl10* and *Ccl2* levels were elevated (Xia et al., 1997). Although this hypothesis remains to be examined, BIRC2 and BIRC3 may control islet inflammation by suppressing basal non-canonical NF-κB activation to prevent constitutive chemokine expression. Thus, it would be interesting to assess p100 processing and NIK levels in *Birc2*$^{-/-}$*Birc3*$^{-/-}$ islets.

6.1.3 A possible role for TRAF2 in regulating basal IKK activity.

BIRC2 and BIRC3 are involved in TNFR1 signalling through their interaction with TRAF2 (Rothe et al., 1995; Shu et al., 1996). TRAF proteins upstream of BIRC2 and BIRC3 in the TNF signalling pathway are also important for driving TNFα-mediated NF-κB signalling (Sakurai et al., 2003; Tada et al., 2001). Hence, it is possible that TRAF2 and TRAF5 may contribute to the residual NF-κB signalling in our *Birc2*$^{-/-}$*Birc3*$^{-/-}$ islets. In particular, TRAF2 has been reported to suppress basal IKK activity in resting cells (Zhang et al., 2009). Furthermore, the importance of TRAF2 and TRAF5 in the regulation basal gene expression has been demonstrated in MEF cells (Zhang et al., 2009). *Traf2*$^{-/-}$*Traf5*$^{-/-}$ MEF cells were found to express higher basal levels of NF-κB-target genes such as *Cxcl10, Rantes* and *Ikba*, a

phenotype that is reminiscent to our *Birc2*$^{-/-}$*Birc3*$^{-/-}$ islets. Additionally, increased gene expression in TRAF-deficient MEF cells was associated with increased basal IKK activity (Zhang et al., 2009). Since it has been demonstrated that recruitment of BIRC2 and BIRC3 by TRAF2 to the TNFR1 is required to maintain normal NF-κB signalling (Vince et al., 2009), therefore one possibility is that TRAF2 is not able to recruit BIRC2 and BIRC3 in *Birc2*$^{-/-}$*Birc3*$^{-/}$ islets and initiate proper suppression of basal IKK activity to prevent basal NF-κB-target gene expression.

6.1.4 A possible role for TRAF2 in suppressing NIK activation

Of interest, the addition of TNFα to *Traf2*$^{-/-}$*Traf5*$^{-/-}$ MEFs led to further increase in IKK activity and increase in NF-κB-regulated gene expression (Zhang et al., 2009). As a result of impaired recruitment of anti-apoptotic genes to the TNFR1, these cells were sensitive to TNFα-mediated apoptosis, thus suggesting that TRAF2 and TRAF5 are required to protect cells from TNFα-mediated apoptosis. In resting cells, TRAF2 forms part of an inhibitory complex with TRAF3-BIRC2/3 to suppress NIK degradation and activation, thus preventing unwanted constitutive activation of the non-canonical NF-κB pathway (Vallabhapurapu et al., 2008; Zarnegar et al., 2008b). When the pathway is induced, NIK degradation activates downstream IKK and result in the activation of the non-canonical NF-κB pathway. Expectedly, inhibition of NIK activity in *Traf2*$^{-/-}$*Traf5*$^{-/-}$ MEFs restored basal IKK activity and downstream gene expression to WT levels (Zhang et al., 2009). These findings indicated that increased expression of TNFα-induced NF-κB-dependent genes seen in *Birc2*$^{-/-}$*Birc3*$^{-/}$ islets could be attributed to the lost of TRAF2's suppressive function on NIK-activation (Bonizzi and Karin, 2004; Zarnegar et al., 2008a).

6.1.5 BIRC2 may provide control over TRAF2 and ASK1 for JNK signalling

Our study found that the increase in basal gene expression in our DKO islets, involved a mechanism of increase in JNK activation. Some studies demonstrate that TRAF2 is important for TNFα-mediated JNK signalling but is redundant for the NF-κB or p-38-MAPK pathways (Habelhah et al., 2004; Lee et al., 1997; Lee et al., 2003; Yeh et al., 1997). Others suggest that TRAF2 phosphorylation is required to

mediate full activation of TNFα-induced JNK signalling and for efficient expression of the NF-κB-target genes such as *Icam-1* (Blackwell et al., 2009). Further, it has also been reported that BIRC2 can modulate the duration of TNFα-induced p38 and JNK signalling by targeting TRAF2 and the MAPKKK protein, ASK1, for proteosomal degradation (Dupoux et al., 2009; Samuel et al., 2006; Zhao et al., 2007). In B lymphocytes, loss of BIRC2 leads to prolonged TNFα-mediated JNK and p38 signalling (Zhao et al., 2007). Hence, it is also possible that without BIRC2 and BIRC3, islets cannot provide normal control over TRAF2 and ASK1 levels or their activity. As a result the JNK pathway becomes hyper-activated in the absence of an overt signal. Further, it has also been shown that BIRC proteins can function as ubiquitin ligases that target proteins for proteosomal degradation (Hu and Yang, 2003; Huang et al., 2000; Li et al., 2002). Our data showed that proteosomal inhibition on MIN6 cells led to the same increased basal gene expression and c-Jun hyper-phosphorylation seen in DKO-RIP islets in the absence of stimulus. Thus, it is possible that the increased basal JNK hyper-activation in DKO beta cells occurred as a result of loss of regulation on TRAF2 and ASK1. Thus, one of the outcomes of this study would be to better understand the role of TRAF2 in beta cells to further improve our knowledge on how beta cells respond to TNFα.

6.2 Future work

Our present study provided the platform to further investigate the function of BIRC proteins and TNF signalling in beta cells. Firstly, we have shown that *Birc3* expression can also be induced by pathogenic cytokines implicated in T1D such as IL-1β and IFNγ. As these cytokines can activate signalling pathways different to TNFα, determining the role of BIRC proteins in IL-1β- and TNFα+IL-1β+IFNγ-stimulated beta cells, may help identify novel roles of these proteins and also contribute to better understanding of these pathways in beta cells. In addition, BIRC2 and BIRC3 are generally regarded as weak caspase inhibitors. However, several evidences have point towards the role of BIRC2 and BIRC3 as anti-apoptotic genes. This study did not directly determine the anti-apoptotic function of BIRC2 and BIRC3 in beta cells. It will be interesting to test whether overexpression of BIRC2 or BIRC3 can protect beta cells cytokine-mediated apoptosis.

This thesis is the first to report the role of BIRC2 and BIRC3 as negative regulators of basal JNK activation. Although immediate TNFα-induced JNK activation is associated with cell survival and proliferation, extended JNK activation results in TNFα-induced apoptosis (Chen et al., 1996; Guo et al., 1998; Tang et al., 2002). It has been shown that suppression of JNK activation prior to islet transplantation is found to be beneficial in preserving islet function and lead to enhanced graft survival. For this study, we have performed islet transplantation of *Birc2*$^{-/-}$*Birc3*$^{-/-}$ islets under the kidney capsule of allogeneic and streptozotocin-induced diabetic recipients. We found islets without BIRC2 and BIRC3 were still able to restore early graft function as WT islets, suggesting that the islets were not sensitive to islet isolation and transplantation procedure stress without BIRC2 and BIRC3. However, when subjected to prolonged immune attack, *Birc2*$^{-/-}$*Birc3*$^{-/-}$ islets do worse and rejected faster than WT islets *in vivo*. These findings presented an interesting aspect to examine whether JNK-hyperactivation and increased inflammatory gene expression in our *Birc2*$^{-/-}$*Birc3*$^{-/-}$ islets directly affects beta cell function and viability. Conversely, we can aslo examine whether the gain-of-

function of BIRC2 or BIRC3 respectively, can provide suppression on JNK activation and improve islet transplantation outcomes as part of the further work.

Another area of investigation is the regulation of NF-κB on JNK activation. It has been demonstrated that NF-κB can prevent JNK-mediated apoptosis by modulating the duration of JNK activation (Liu et al., 2002; Tang et al., 2002; Zhang et al., 2004). NF-κB prevents the cytotoxic effects of JNK activation by inducing JNK-inhibitory genes and controlling ROS production (Bubici et al., 2006; Sakon et al., 2003; Tang et al., 2001). Our study suggested that BIRC2 and BIRC3 are required to provide proper NF-κB activation kinetics, which may be needed to control the duration of JNK activation and prevent JNK-induced apoptosis. The possibility of NF-κB's control on JNK activation can be examined by analysing the TNFα-induced JNK activation kinetics in our DKO-RIP islets, to determine whether the delayed NF-κB activation can result in prolonged JNK activation. These findings may provide further explanation for the increased TNF-regulated genes expression seen in our DKO-islets.

Last but not least, this study provided a platform to further explore the roles of BIRC2 and BIRC3 in animal models of T1D and T2D. To study the effects of BIRC on T1D, subjecting the DKO and BIRC-transgenic mice to streptozotocin-induced diabetes and creating BIRC-transgenic NOD or BIRC-deleted NOD mice would help determine: firstly, the direct function of BIRCs on the beta cell's fate in T1D and secondly, whether BIRC proteins may be a suitable therapeutic target for T1D. In the context of T2D, this can be studied by subjecting DKO mice to prolonged high fat diet. As JNK is found to be hyper-activated under conditions of ER stress and the induction of BIRC proteins have been implicated to delay the occurrence of ER stress-induced caspase activation and apoptosis in MEFs *in vitro* (Hamanaka et al., 2009), thus BIRC-deletion may render islets susceptible to stress-induced by high fat diet. Conversely, overexpression of BIRC proteins in this case may provide protection against diet-induced islet stress and could be an avenue of target for T2D treatment.

6.3 Concluding remarks

In conclusion, this present study provided the first molecular insights into the complex roles played by BIRC proteins in TNF-signalling in primary pancreatic islets and beta cells (Figure 6.1). Analysis of four TNF-target genes showed that BIRC2 and BIRC3 perform different and overlapping functions in these cells. TNFα stimulation induces NF-κB activation (Figure 6.1A), which drives the early-immediate expression of *Birc3* (Figure 6.1B). The increased levels of BIRC3 may be required to fine-tune TNF-induced inflammatory signalling for full expression of select TNF-α-induced NF-κB-target genes (Figure 6.1A). Additionally, increased BIRC3, in combination with BIRC2, provided regulatory control over the JNK signalling axis – preventing activation of this pathway in the absence of an overt extra-cellular signal normally provided by TNFα (Figure 6.1D). This is the first time such a role for BIRC proteins has been demonstrated. Dysregulated control of the *Cxcl10*, *Ccl2* and *Icam-1* has been associated with beta cell failure and destruction in diabetes (Figure 6.1F) (Martin et al., 2008; Martin et al., 2001; Schulthess et al., 2009; Yagi et al., 1995), demonstrating the importance of this regulatory control loop in beta cells - expression of these genes were exacerbated with combined BIRC2 and BIRC3 loss. Thus, under inflammatory conditions, pancreatic islets rapidly induce BIRC3 to fine-tune NF-κB and JNK signalling pathways to ensure the beta cell's transcriptional responses are appropriately matched to extra-cellular inputs. The balance of these pathways may be critical for regulating beta cell function under conditions of cellular stress.

Importantly, we showed that antagonizing TNF signalling (with neutralizing antibodies) or targeting BIRC proteins (with BIRC antagonist) for cancer, diabetes or other autoimmune diseases need to be used with caution. Contrary to believe, targeting BIRC deletion may not just result in impaired TNFα-mediated NF-κB signalling but may also lift the suppression of BIRCs on basal JNK activation mediated under conditions of stress. While targeting BIRC proteins may serve as a possibility for eradicating cancers where high levels of BIRC proteins have been detected, especially since BIRC antagonist have shown to induce apoptosis on a variety of cancer cells, we recommend further testing before taking these drugs into clinical trials.

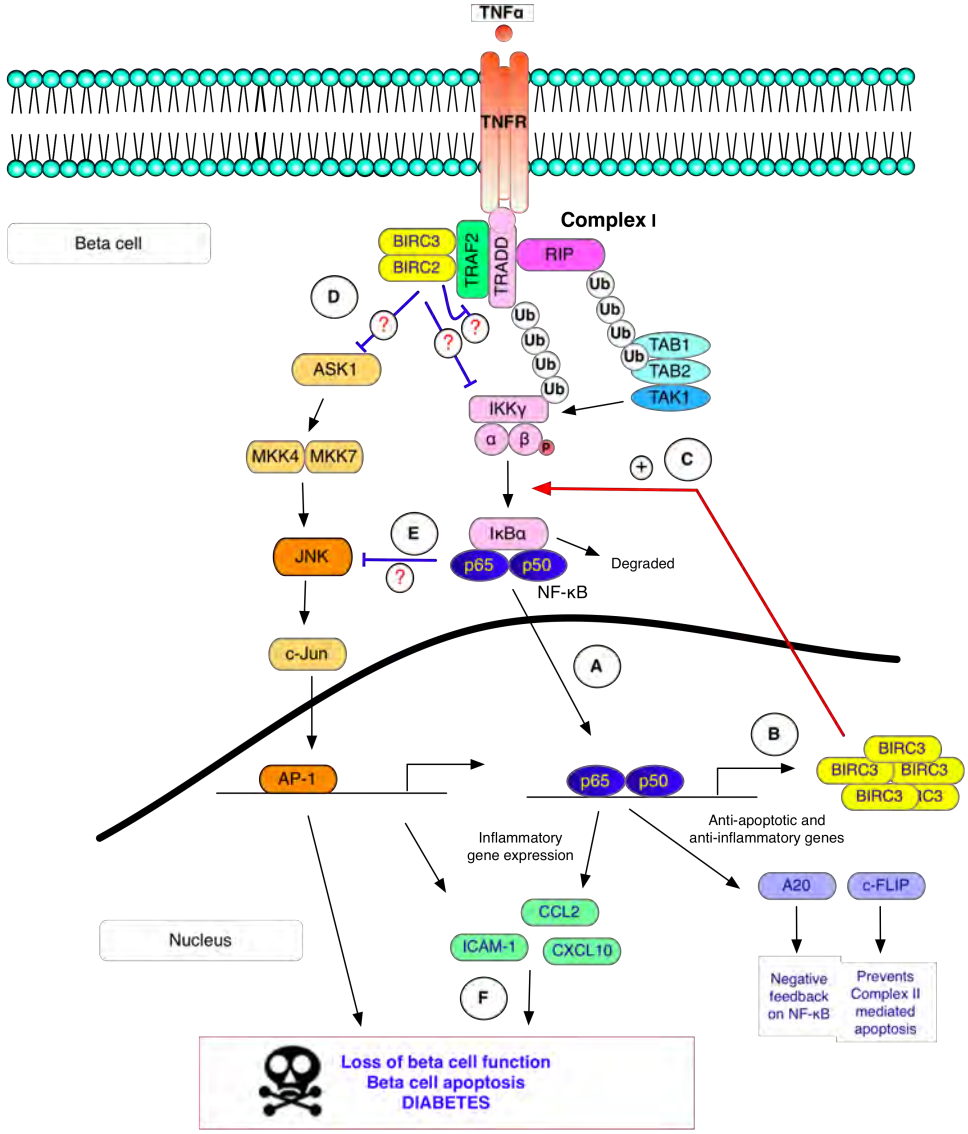


Figure 6.1. Schematic of complex roles played by BIRC proteins in TNF-signalling in primary beta cells proposed in this thesis. (A) Upon TNFα stimulation, a complex signalling cascade involving the sequential recruitment and activation of signalling proteins occurs at the cytoplasmic domain of TNFR1. TRADD recruits TRAF2. TRAF2 recruits BIRC2 and BIRC3 thought to ubiquitinate RIPK1. RIPK1 ubiquitinates and activates TAK1 resulting in IKK phosphorylation and activation. These events lead to the activation of NF-κB signalling which drives the expression of inflammatory genes including *Ccl2, Icam-1* and *Cxcl10* and anti-apoptotic genes such as *A20* and *c-flip*, as well as (B) *Birc3,* which participates in (C) a positive feedback loop in TNFα-induced NF-κB activation required to fine-tune the expression of down-stream NF-κB-regulated genes. (D) BIRC2 and BIRC3 were found to negatively regulate basal JNK activation. However the direct mechanism of BIRC2's suppression of JNK in beta cells remains to be studied. We propose that downstream JNK activation may be modulated in a process involving TRAF2 and BIRC2. BIRC2 is suggested to prevent prolonged JNK activation by targeting TRAF2 and the MAPKKK protein, ASK1, for proteosomal degradation. (E) Further, NF-κB is thought to prevent JNK-mediated apoptosis by suppressing the prolonged phase of JNK activation. This mechanism remains to be elucidated in beta cells. Overall, BIRC2 and BIRC3 are thought fine-tune NF-κB signalling and negatively regulate the JNK pathway to protect beta cells from JNK-mediated apoptosis and (F) to prevent over-production of inflammatory cytokines.

7 References

Aikin, R., Maysinger, D., and Rosenberg, L. (2004). Cross-talk between phosphatidylinositol 3-kinase/AKT and c-jun NH2-terminal kinase mediates survival of isolated human islets. Endocrinology *145*, 4522-4531.

Ainbinder, E., Revach, M., Wolstein, O., Moshonov, S., Diamant, N., and Dikstein, R. (2002). Mechanism of rapid transcriptional induction of tumor necrosis factor alpha-responsive genes by NF-kappaB. Molecular and Cellular Biology *22*, 6354-6362.

Akutsu, T., Nishio, H., Sumino, K., Takeshima, Y., Tsuneishi, S., Wada, H., Takada, S., Matsuo, M., and Nakamura, H. (2002). Molecular genetics of spinal muscular atrophy: contribution of the NAIP gene to clinical severity. The Kobe journal of medical sciences *48*, 25-31.

Allison, J., Thomas, H., Beck, D., Brady, J.L., Lew, A.M., Elefanty, A., Kosaka, H., Kay, T.W., Huang, D.C., and Strasser, A. (2000). Transgenic overexpression of human Bcl-2 in islet beta cells inhibits apoptosis but does not prevent autoimmune destruction. International immunology *12*, 9-17.

Alrashdan, Y.A., Alkhouri, H., Chen, E., Lalor, D.J., Poniris, M., Henness, S., Brightling, C.E., Burgess, J.K., Armour, C.L., Ammit, A.J., and Margaret Hughes, J. (2012). Asthmatic airway smooth muscle CXCL10 production: mitogen-activated protein kinase JNK involvement. American journal of physiology. Lung cellular and molecular physiology *302*, L1118-1127.

Altieri, D.C. (2003). Validating survivin as a cancer therapeutic target. Nature reviews. Cancer *3*, 46-54.

Altieri, D.C. (2006). The case for survivin as a regulator of microtubule dynamics and cell-death decisions. Current opinion in cell biology *18*, 609-615.

Amano, K., and Yoon, J.W. (1990). Studies on autoimmunity for initiation of beta-cell destruction. V. Decrease of macrophage-dependent T lymphocytes and natural killer cytotoxicity in silica-treated BB rats. Diabetes *39*, 590-596.

Ammendrup, A., Maillard, A., Nielsen, K., Aabenhus Andersen, N., Serup, P., Dragsbaek Madsen, O., Mandrup-Poulsen, T., and Bonny, C. (2000). The c-Jun amino-terminal kinase pathway is preferentially activated by interleukin-1 and controls apoptosis in differentiating pancreatic beta-cells. Diabetes *49*, 1468-1476.

Amrani, A., Verdaguer, J., Thiessen, S., Bou, S., and Santamaria, P. (2000). IL-1alpha, IL-1beta, and IFN-gamma mark beta cells for Fas-dependent destruction by diabetogenic CD4(+) T lymphocytes. J Clin Invest *105*, 459-468.

Ankarcrona, M., Dypbukt, J.M., Brune, B., and Nicotera, P. (1994). Interleukin-1 beta-induced nitric oxide production activates apoptosis in pancreatic RINm5F cells. Experimental cell research *213*, 172-177.

Anrather, J., Csizmadia, V., Brostjan, C., Soares, M.P., Bach, F.H., and Winkler, H. (1997). Inhibition of bovine endothelial cell activation in vitro by regulated expression of a transdominant inhibitor of NF-kappa B. J Clin Invest *99*, 763-772.

Appels, B., Burkart, V., Kantwerk-Funke, G., Funda, J., Kolb-Bachofen, V., and Kolb, H. (1989). Spontaneous cytotoxicity of macrophages against pancreatic islet cells. Journal of immunology *142*, 3803-3808.

Arif, S., Cox, P., Afzali, B., Lombardi, G., Lechler, R.I., Peakman, M., and Mirenda, V. (2010). Anti-TNFalpha therapy--killing two birds with one stone? Lancet *375*, 2278.

Arnush, M., Scarim, A.L., Heitmeier, M.R., Kelly, C.B., and Corbett, J.A. (1998). Potential role of resident islet macrophage activation in the initiation of autoimmune diabetes. J Immunol *160*, 2684-2691.

Ashburner, B.P., Westerheide, S.D., and Baldwin, A.S., Jr. (2001). The p65 (RelA) subunit of NF-kappaB interacts with the histone deacetylase (HDAC) corepressors HDAC1 and HDAC2 to negatively regulate gene expression. Molecular and Cellular Biology *21*, 7065-7077.

Babaya, N., Ikegami, H., Fujisawa, T., Ueda, H., Nojima, K., Itoi-Babaya, M., Yamada, K., Kawaguchi, Y., Yamato, E., Makino, S., and Ogihara, T. (2002). Sequence analysis of Tnf as a candidate for Idd16. Autoimmunity *35*, 63-66.

Bach, F.H., Hancock, W.W., and Ferran, C. (1997). Protective genes expressed in endothelial cells: a regulatory response to injury. Immunology today *18*, 483-486.

Baeuerle, P.A., and Baltimore, D. (1996). NF-kappa B: ten years after. Cell *87*, 13-20.

Baker, M.S., Chen, X., Cao, X.C., and Kaufman, D.B. (2001). Expression of a dominant negative inhibitor of NF-kappaB protects MIN6 beta-cells from cytokine-induced apoptosis. The Journal of surgical research *97*, 117-122.

Baldeon, M.E., Chun, T., and Gaskins, H.R. (1998). Interferon-alpha and interferon-gamma differentially affect pancreatic beta-cell phenotype and function. The American journal of physiology *275*, C25-32.

Baldeon, M.E., Neece, D.J., Nandi, D., Monaco, J.J., and Gaskins, H.R. (1997). Interferon-gamma independently activates the MHC class I antigen processing pathway and diminishes glucose responsiveness in pancreatic beta-cell lines. Diabetes *46*, 770-778.

Banatvala, J.E., Bryant, J., Schernthaner, G., Borkenstein, M., Schober, E., Brown, D., De Silva, L.M., Menser, M.A., and Silink, M. (1985). Coxsackie B, mumps, rubella, and cytomegalovirus specific IgM responses in patients with juvenile-onset insulin-dependent diabetes mellitus in Britain, Austria, and Australia. Lancet *1*, 1409-1412.

Banting, F.G., and Best, C.H. (1990). Pancreatic extracts. 1922. The Journal of laboratory and clinical medicine *115*, 254-272.

Baquerizo, H., and Rabinovitch, A. (1990). Interferon-gamma sensitizes rat pancreatic islet cells to lysis by cytokines and cytotoxic cells. J Autoimmun *3 Suppl 1*, 123-130.

Bauler, L.D., Duckett, C.S., and O'Riordan, M.X. (2008). XIAP regulates cytosol-specific innate immunity to Listeria infection. PLoS pathogens *4*, e1000142.

Beg, A.A., Finco, T.S., Nantermet, P.V., and Baldwin, A.S., Jr. (1993). Tumor necrosis factor and interleukin-1 lead to phosphorylation and loss of I kappa B alpha: a mechanism for NF-kappa B activation. Molecular and Cellular Biology *13*, 3301-3310.

Beg, A.A., Ruben, S.M., Scheinman, R.I., Haskill, S., Rosen, C.A., and Baldwin, A.S., Jr. (1992). I kappa B interacts with the nuclear localization sequences of the subunits of NF-kappa B: a mechanism for cytoplasmic retention. Genes & Development *6*, 1899-1913.

Beg, A.A., Sha, W.C., Bronson, R.T., Ghosh, S., and Baltimore, D. (1995). Embryonic lethality and liver degeneration in mice lacking the RelA component of NF-kappa B. Nature *376*, 167-170.

Bell, G.I., Horita, S., and Karam, J.H. (1984). A polymorphic locus near the human insulin gene is associated with insulin-dependent diabetes mellitus. Diabetes *33*, 176-183.

Benson, D.A., Karsch-Mizrachi, I., Lipman, D.J., Ostell, J., and Sayers, E.W. (2011). GenBank. Nucleic acids research *39*, D32-37.

Berger, S.L. (2002). Histone modifications in transcriptional regulation. Current opinion in genetics & development *12*, 142-148.

Bergholdt, R., Heding, P., Nielsen, K., Nolsoe, R., Sparre, T., Stoerling, J., Nerup, J., Pociot, F., and Mandrup-Poulsen, T. (2004). Type 1 diabetes mellitus: An inflammatory disease of the islet. In Immunology of Type 1 Diabetes (Eisenbarth, G. S., ed) pp.129– 153 (Eurekah.com and Kluwer Academic/Plenum Publishers, Amsterdam, The Netherlands).

Bergmann, L., Kroncke, K.D., Suschek, C., Kolb, H., and Kolb-Bachofern, V. (1992). Cytotoxic action of IL-1 beta against pancreatic islets is mediated via nitric oxide formation and is inhibited by NG-monomethyl-L-arginine. FEBS letters *299*, 103-106.

Bertrand, M.J., Doiron, K., Labbe, K., Korneluk, R.G., Barker, P.A., and Saleh, M. (2009). Cellular inhibitors of apoptosis cIAP1 and cIAP2 are required for innate immunity signaling by the pattern recognition receptors NOD1 and NOD2. Immunity *30*, 789-801.

Bertrand, M.J., Lippens, S., Staes, A., Gilbert, B., Roelandt, R., De Medts, J., Gevaert, K., Declercq, W., and Vandenabeele, P. (2011). cIAP1/2 are direct E3 ligases conjugating diverse types of ubiquitin chains to receptor interacting proteins kinases 1 to 4 (RIP1-4). PLoS One *6*, e22356.

Bertrand, M.J.M., Milutinovic, S., Dickson, K.M., Ho, W.C., Boudreault, A., Durkin, J., Gillard, J.W., Jaquith, J.B., Morris, S.J., and Barker, P.A. (2008). cIAP1 and cIAP2 facilitate cancer cell survival by functioning as E3 ligases that promote RIP1 ubiquitination. Molecular Cell *30*, 689-700.

Besse, A., Lamothe, B., Campos, A.D., Webster, W.K., Maddineni, U., Lin, S.C., Wu, H., and Darnay, B.G. (2007). TAK1-dependent signaling requires functional interaction with TAB2/TAB3. J Biol Chem *282*, 3918-3928.

Biarnes, M., Montolio, M., Nacher, V., Raurell, M., Soler, J., and Montanya, E. (2002). Beta-cell death and mass in syngeneically transplanted islets exposed to short- and long-term hyperglycemia. Diabetes *51*, 66-72.

Birnbaum, M.J., Clem, R.J., and Miller, L.K. (1994). An apoptosis-inhibiting gene from a nuclear polyhedrosis virus encoding a polypeptide with Cys/His sequence motifs. Journal of virology *68*, 2521-2528.

Blackwell, K., Zhang, L., Thomas, G.S., Sun, S., Nakano, H., and Habelhah, H. (2009). TRAF2 phosphorylation modulates tumor necrosis factor alpha-induced gene expression and cell resistance to apoptosis. Mol Cell Biol *29*, 303-314.

Blankenship, J.W., Varfolomeev, E., Goncharov, T., Fedorova, A.V., Kirkpatrick, D.S., Izrael-Tomasevic, A., Phu, L., Arnott, D., Aghajan, M., Zobel, K*., et al.* (2009). Ubiquitin binding modulates IAP antagonist-stimulated proteasomal degradation of c-IAP1 and c-IAP2(1). The Biochemical journal *417*, 149-160.

Bliss, M. (1993). The history of insulin. Diabetes care *16 Suppl 3*, 4-7.

Blonska, M., Shambharkar, P.B., Kobayashi, M., Zhang, D., Sakurai, H., Su, B., and Lin, X. (2005). TAK1 is recruited to the tumor necrosis factor-alpha (TNF-alpha) receptor 1 complex in a receptor-interacting protein (RIP)-dependent manner and

cooperates with MEKK3 leading to NF-kappaB activation. J Biol Chem *280*, 43056-43063.

Bloom, B.J. (2000). Development of diabetes mellitus during etanercept therapy in a child with systemic-onset juvenile rheumatoid arthritis. Arthritis and rheumatism *43*, 2606-2608.

Bone, A.J., Hitchcock, P.R., Gwilliam, D.J., Cunningham, J.M., and Barley, J. (1999). Insulitis and mechanisms of disease resistance: studies in an animal model of insulin dependent diabetes mellitus. Journal of molecular medicine *77*, 57-61.

Bonizzi, G., Bebien, M., Otero, D.C., Johnson-Vroom, K.E., Cao, Y., Vu, D., Jegga, A.G., Aronow, B.J., Ghosh, G., Rickert, R.C., and Karin, M. (2004). Activation of IKKalpha target genes depends on recognition of specific kappaB binding sites by RelB:p52 dimers. The EMBO journal *23*, 4202-4210.

Bonizzi, G., and Karin, M. (2004). The two NF-kappaB activation pathways and their role in innate and adaptive immunity. Trends in immunology *25*, 280-288.

Bottazzo, G.F., Dean, B.M., McNally, J.M., MacKay, E.H., Swift, P.G., and Gamble, D.R. (1985). In situ characterization of autoimmune phenomena and expression of HLA molecules in the pancreas in diabetic insulitis. The New England journal of medicine *313*, 353-360.

Bottini, N., Musumeci, L., Alonso, A., Rahmouni, S., Nika, K., Rostamkhani, M., MacMurray, J., Meloni, G.F., Lucarelli, P., Pellecchia, M*., et al.* (2004). A functional variant of lymphoid tyrosine phosphatase is associated with type I diabetes. Nature genetics *36*, 337-338.

Boulton, J.G., and Bourne, J.T. (2007). Unstable diabetes in a patient receiving anti-TNF-alpha for rheumatoid arthritis. Rheumatology *46*, 178-179.

Bradford, M.M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem *72*, 248-254.

Bradley, L.M., Asensio, V.C., Schioetz, L.K., Harbertson, J., Krahl, T., Patstone, G., Woolf, N., Campbell, I.L., and Sarvetnick, N. (1999). Islet-specific Th1, but not Th2, cells secrete multiple chemokines and promote rapid induction of autoimmune diabetes. Journal of immunology *162*, 2511-2520.

Brennan, F.M., and Feldmann, M. (1992). Cytokines in autoimmunity. Current opinion in immunology *4*, 754-759.

Brockhaus, M., Schoenfeld, H.J., Schlaeger, E.J., Hunziker, W., Lesslauer, W., and Loetscher, H. (1990). Identification of two types of tumor necrosis factor receptors

on human cell lines by monoclonal antibodies. Proceedings of the National Academy of Sciences of the United States of America *87*, 3127-3131.

Brown, G.R., Silva, M.D., Thompson, P.A., and Beutler, B. (1998). Lymphoid hyperplasia, CD45RBhigh to CD45RBlow T-cell imbalance, and suppression of Type I diabetes mellitus result from TNF blockade in NOD-->NOD-scid adoptive T cell transfer. Diabetologia *41*, 1502-1510.

Browning, J.L., Ngam-ek, A., Lawton, P., DeMarinis, J., Tizard, R., Chow, E.P., Hession, C., O'Brine-Greco, B., Foley, S.F., and Ware, C.F. (1993). Lymphotoxin beta, a novel member of the TNF family that forms a heteromeric complex with lymphotoxin on the cell surface. Cell *72*, 847-856.

Bubici, C., Papa, S., Pham, C.G., Zazzeroni, F., and Franzoso, G. (2006). The NF-kappaB-mediated control of ROS and JNK signaling. Histology and histopathology *21*, 69-80.

Burke, S.P., Smith, L., and Smith, J.B. (2010). cIAP1 cooperatively inhibits procaspase-3 activation by the caspase-9 apoptosome. J Biol Chem *285*, 30061-30068.

Burren, O.S., Adlem, E.C., Achuthan, P., Christensen, M., Coulson, R.M., and Todd, J.A. (2011). T1DBase: update 2011, organization and presentation of large-scale data sets for type 1 diabetes research. Nucleic acids research *39*, D997-1001.

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

Cabrera, O., Berman, D.M., Kenyon, N.S., Ricordi, C., Berggren, P.O., and Caicedo, A. (2006). The unique cytoarchitecture of human pancreatic islets has implications for islet cell function. Proceedings of the National Academy of Sciences of the United States of America *103*, 2334-2339.

Camacho, S.A., Heath, W.R., Carbone, F.R., Sarvetnick, N., LeBon, A., Karlsson, L., Peterson, P.A., and Webb, S.R. (2001). A key role for ICAM-1 in generating effector cells mediating inflammatory responses. Nature Immunology *2*, 523-529.

Cameron, M.J., Arreaza, G.A., Grattan, M., Meagher, C., Sharif, S., Burdick, M.D., Strieter, R.M., Cook, D.N., and Delovitch, T.L. (2000). Differential expression of CC chemokines and the CCR5 receptor in the pancreas is associated with progression to type I diabetes. Journal of immunology *165*, 1102-1110.

Campbell, I.L., Cutri, A., Wilkinson, D., Boyd, A.W., and Harrison, L.C. (1989). Intercellular adhesion molecule 1 is induced on isolated endocrine islet cells by cytokines but not by reovirus infection. Proc Natl Acad Sci U S A *86*, 4282-4286.

Cantley, J., Selman, C., Shukla, D., Abramov, A.Y., Forstreuter, F., Esteban, M.A., Claret, M., Lingard, S.J., Clements, M., Harten, S.K.*, et al.* (2009). Deletion of the von Hippel-Lindau gene in pancreatic beta cells impairs glucose homeostasis in mice. J Clin Invest *119*, 125-135.

Cantor, J., and Haskins, K. (2005). Effector function of diabetogenic CD4 Th1 T cell clones: a central role for TNF-alpha. Journal of immunology *175*, 7738-7745.

Cardona, K., Korbutt, G.S., Milas, Z., Lyon, J., Cano, J., Jiang, W., Bello-Laborn, H., Hacquoil, B., Strobert, E., Gangappa, S.*, et al.* (2006). Long-term survival of neonatal porcine islets in nonhuman primates by targeting costimulation pathways. Nature medicine *12*, 304-306.

Cardozo, A.K., Heimberg, H., Heremans, Y., Leeman, R., Kutlu, B., Kruhoffer, M., Orntoft, T., and Eizirik, D.L. (2001a). A comprehensive analysis of cytokine-induced and nuclear factor-kappa B-dependent genes in primary rat pancreatic beta-cells. J Biol Chem *276*, 48879-48886.

Cardozo, A.K., Kruhoffer, M., Leeman, R., Orntoft, T., and Eizirik, D.L. (2001b). Identification of novel cytokine-induced genes in pancreatic beta-cells by high-density oligonucleotide arrays. Diabetes *50*, 909-920.

Cardozo, A.K., Proost, P., Gysemans, C., Chen, M.C., Mathieu, C., and Eizirik, D.L. (2003). IL-1beta and IFN-gamma induce the expression of diverse chemokines and IL-15 in human and rat pancreatic islet cells, and in islets from pre-diabetic NOD mice. Diabetologia *46*, 255-266.

Carpentier, I., Declercq, W., Malinin, N.L., Wallach, D., Fiers, W., and Beyaert, R. (1998). TRAF2 plays a dual role in NF-kappaB-dependent gene activation by mediating the TNF-induced activation of p38 MAPK and IkappaB kinase pathways. FEBS letters *425*, 195-198.

Carswell, E.A., Old, L.J., Kassel, R.L., Green, S., Fiore, N., and Williamson, B. (1975). An endotoxin-induced serum factor that causes necrosis of tumors. Proc Natl Acad Sci U S A *72*, 3666-3670.

Cetkovic-Cvrlje, M., and Eizirik, D.L. (1994). TNF-alpha and IFN-gamma potentiate the deleterious effects of IL-1 beta on mouse pancreatic islets mainly via generation of nitric oxide. Cytokine *6*, 399-406.

Chai, J., Shiozaki, E., Srinivasula, S.M., Wu, Q., Datta, P., Alnemri, E.S., and Shi, Y. (2001). Structural basis of caspase-7 inhibition by XIAP. Cell *104*, 769-780.

Chamaillard, M., Girardin, S.E., Viala, J., and Philpott, D.J. (2003). Nods, Nalps and Naip: intracellular regulators of bacterial-induced inflammation. Cellular microbiology *5*, 581-592.

Chang, I., Kim, S., Kim, J.Y., Cho, N., Kim, Y.H., Kim, H.S., Lee, M.K., Kim, K.W., and Lee, M.S. (2003). Nuclear factor kappaB protects pancreatic beta-cells from tumor necrosis factor-alpha-mediated apoptosis. Diabetes *52*, 1169-1175.

Chang, L., Kamata, H., Solinas, G., Luo, J.L., Maeda, S., Venuprasad, K., Liu, Y.C., and Karin, M. (2006). The E3 ubiquitin ligase itch couples JNK activation to TNFalpha-induced cell death by inducing c-FLIP(L) turnover. Cell *124*, 601-613.

Chang, L., and Karin, M. (2001). Mammalian MAP kinase signalling cascades. Nature *410*, 37-40.

Chawla-Sarkar, M., Bae, S.I., Reu, F.J., Jacobs, B.S., Lindner, D.J., and Borden, E.C. (2004). Downregulation of Bcl-2, FLIP or IAPs (XIAP and survivin) by siRNAs sensitizes resistant melanoma cells to Apo2L/TRAIL-induced apoptosis. Cell death and differentiation *11*, 915-923.

Cheatham, B., and Kahn, C.R. (1995). Insulin action and the insulin signaling network. Endocrine reviews *16*, 117-142.

Chen, C.C., and Manning, A.M. (1995). Transcriptional regulation of endothelial cell adhesion molecules: a dominant role for NF-kappa B. Agents Actions Suppl *47*, 135-141.

Chen, M.C., Proost, P., Gysemans, C., Mathieu, C., and Eizirik, D.L. (2001). Monocyte chemoattractant protein-1 is expressed in pancreatic islets from prediabetic NOD mice and in interleukin-1 beta-exposed human and rat islet cells. Diabetologia *44*, 325-332.

Chen, Y.M., Chiang, W.C., Lin, S.L., Wu, K.D., Tsai, T.J., and Hsieh, B.S. (2004). Dual regulation of tumor necrosis factor-alpha-induced CCL2/monocyte chemoattractant protein-1 expression in vascular smooth muscle cells by nuclear factor-kappaB and activator protein-1: modulation by type III phosphodiesterase inhibition. J Pharmacol Exp Ther *309*, 978-986.

Chen, Y.R., Wang, X., Templeton, D., Davis, R.J., and Tan, T.H. (1996). The role of c-Jun N-terminal kinase (JNK) in apoptosis induced by ultraviolet C and gamma radiation. Duration of JNK activation may determine cell death and proliferation. J Biol Chem *271*, 31929-31936.

Chen, Z., Hagler, J., Palombella, V.J., Melandri, F., Scherer, D., Ballard, D., and Maniatis, T. (1995). Signal-induced site-specific phosphorylation targets I kappa B alpha to the ubiquitin-proteasome pathway. Genes & Development *9*, 1586-1597.

Chen, Z., Naito, M., Hori, S., Mashima, T., Yamori, T., and Tsuruo, T. (1999). A human IAP-family gene, apollon, expressed in human brain cancer cells. Biochemical and Biophysical Research Communications *264*, 847-854.

Cheung, A.T., Dayanandan, B., Lewis, J.T., Korbutt, G.S., Rajotte, R.V., Bryer-Ash, M., Boylan, M.O., Wolfe, M.M., and Kieffer, T.J. (2000a). Glucose-dependent insulin release from genetically engineered K cells. Science *290*, 1959-1962.

Cheung, H.H., Lynn Kelly, N., Liston, P., and Korneluk, R.G. (2006). Involvement of caspase-2 and caspase-9 in endoplasmic reticulum stress-induced apoptosis: a role for the IAPs. Experimental cell research *312*, 2347-2357.

Cheung, H.H., Plenchette, S., Kern, C.J., Mahoney, D.J., and Korneluk, R.G. (2008). The RING domain of cIAP1 mediates the degradation of RING-bearing inhibitor of apoptosis proteins by distinct pathways. Mol Biol Cell *19*, 2729-2740.

Cheung, P., Allis, C.D., and Sassone-Corsi, P. (2000b). Signaling to chromatin through histone modifications. Cell *103*, 263-271.

Choi, Y.E., Butterworth, M., Malladi, S., Duckett, C.S., Cohen, G.M., and Bratton, S.B. (2009). The E3 ubiquitin ligase cIAP1 binds and ubiquitinates caspase-3 and -7 via unique mechanisms at distinct steps in their processing. J Biol Chem *284*, 12772-12782.

Choudhury, A.I., Heffron, H., Smith, M.A., Al-Qassab, H., Xu, A.W., Selman, C., Simmgen, M., Clements, M., Claret, M., Maccoll, G.*, et al.* (2005). The role of insulin receptor substrate 2 in hypothalamic and beta cell function. J Clin Invest *115*, 940-950.

Chu, Z.L., McKinsey, T.A., Liu, L., Gentry, J.J., Malim, M.H., and Ballard, D.W. (1997). Suppression of tumor necrosis factor-induced cell death by inhibitor of apoptosis c-IAP2 is under NF-kappaB control. Proc Natl Acad Sci U S A *94*, 10057-10062.

Chua, C.C., Gao, J., Ho, Y.S., Xiong, Y., Xu, X., Chen, Z., Hamdy, R.C., and Chua, B.H. (2007). Overexpression of IAP-2 attenuates apoptosis and protects against myocardial ischemia/reperfusion injury in transgenic mice. Biochim Biophys Acta *1773*, 577-583.

Clarke, D.L., Clifford, R.L., Jindarat, S., Proud, D., Pang, L., Belvisi, M., and Knox, A.J. TNFalpha and IFNgamma synergistically enhance transcriptional activation of CXCL10 in human airway smooth muscle cells via STAT-1, NF-kappaB, and the transcriptional coactivator CREB-binding protein. J Biol Chem *285*, 29101-29110.

Clem, R.J., and Miller, L.K. (1994). Control of programmed cell death by the baculovirus genes p35 and iap. Molecular and Cellular Biology *14*, 5212-5222.

Clements, G.B., Galbraith, D.N., and Taylor, K.W. (1995). Coxsackie B virus infection and onset of childhood diabetes. Lancet *346*, 221-223.

Clerk, A., Fuller, S.J., Michael, A., and Sugden, P.H. (1998). Stimulation of "stress-regulated" mitogen-activated protein kinases (stress-activated protein kinases/c-Jun N-terminal kinases and p38-mitogen-activated protein kinases) in perfused rat hearts by oxidative and other stresses. J Biol Chem *273*, 7228-7234.

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

Conte, D., Holcik, M., Lefebvre, C.A., LaCasse, E., Picketts, D.J., Wright, K.E., and Korneluk, R.G. (2006). Inhibitor of apoptosis protein cIAP2 is essential for lipopolysaccharide-induced macrophage survival. Molecular and Cellular Biology *26*, 699-708.

Conte, D., Liston, P., Wong, J.W., Wright, K.E., and Korneluk, R.G. (2001). Thymocyte-targeted overexpression of xiap transgene disrupts T lymphoid apoptosis and maturation. Proceedings of the National Academy of Sciences of the United States of America *98*, 5049-5054.

Conze, D.B., Albert, L., Ferrick, D.A., Goeddel, D.V., Yeh, W.C., Mak, T., and Ashwell, J.D. (2005). Posttranscriptional downregulation of c-IAP2 by the ubiquitin protein ligase c-IAP1 in vivo. Mol Cell Biol *25*, 3348-3356.

Coppieters, K., Roep, B., and von Herrath, M. (2011). Beta cells under attack: toward a better understanding of type 1 diabetes immunopathology. Seminars in immunopathology *33*, 1-8.

Coppieters, K.T., Dotta, F., Amirian, N., Campbell, P.D., Kay, T.W., Atkinson, M.A., Roep, B.O., and von Herrath, M.G. (2012). Demonstration of islet-autoreactive CD8 T cells in insulitic lesions from recent onset and long-term type 1 diabetes patients. The Journal of experimental medicine *209*, 51-60.

Corbett, J.A., Kwon, G., Turk, J., and McDaniel, M.L. (1993a). IL-1 beta induces the coexpression of both nitric oxide synthase and cyclooxygenase by islets of Langerhans: activation of cyclooxygenase by nitric oxide. Biochemistry *32*, 13767-13770.

Corbett, J.A., and McDaniel, M.L. (1995). Intraislet release of interleukin 1 inhibits beta cell function by inducing beta cell expression of inducible nitric oxide synthase. The Journal of experimental medicine *181*, 559-568.

Corbett, J.A., Sweetland, M.A., Lancaster, J.R., Jr., and McDaniel, M.L. (1993b). A 1-hour pulse with IL-1 beta induces formation of nitric oxide and inhibits insulin secretion by rat islets of Langerhans: evidence for a tyrosine kinase signaling mechanism. FASEB journal : official publication of the Federation of American Societies for Experimental Biology *7*, 369-374.

Corbett, J.A., Wang, J.L., Hughes, J.H., Wolf, B.A., Sweetland, M.A., Lancaster, J.R., Jr., and McDaniel, M.L. (1992). Nitric oxide and cyclic GMP formation induced by interleukin 1 beta in islets of Langerhans. Evidence for an effector role of nitric oxide in islet dysfunction. The Biochemical journal *287 (Pt 1)*, 229-235.

Cottet, S., Dupraz, P., Hamburger, F., Dolci, W., Jaquet, M., and Thorens, B. (2002). cFLIP protein prevents tumor necrosis factor-alpha-mediated induction of caspase-8-dependent apoptosis in insulin-secreting betaTc-Tet cells. Diabetes *51*, 1805-1814.

Crisa, L., Mordes, J.P., and Rossini, A.A. (1992). Autoimmune diabetes mellitus in the BB rat. Diabetes/metabolism reviews *8*, 4-37.

Crook, N.E., Clem, R.J., and Miller, L.K. (1993). An apoptosis-inhibiting baculovirus gene with a zinc finger-like motif. Journal of virology *67*, 2168-2174.

Csomos, R.A., Brady, G.F., and Duckett, C.S. (2009). Enhanced cytoprotective effects of the inhibitor of apoptosis protein cellular IAP1 through stabilization with TRAF2. J Biol Chem *284*, 20531-20539.

Cummins, J.M., Kohli, M., Rago, C., Kinzler, K.W., Vogelstein, B., and Bunz, F. (2004). X-linked inhibitor of apoptosis protein (XIAP) is a nonredundant modulator of tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)-mediated apoptosis in human cancer cells. Cancer Research *64*, 3006-3008.

Dahlen, E., Dawe, K., Ohlsson, L., and Hedlund, G. (1998). Dendritic cells and macrophages are the first and major producers of TNF-alpha in pancreatic islets in the nonobese diabetic mouse. Journal of immunology *160*, 3585-3593.

Damiano, J.S., Oliveira, V., Welsh, K., and Reed, J.C. (2004). Heterotypic interactions among NACHT domains: implications for regulation of innate immune responses. The Biochemical journal *381*, 213-219.

Dandona, P., Weinstock, R., Thusu, K., Abdel-Rahman, E., Aljada, A., and Wadden, T. (1998). Tumor necrosis factor-alpha in sera of obese patients: fall with weight loss. The Journal of clinical endocrinology and metabolism *83*, 2907-2910.

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

Daniel, S., Arvelo, M.B., Patel, V.I., Longo, C.R., Shrikhande, G., Shukri, T., Mahiou, J., Sun, D.W., Mottley, C., Grey, S.T., and Ferran, C. (2004). A20 protects endothelial cells from TNF-, Fas-, and NK-mediated cell death by inhibiting caspase 8 activation. Blood *104*, 2376-2384.

Darville, M.I., and Eizirik, D.L. (2001). Cytokine induction of Fas gene expression in insulin-producing cells requires the transcription factors NF-kappaB and C/EBP. Diabetes *50*, 1741-1748.

Dasgupta, A., Alvarado, C.S., Xu, Z., and Findley, H.W. (2010). Expression and functional role of inhibitor-of-apoptosis protein livin (BIRC7) in neuroblastoma. Biochemical and Biophysical Research Communications *400*, 53-59.

Davis, R.J. (2000). Signal transduction by the JNK group of MAP kinases. Cell *103*, 239-252.

Davoodi, J., Ghahremani, M.H., Es-Haghi, A., Mohammad-Gholi, A., and Mackenzie, A. (2010). Neuronal apoptosis inhibitory protein, NAIP, is an inhibitor of procaspase-9. The international journal of biochemistry & cell biology *42*, 958-964.

Delaney, C.A., Green, M.H., Lowe, J.E., and Green, I.C. (1993). Endogenous nitric oxide induced by interleukin-1 beta in rat islets of Langerhans and HIT-T15 cells causes significant DNA damage as measured by the 'comet' assay. FEBS letters *333*, 291-295.

Delovitch, T.L., and Singh, B. (1997). The nonobese diabetic mouse as a model of autoimmune diabetes: immune dysregulation gets the NOD. Immunity *7*, 727-738.

Deng, Y., Ren, X., Yang, L., Lin, Y., and Wu, X. (2003). A JNK-dependent pathway is required for TNFalpha-induced apoptosis. Cell *115*, 61-70.

Derijard, B., Hibi, M., Wu, I.H., Barrett, T., Su, B., Deng, T., Karin, M., and Davis, R.J. (1994). JNK1: a protein kinase stimulated by UV light and Ha-Ras that binds and phosphorylates the c-Jun activation domain. Cell *76*, 1025-1037.

Deveraux, Q.L., Roy, N., Stennicke, H.R., Van Arsdale, T., Zhou, Q., Srinivasula, S.M., Alnemri, E.S., Salvesen, G.S., and Reed, J.C. (1998). IAPs block apoptotic events induced by caspase-8 and cytochrome c by direct inhibition of distinct caspases. Embo J *17*, 2215-2223.

Deveraux, Q.L., Takahashi, R., Salvesen, G.S., and Reed, J.C. (1997). X-linked IAP is a direct inhibitor of cell-death proteases. Nature *388*, 300-304.

Devin, A., Cook, A., Lin, Y., Rodriguez, Y., Kelliher, M., and Liu, Z. (2000). The distinct roles of TRAF2 and RIP in IKK activation by TNF-R1: TRAF2 recruits IKK to TNF-R1 while RIP mediates IKK activation. Immunity *12*, 419-429.

DiDonato, J.A., Mercurio, F., and Karin, M. (1995). Phosphorylation of I kappa B alpha precedes but is not sufficient for its dissociation from NF-kappa B. Molecular and Cellular Biology *15*, 1302-1311.

Dinarello, C.A. (1991). Interleukin-1 and interleukin-1 antagonism. Blood *77*, 1627-1652.

Ding, X., Wang, X., Xue, W., Tian, X., Li, Y., Jiao, F., Feng, X., and Zheng, J. (2012). Blockade of the nuclear factor kappa B pathway prolonged islet allograft survival. Artificial organs *36*, E21-27.

Dittrich, H.M. (1989). [History of the discovery of pancreatic diabetes by von Mering and Minkowski 1889. A historical overview on the occasion of the 100th anniversary]. Zeitschrift fur die gesamte innere Medizin und ihre Grenzgebiete *44*, 335-340.

Dogan, T., Harms, G.S., Hekman, M., Karreman, C., Oberoi, T.K., Alnemri, E.S., Rapp, U.R., and Rajalingam, K. (2008). X-linked and cellular IAPs modulate the stability of C-RAF kinase and cell motility. Nature cell biology *10*, 1447-1455.

Dohi, T., Salz, W., Costa, M., Ariyan, C., Basadonna, G.P., and Altieri, D.C. (2006). Inhibition of apoptosis by survivin improves transplantation of pancreatic islets for treatment of diabetes in mice. EMBO reports *7*, 438-443.

Donath, M.Y., Boni-Schnetzler, M., Ellingsgaard, H., and Ehses, J.A. (2009). Islet inflammation impairs the pancreatic beta-cell in type 2 diabetes. Physiology (Bethesda) *24*, 325-331.

Donath, M.Y., Schumann, D.M., Faulenbach, M., Ellingsgaard, H., Perren, A., and Ehses, J.A. (2008). Islet inflammation in type 2 diabetes: from metabolic stress to therapy. Diabetes Care *31 Suppl 2*, S161-164.

Dong, Z., Wang, J.Z., Yu, F., and Venkatachalam, M.A. (2003). Apoptosis-resistance of hypoxic cells: multiple factors involved and a role for IAP-2. The American journal of pathology *163*, 663-671.

Duckett, C.S., Nava, V.E., Gedrich, R.W., Clem, R.J., Van Dongen, J.L., Gilfillan, M.C., Shiels, H., Hardwick, J.M., and Thompson, C.B. (1996). A conserved family of cellular genes related to the baculovirus iap gene and encoding apoptosis inhibitors. EMBO J *15*, 2685-2694.

Duffy, M.J., O'Donovan, N., Brennan, D.J., Gallagher, W.M., and Ryan, B.M. (2007). Survivin: a promising tumor biomarker. Cancer letters *249*, 49-60.

Dungan, K.M., Buse, J.B., and Ratner, R.E. (2009). Effects of therapy in type 1 and type 2 diabetes mellitus with a peptide derived from islet neogenesis associated protein (INGAP). Diabetes/metabolism research and reviews *25*, 558-565.

Dupoux, A., Cartier, J., Cathelin, S., Filomenko, R., Solary, E., and Dubrez-Daloz, L. (2009). cIAP1-dependent TRAF2 degradation regulates the differentiation of

monocytes into macrophages and their response to CD40 ligand. Blood *113*, 175-185.

Eberhardy, S.R., D'Cunha, C.A., and Farnham, P.J. (2000). Direct examination of histone acetylation on Myc target genes using chromatin immunoprecipitation. J Biol Chem *275*, 33798-33805.

Eckelman, B.P., and Salvesen, G.S. (2006). The human anti-apoptotic proteins cIAP1 and cIAP2 bind but do not inhibit caspases. J Biol Chem *281*, 3254-3260.

Eckelman, B.P., Salvesen, G.S., and Scott, F.L. (2006). Human inhibitor of apoptosis proteins: why XIAP is the black sheep of the family. EMBO reports *7*, 988-994.

Efrat, S., Linde, S., Kofod, H., Spector, D., Delannoy, M., Grant, S., Hanahan, D., and Baekkeskov, S. (1988). Beta-cell lines derived from transgenic mice expressing a hybrid insulin gene-oncogene. Proc Natl Acad Sci U S A *85*, 9037-9041.

Ehses, J.A., Ellingsgaard, H., Boni-Schnetzler, M., and Donath, M.Y. (2009). Pancreatic islet inflammation in type 2 diabetes: from alpha and beta cell compensation to dysfunction. Arch Physiol Biochem *115*, 240-247.

Eisenbarth, G.S. (1986). Type I diabetes mellitus. A chronic autoimmune disease. The New England journal of medicine *314*, 1360-1368.

Eisenbarth GS, P.K., Buse JB. (2008). Type 1 Diabetes Mellitus. *In: Kronenberg: Williams Textbook of Endocrinology, Kronenberg HM, Melmed S, Polonsky KS, Larsen PR.* , Vol Chapter 31, 11th ed. edn (Philadelphia, Pa: Saunders Elsevier, Chapter 31).

Eizirik, D.L., Bjorklund, A., and Welsh, N. (1993). Interleukin-1-induced expression of nitric oxide synthase in insulin-producing cells is preceded by c-fos induction and depends on gene transcription and protein synthesis. FEBS letters *317*, 62-66.

Eizirik, D.L., Cagliero, E., Bjorklund, A., and Welsh, N. (1992). Interleukin-1 beta induces the expression of an isoform of nitric oxide synthase in insulin-producing cells, which is similar to that observed in activated macrophages. FEBS letters *308*, 249-252.

Eizirik, D.L., Sammeth, M., Bouckenooghe, T., Bottu, G., Sisino, G., Igoillo-Esteve, M., Ortis, F., Santin, I., Colli, M.L., Barthson, J.*, et al.* (2012). The human pancreatic islet transcriptome: expression of candidate genes for type 1 diabetes and the impact of pro-inflammatory cytokines. PLoS genetics *8*, e1002552.

Eldor, R., Yeffet, A., Baum, K., Doviner, V., Amar, D., Ben-Neriah, Y., Christofori, G., Peled, A., Carel, J.C., Boitard, C.*, et al.* (2006). Conditional and specific NF-kappaB blockade protects pancreatic beta cells from diabetogenic agents. Proceedings of the National Academy of Sciences of the United States of America *103*, 5072-5077.

Emamaullee, J.A., Rajotte, R.V., Liston, P., Korneluk, R.G., Lakey, J.R., Shapiro, A.M., and Elliott, J.F. (2005). XIAP overexpression in human islets prevents early posttransplant apoptosis and reduces the islet mass needed to treat diabetes. Diabetes *54*, 2541-2548.

Endo, K., Kohnoe, S., Tsujita, E., Watanabe, A., Nakashima, H., Baba, H., and Maehara, Y. (2005). Modulation of anti-apoptosis by endogenous IAP expression in MKN45 human gastric cancer cells. Anticancer research *25*, 2713-2717.

Essawi, M.L., Effat, L.K., Shanab, G.M., Al-Ettribi, G.M., El-Haronui, A.A., and Karim, A.M. (2007). Molecular analysis of SMN1 and NAIP genes in Egyptian patients with spinal muscular atrophy. Bratislavske lekarske listy *108*, 133-137.

Fan, T.J., Han, L.H., Cong, R.S., and Liang, J. (2005). Caspase family proteases and apoptosis. Acta biochimica et biophysica Sinica *37*, 719-727.

Farre, D., Roset, R., Huerta, M., Adsuara, J.E., Rosello, L., Alba, M.M., and Messeguer, X. (2003). Identification of patterns in biological sequences at the ALGGEN server: PROMO and MALGEN. Nucleic Acids Res *31*, 3651-3653.

Fex, M., Wierup, N., Nitert, M.D., Ristow, M., and Mulder, H. (2007). Rat insulin promoter 2-Cre recombinase mice bred onto a pure C57BL/6J background exhibit unaltered glucose tolerance. The Journal of endocrinology *194*, 551-555.

Filippi, C.M., and von Herrath, M.G. (2008). Viral trigger for type 1 diabetes: pros and cons. Diabetes *57*, 2863-2871.

Fornoni, A., Pileggi, A., Molano, R.D., Sanabria, N.Y., Tejada, T., Gonzalez-Quintana, J., Ichii, H., Inverardi, L., Ricordi, C., and Pastori, R.L. (2008). Inhibition of c-jun N terminal kinase (JNK) improves functional beta cell mass in human islets and leads to AKT and glycogen synthase kinase-3 (GSK-3) phosphorylation. Diabetologia *51*, 298-308.

Foulis, A.K. (1987). C. L. Oakley lecture (1987). The pathogenesis of beta cell destruction in type I (insulin-dependent) diabetes mellitus. The Journal of pathology *152*, 141-148.

Foulis, A.K., and Stewart, J.A. (1984). The pancreas in recent-onset type 1 (insulin-dependent) diabetes mellitus: insulin content of islets, insulitis and associated changes in the exocrine acinar tissue. Diabetologia *26*, 456-461.

Frigerio, S., Junt, T., Lu, B., Gerard, C., Zumsteg, U., Hollander, G.A., and Piali, L. (2002). Beta cells are responsible for CXCR3-mediated T-cell infiltration in insulitis. Nature medicine *8*, 1414-1420.

Fritz, J.H., Ferrero, R.L., Philpott, D.J., and Girardin, S.E. (2006). Nod-like proteins in immunity, inflammation and disease. Nature Immunology *7*, 1250-1257.

Fukuda, K., Tesch, G.H., and Nikolic-Paterson, D.J. (2008). c-Jun amino terminal kinase 1 deficient mice are protected from streptozotocin-induced islet injury. Biochemical and Biophysical Research Communications *366*, 710-716.

Fulda, S., and Vucic, D. (2012). Targeting IAP proteins for therapeutic intervention in cancer. Nature reviews. Drug discovery *11*, 109-124.

Furumai, R., Ito, A., Ogawa, K., Maeda, S., Saito, A., Nishino, N., Horinouchi, S., and Yoshida, M. (2011). Histone deacetylase inhibitors block nuclear factor-kappaB-dependent transcription by interfering with RNA polymerase II recruitment. Cancer science *102*, 1081-1087.

Furusu, A., Nakayama, K., Xu, Q., Konta, T., Sugiyama, H., and Kitamura, M. (2001). Expression, regulation, and function of inhibitor of apoptosis family genes in rat mesangial cells. Kidney international *60*, 579-586.

Furusu, A., Nakayama, K., Xu, Q.H., Konta, T., and Kitamura, M. (2007). MAP kinase-dependent, NF-kappa B-independent regulation of inhibitor of apoptosis protein genes by TNF-alpha. Journal of Cellular Physiology *210*, 703-710.

Gagnon, V., St-Germain, M.E., Parent, S., and Asselin, E. (2003). Akt activity in endometrial cancer cells: regulation of cell survival through cIAP-1. International journal of oncology *23*, 803-810.

Gamble, D.R., Kinsley, M.L., FitzGerald, M.G., Bolton, R., and Taylor, K.W. (1969). Viral antibodies in diabetes mellitus. British medical journal *3*, 627-630.

Ganchi, P.A., Sun, S.C., Greene, W.C., and Ballard, D.W. (1992). I kappa B/MAD-3 masks the nuclear localization signal of NF-kappa B p65 and requires the transactivation domain to inhibit NF-kappa B p65 DNA binding. Molecular Biology of the Cell *3*, 1339-1352.

Gannon, M., Shiota, C., Postic, C., Wright, C.V., and Magnuson, M. (2000). Analysis of the Cre-mediated recombination driven by rat insulin promoter in embryonic and adult mouse pancreas. Genesis *26*, 139-142.

Gao, Y.J., Zhang, L., Samad, O.A., Suter, M.R., Yasuhiko, K., Xu, Z.Z., Park, J.Y., Lind, A.L., Ma, Q., and Ji, R.R. (2009). JNK-induced MCP-1 production in spinal cord astrocytes contributes to central sensitization and neuropathic pain. J Neurosci *29*, 4096-4108.

Gardam, S., Turner, V.M., Anderton, H., Limaye, S., Basten, A., Koentgen, F., Vaux, D.L., Silke, J., and Brink, R. Deletion of cIAP1 and cIAP2 in murine B lymphocytes

constitutively activates cell survival pathways and inactivates the germinal center response. Blood.

Ge, B., Gram, H., Di Padova, F., Huang, B., New, L., Ulevitch, R.J., Luo, Y., and Han, J. (2002). MAPKK-independent activation of p38alpha mediated by TAB1-dependent autophosphorylation of p38alpha. Science *295*, 1291-1294.

Ge, B., Xiong, X., Jing, Q., Mosley, J.L., Filose, A., Bian, D., Huang, S., and Han, J. (2003). TAB1beta (transforming growth factor-beta-activated protein kinase 1-binding protein 1beta), a novel splicing variant of TAB1 that interacts with p38alpha but not TAK1. J Biol Chem *278*, 2286-2293.

Gentle, I.E., Wong, W.W., Evans, J.M., Bankovacki, A., Cook, W.D., Khan, N.R., Nachbur, U., Rickard, J., Anderton, H., Moulin, M.*, et al.* In TNF-stimulated cells, RIPK1 promotes cell survival by stabilizing TRAF2 and cIAP1, which limits induction of non-canonical NF-(kappa)B and activation of caspase-8. J Biol Chem.

Gepts, W. (1965). Pathologic anatomy of the pancreas in juvenile diabetes mellitus. Diabetes *14*, 619-633.

Gepts, W., and Lecompte, P.M. (1981). The pancreatic islets in diabetes. The American journal of medicine *70*, 105-115.

Giannoukakis, N., Rudert, W.A., Trucco, M., and Robbins, P.D. (2000). Protection of human islets from the effects of interleukin-1beta by adenoviral gene transfer of an Ikappa B repressor. J Biol Chem *275*, 36509-36513.

Gill, C., Dowling, C., O'Neill, A.J., and Watson, R.W. (2009). Effects of cIAP-1, cIAP-2 and XIAP triple knockdown on prostate cancer cell susceptibility to apoptosis, cell survival and proliferation. Mol Cancer *8*, 39.

Gilmore, T.D. (2006). Introduction to NF-kappaB: players, pathways, perspectives. Oncogene *25*, 6680-6684.

Goldberg, I.H., Rabinowitz, M., and Reich, E. (1962). Basis of actinomycin action. I. DNA binding and inhibition of RNA-polymerase synthetic reactions by actinomycin. Proc Natl Acad Sci U S A *48*, 2094-2101.

Goldfeld, A.E., Strominger, J.L., and Doyle, C. (1991). Human tumor necrosis factor alpha gene regulation in phorbol ester stimulated T and B cell lines. The Journal of experimental medicine *174*, 73-81.

Goping, I.S., Barry, M., Liston, P., Sawchuk, T., Constantinescu, G., Michalak, K.M., Shostak, I., Roberts, D.L., Hunter, A.M., Korneluk, R., and Bleackley, R.C. (2003). Granzyme B-induced apoptosis requires both direct caspase activation and relief of caspase inhibition. Immunity *18*, 355-365.

Graham, F.L., Smiley, J., Russell, W.C., and Nairn, R. (1977). Characteristics of a human cell line transformed by DNA from human adenovirus type 5. J Gen Virol *36*, 59-74.

Green, E.A., Eynon, E.E., and Flavell, R.A. (1998). Local expression of TNFalpha in neonatal NOD mice promotes diabetes by enhancing presentation of islet antigens. Immunity *9*, 733-743.

Green, E.A., and Flavell, R.A. (1999). Tumor necrosis factor-alpha and the progression of diabetes in non-obese diabetic mice. Immunological reviews *169*, 11-22.

Grell, M., Douni, E., Wajant, H., Lohden, M., Clauss, M., Maxeiner, B., Georgopoulos, S., Lesslauer, W., Kollias, G., Pfizenmaier, K., and Scheurich, P. (1995). The transmembrane form of tumor necrosis factor is the prime activating ligand of the 80 kDa tumor necrosis factor receptor. Cell *83*, 793-802.

Grey, S.T., Arvelo, M.B., Hasenkamp, W., Bach, F.H., and Ferran, C. (1999). A20 inhibits cytokine-induced apoptosis and nuclear factor kappaB-dependent gene activation in islets. J Exp Med *190*, 1135-1146.

Grey, S.T., Longo, C., Shukri, T., Patel, V.I., Csizmadia, E., Daniel, S., Arvelo, M.B., Tchipashvili, V., and Ferran, C. (2003). Genetic engineering of a suboptimal islet graft with A20 preserves beta cell mass and function. Journal of immunology *170*, 6250-6256.

Grunnet, L.G., Aikin, R., Tonnesen, M.F., Paraskevas, S., Blaabjerg, L., Storling, J., Rosenberg, L., Billestrup, N., Maysinger, D., and Mandrup-Poulsen, T. (2009). Proinflammatory cytokines activate the intrinsic apoptotic pathway in beta-cells. Diabetes *58*, 1807-1815.

Gu, H., Zou, Y.R., and Rajewsky, K. (1993). Independent control of immunoglobulin switch recombination at individual switch regions evidenced through Cre-loxP-mediated gene targeting. Cell *73*, 1155-1164.

Guo, Y.L., Baysal, K., Kang, B., Yang, L.J., and Williamson, J.R. (1998). Correlation between sustained c-Jun N-terminal protein kinase activation and apoptosis induced by tumor necrosis factor-alpha in rat mesangial cells. J Biol Chem *273*, 4027-4034.

Gupta, S., Barrett, T., Whitmarsh, A.J., Cavanagh, J., Sluss, H.K., Derijard, B., and Davis, R.J. (1996). Selective interaction of JNK protein kinase isoforms with transcription factors. The EMBO journal *15*, 2760-2770.

Gyrd-Hansen, M., Darding, M., Miasari, M., Santoro, M.M., Zender, L., Xue, W., Tenev, T., da Fonseca, P.C., Zvelebil, M., Bujnicki, J.M., *et al.* (2008). IAPs contain an

evolutionarily conserved ubiquitin-binding domain that regulates NF-kappaB as well as cell survival and oncogenesis. Nature cell biology *10*, 1309-1317.

Gyrd-Hansen, M., and Meier, P. (2010). IAPs: from caspase inhibitors to modulators of NF-kappaB, inflammation and cancer. Nature reviews. Cancer *10*, 561-574.

Haag, C., Stadel, D., Zhou, S., Bachem, M.G., Moller, P., Debatin, K.M., and Fulda, S. Identification of c-FLIP(L) and c-FLIP(S) as critical regulators of death receptor-induced apoptosis in pancreatic cancer cells. Gut *60*, 225-237.

Haas, T.L., Emmerich, C.H., Gerlach, B., Schmukle, A.C., Cordier, S.M., Rieser, E., Feltham, R., Vince, J., Warnken, U., Wenger, T.*, et al.* (2009). Recruitment of the linear ubiquitin chain assembly complex stabilizes the TNF-R1 signaling complex and is required for TNF-mediated gene induction. Molecular Cell *36*, 831-844.

Habelhah, H., Takahashi, S., Cho, S.G., Kadoya, T., Watanabe, T., and Ronai, Z. (2004). Ubiquitination and translocation of TRAF2 is required for activation of JNK but not of p38 or NF-kappaB. EMBO J *23*, 322-332.

Hadjiyanni, I., Baggio, L.L., Poussier, P., and Drucker, D.J. (2008). Exendin-4 modulates diabetes onset in nonobese diabetic mice. Endocrinology *149*, 1338-1349.

Halban, P.A. (2004). Cellular sources of new pancreatic beta cells and therapeutic implications for regenerative medicine. Nature cell biology *6*, 1021-1025.

Hamaguchi, H., Takano, N., Saito, K., Enokihara, H., Furusawa, S., and Shishido, H. (1990). Interaction of monocytes and T cells in the regulation of normal human megakaryocytopoiesis in vitro: role of IL-1 and IL-2. British journal of haematology *76*, 12-20.

Hamanaka, R.B., Bobrovnikova-Marjon, E., Ji, X., Liebhaber, S.A., and Diehl, J.A. (2009). PERK-dependent regulation of IAP translation during ER stress. Oncogene *28*, 910-920.

Hamilton-Williams, E.E., Palmer, S.E., Charlton, B., and Slattery, R.M. (2003). Beta cell MHC class I is a late requirement for diabetes. Proceedings of the National Academy of Sciences of the United States of America *100*, 6688-6693.

Hammonds, P., Beggs, M., Beresford, G., Espinal, J., Clarke, J., and Mertz, R.J. (1990). Insulin-secreting beta-cells possess specific receptors for interleukin-1 beta. FEBS letters *261*, 97-100.

Hanahan, D. (1985). Heritable formation of pancreatic beta-cell tumours in transgenic mice expressing recombinant insulin/simian virus 40 oncogenes. Nature *315*, 115-122.

Hanenberg, H., Kolb-Bachofen, V., Kantwerk-Funke, G., and Kolb, H. (1989). Macrophage infiltration precedes and is a prerequisite for lymphocytic insulitis in pancreatic islets of pre-diabetic BB rats. Diabetologia *32*, 126-134.

Hanninen, A., Jalkanen, S., Salmi, M., Toikkanen, S., Nikolakaros, G., and Simell, O. (1992). Macrophages, T cell receptor usage, and endothelial cell activation in the pancreas at the onset of insulin-dependent diabetes mellitus. J Clin Invest *90*, 1901-1910.

Hao, Y., Sekine, K., Kawabata, A., Nakamura, H., Ishioka, T., Ohata, H., Katayama, R., Hashimoto, C., Zhang, X., Noda, T.*, et al.* (2004). Apollon ubiquitinates SMAC and caspase-9, and has an essential cytoprotection function. Nature cell biology *6*, 849-860.

Hauser, H.P., Bardroff, M., Pyrowolakis, G., and Jentsch, S. (1998). A giant ubiquitin-conjugating enzyme related to IAP apoptosis inhibitors. The Journal of cell biology *141*, 1415-1422.

Hay, B.A., Wassarman, D.A., and Rubin, G.M. (1995). Drosophila homologs of baculovirus inhibitor of apoptosis proteins function to block cell death. Cell *83*, 1253-1262.

Hay, C.W., and Docherty, K. (2006). Comparative analysis of insulin gene promoters: implications for diabetes research. Diabetes *55*, 3201-3213.

Hayden, M.S., and Ghosh, S. (2008). Shared principles in NF-kappaB signaling. Cell *132*, 344-362.

Heimberg, H., Heremans, Y., Jobin, C., Leemans, R., Cardozo, A.K., Darville, M., and Eizirik, D.L. (2001). Inhibition of cytokine-induced NF-kappaB activation by adenovirus-mediated expression of a NF-kappaB super-repressor prevents beta-cell apoptosis. Diabetes *50*, 2219-2224.

Held, W., MacDonald, H.R., Weissman, I.L., Hess, M.W., and Mueller, C. (1990). Genes encoding tumor necrosis factor alpha and granzyme A are expressed during development of autoimmune diabetes. Proceedings of the National Academy of Sciences of the United States of America *87*, 2239-2243.

Hering, B.J., Wijkstrom, M., Graham, M.L., Hardstedt, M., Aasheim, T.C., Jie, T., Ansite, J.D., Nakano, M., Cheng, J., Li, W.*, et al.* (2006). Prolonged diabetes reversal after intraportal xenotransplantation of wild-type porcine islets in immunosuppressed nonhuman primates. Nature medicine *12*, 301-303.

Hilbrands, R., Huurman, V., Gillard, P., Velthuis, J., De Waele, M., Mathieu, C., Kaufman, L., Pipeleers-Marichal, M., Ling, Z., Movahedi, B.*, et al.* (2009). Differences in baseline lymphocyte counts and autoreactivity are associated with differences

in outcome of islet cell transplantation in type 1 diabetic patients. Diabetes *58*, 2267-2343.

Hinds, M.G., Norton, R.S., Vaux, D.L., and Day, C.L. (1999). Solution structure of a baculoviral inhibitor of apoptosis (IAP) repeat. Nat Struct Biol *6*, 648-651.

Hirai, H., Kaino, Y., Ito, T., and Kida, K. (2000). Analysis of cytokine mRNA expression in pancreatic islets of nonobese diabetic mice. Journal of pediatric endocrinology & metabolism : JPEM *13*, 91-98.

Ho, A.W., Wong, C.K., and Lam, C.W. (2008). Tumor necrosis factor-alpha up-regulates the expression of CCL2 and adhesion molecules of human proximal tubular epithelial cells through MAPK signaling pathways. Immunobiology *213*, 533-544.

Hoffmann, A., and Baltimore, D. (2006). Circuitry of nuclear factor kappaB signaling. Immunological reviews *210*, 171-186.

Hohmann, H.P., Remy, R., Brockhaus, M., and van Loon, A.P. (1989). Two different cell types have different major receptors for human tumor necrosis factor (TNF alpha). J Biol Chem *264*, 14927-14934.

Hong, S.Y., Yoon, W.H., Park, J.H., Kang, S.G., Ahn, J.H., and Lee, T.H. (2000). Involvement of two NF-kappa B binding elements in tumor necrosis factor alpha -, CD40-, and epstein-barr virus latent membrane protein 1-mediated induction of the cellular inhibitor of apoptosis protein 2 gene. J Biol Chem *275*, 18022-18028.

Hotamisligil, G.S. (1999). The role of TNFalpha and TNF receptors in obesity and insulin resistance. Journal of internal medicine *245*, 621-625.

Hotamisligil, G.S. (2005). Role of endoplasmic reticulum stress and c-Jun NH2-terminal kinase pathways in inflammation and origin of obesity and diabetes. Diabetes *54 Suppl 2*, S73-78.

Hsu, H., Shu, H.B., Pan, M.G., and Goeddel, D.V. (1996). TRADD-TRAF2 and TRADD-FADD interactions define two distinct TNF receptor 1 signal transduction pathways. Cell *84*, 299-308.

Hsu, H., Xiong, J., and Goeddel, D.V. (1995). The TNF receptor 1-associated protein TRADD signals cell death and NF-kappa B activation. Cell *81*, 495-504.

Hu, S., and Yang, X. (2003). Cellular inhibitor of apoptosis 1 and 2 are ubiquitin ligases for the apoptosis inducer Smac/DIABLO. J Biol Chem *278*, 10055-10060.

Huang, H., Joazeiro, C.A., Bonfoco, E., Kamada, S., Leverson, J.D., and Hunter, T. (2000). The inhibitor of apoptosis, cIAP2, functions as a ubiquitin-protein ligase

and promotes in vitro monoubiquitination of caspases 3 and 7. J Biol Chem *275*, 26661-26664.

Huang, Y., Park, Y.C., Rich, R.L., Segal, D., Myszka, D.G., and Wu, H. (2001). Structural basis of caspase inhibition by XIAP: differential roles of the linker versus the BIR domain. Cell *104*, 781-790.

Hughes, J.H., Colca, J.R., Easom, R.A., Turk, J., and McDaniel, M.L. (1990). Interleukin 1 inhibits insulin secretion from isolated rat pancreatic islets by a process that requires gene transcription and mRNA translation. J Clin Invest *86*, 856-863.

Hui, H., Khoury, N., Zhao, X., Balkir, L., D'Amico, E., Bullotta, A., Nguyen, E.D., Gambotto, A., and Perfetti, R. (2005). Adenovirus-mediated XIAP gene transfer reverses the negative effects of immunosuppressive drugs on insulin secretion and cell viability of isolated human islets. Diabetes *54*, 424-433.

Hultgren, B., Huang, X., Dybdal, N., and Stewart, T.A. (1996). Genetic absence of gamma-interferon delays but does not prevent diabetes in NOD mice. Diabetes *45*, 812-817.

Hussain, M.J., Peakman, M., Gallati, H., Lo, S.S., Hawa, M., Viberti, G.C., Watkins, P.J., Leslie, R.D., and Vergani, D. (1996). Elevated serum levels of macrophage-derived cytokines precede and accompany the onset of IDDM. Diabetologia *39*, 60-69.

Ikegami, H., and Ogihara, T. (1996). Genetics of insulin-dependent diabetes mellitus. Endocrine journal *43*, 605-613.

Imoto, I., Tsuda, H., Hirasawa, A., Miura, M., Sakamoto, M., Hirohashi, S., and Inazawa, J. (2002). Expression of cIAP1, a target for 11q22 amplification, correlates with resistance of cervical cancers to radiotherapy. Cancer Research *62*, 4860-4866.

Ishihara, H., Asano, T., Tsukuda, K., Katagiri, H., Inukai, K., Anai, M., Kikuchi, M., Yazaki, Y., Miyazaki, J.I., and Oka, Y. (1993). Pancreatic beta cell line MIN6 exhibits characteristics of glucose metabolism and glucose-stimulated insulin secretion similar to those of normal islets. Diabetologia *36*, 1139-1145.

Ivakine, E.A., Gulban, O.M., Mortin-Toth, S.M., Wankiewicz, E., Scott, C., Spurrell, D., Canty, A., and Danska, J.S. (2006). Molecular genetic analysis of the Idd4 locus implicates the IFN response in type 1 diabetes susceptibility in nonobese diabetic mice. Journal of immunology *176*, 2976-2990.

Iwahashi, H., Hanafusa, T., Eguchi, Y., Nakajima, H., Miyagawa, J., Itoh, N., Tomita, K., Namba, M., Kuwajima, M., Noguchi, T.*, et al.* (1996). Cytokine-induced apoptotic cell death in a mouse pancreatic beta-cell line: inhibition by Bcl-2. Diabetologia *39*, 530-536.

Jaattela, M., Mouritzen, H., Elling, F., and Bastholm, L. (1996). A20 zinc finger protein inhibits TNF and IL-1 signaling. J Immunol *156*, 1166-1173.

Jahromi, M.M., and Eisenbarth, G.S. (2007). Cellular and molecular pathogenesis of type 1A diabetes. Cellular and molecular life sciences : CMLS *64*, 865-872.

Jansen, A., Homo-Delarche, F., Hooijkaas, H., Leenen, P.J., Dardenne, M., and Drexhage, H.A. (1994). Immunohistochemical characterization of monocytes-macrophages and dendritic cells involved in the initiation of the insulitis and beta-cell destruction in NOD mice. Diabetes *43*, 667-675.

Jeyaprakash, A.A., Klein, U.R., Lindner, D., Ebert, J., Nigg, E.A., and Conti, E. (2007). Structure of a Survivin-Borealin-INCENP core complex reveals how chromosomal passengers travel together. Cell *131*, 271-285.

Jin, H.S., Lee, D.H., Kim, D.H., Chung, J.H., Lee, S.J., and Lee, T.H. (2009). cIAP1, cIAP2, and XIAP act cooperatively via nonredundant pathways to regulate genotoxic stress-induced nuclear factor-kappaB activation. Cancer Research *69*, 1782-1791.

Jin, H.S., and Lee, T.H. (2006). Cell cycle-dependent expression of cIAP2 at G2/M phase contributes to survival during mitotic cell cycle arrest. Biochem J *399*, 335-342.

Jun, H.S., Santamaria, P., Lim, H.W., Zhang, M.L., and Yoon, J.W. (1999a). Absolute requirement of macrophages for the development and activation of beta-cell cytotoxic CD8+ T-cells in T-cell receptor transgenic NOD mice. Diabetes *48*, 34-42.

Jun, H.S., Yoon, C.S., Zbytnuik, L., van Rooijen, N., and Yoon, J.W. (1999b). The role of macrophages in T cell-mediated autoimmune diabetes in nonobese diabetic mice. The Journal of experimental medicine *189*, 347-358.

Juvekar, A., Manna, S., Ramaswami, S., Chang, T.P., Vu, H.Y., Ghosh, C.C., Celiker, M.Y., and Vancurova, I. (2011). Bortezomib induces nuclear translocation of IkappaBalpha resulting in gene-specific suppression of NF-kappaB--dependent transcription and induction of apoptosis in CTCL. Mol Cancer Res *9*, 183-194.

Kagi, D., Ho, A., Odermatt, B., Zakarian, A., Ohashi, P.S., and Mak, T.W. (1999). TNF receptor 1-dependent beta cell toxicity as an effector pathway in autoimmune diabetes. Journal of immunology *162*, 4598-4605.

Kanagawa, O., Xu, G., Tevaarwerk, A., and Vaupel, B.A. (2000). Protection of nonobese diabetic mice from diabetes by gene(s) closely linked to IFN-gamma receptor loci. Journal of immunology *164*, 3919-3923.

Kanayama, A., Seth, R.B., Sun, L., Ea, C.K., Hong, M., Shaito, A., Chiu, Y.H., Deng, L., and Chen, Z.J. (2004). TAB2 and TAB3 activate the NF-kappaB pathway through binding to polyubiquitin chains. Molecular Cell *15*, 535-548.

Karin, M., Liu, Z., and Zandi, E. (1997). AP-1 function and regulation. Current opinion in cell biology *9*, 240-246.

Kaur, S., Wang, F., Venkatraman, M., and Arsura, M. (2005). X-linked inhibitor of apoptosis (XIAP) inhibits c-Jun N-terminal kinase 1 (JNK1) activation by transforming growth factor beta1 (TGF-beta1) through ubiquitin-mediated proteosomal degradation of the TGF-beta1-activated kinase 1 (TAK1). J Biol Chem *280*, 38599-38608.

Kay, T.W., Campbell, I.L., Oxbrow, L., and Harrison, L.C. (1991). Overexpression of class I major histocompatibility complex accompanies insulitis in the non-obese diabetic mouse and is prevented by anti-interferon-gamma antibody. Diabetologia *34*, 779-785.

Keats, J.J., Fonseca, R., Chesi, M., Schop, R., Baker, A., Chng, W.J., Van Wier, S., Tiedemann, R., Shi, C.X., Sebag, M.*, et al.* (2007). Promiscuous mutations activate the noncanonical NF-kappaB pathway in multiple myeloma. Cancer Cell *12*, 131-144.

Kelliher, M.A., Grimm, S., Ishida, Y., Kuo, F., Stanger, B.Z., and Leder, P. (1998). The death domain kinase RIP mediates the TNF-induced NF-kappaB signal. Immunity *8*, 297-303.

Keshet, Y., and Seger, R. (2010). The MAP kinase signaling cascades: a system of hundreds of components regulates a diverse array of physiological functions. Methods in molecular biology *661*, 3-38.

Kim, A., Miller, K., Jo, J., Kilimnik, G., Wojcik, P., and Hara, M. (2009). Islet architecture: A comparative study. Islets *1*, 129-136.

Kim, H.S., Kim, S., and Lee, M.S. (2005). IFN-gamma sensitizes MIN6N8 insulinoma cells to TNF-alpha-induced apoptosis by inhibiting NF-kappaB-mediated XIAP upregulation. Biochemical and Biophysical Research Communications *336*, 847-853.

Kim, M.S., and Polychronakos, C. (2005). Immunogenetics of type 1 diabetes. Hormone research *64*, 180-188.

Krieg, A., Correa, R.G., Garrison, J.B., Le Negrate, G., Welsh, K., Huang, Z., Knoefel, W.T., and Reed, J.C. (2009). XIAP mediates NOD signaling via interaction with RIP2. Proceedings of the National Academy of Sciences of the United States of America *106*, 14524-14529.

Kumar, S., and Gelinas, C. (1993). I kappa B alpha-mediated inhibition of v-Rel DNA binding requires direct interaction with the RXXRXRXXC Rel/kappa B DNA-binding motif. Proceedings of the National Academy of Sciences of the United States of America *90*, 8962-8966.

Kwon, G., Corbett, J.A., Rodi, C.P., Sullivan, P., and McDaniel, M.L. (1995). Interleukin-1 beta-induced nitric oxide synthase expression by rat pancreatic beta-cells: evidence for the involvement of nuclear factor kappa B in the signaling mechanism. Endocrinology *136*, 4790-4795.

Kyriakis, J.M., Banerjee, P., Nikolakaki, E., Dai, T., Rubie, E.A., Ahmad, M.F., Avruch, J., and Woodgett, J.R. (1994). The stress-activated protein kinase subfamily of c-Jun kinases. Nature *369*, 156-160.

Kyvik, K.O., Green, A., and Beck-Nielsen, H. (1995). Concordance rates of insulin dependent diabetes mellitus: a population based study of young Danish twins. BMJ *311*, 913-917.

Laing, S.P., Swerdlow, A.J., Carpenter, L.M., Slater, S.D., Burden, A.C., Botha, J.L., Morris, A.D., Waugh, N.R., Gatling, W., Gale, E.A.*, et al.* (2003a). Mortality from cerebrovascular disease in a cohort of 23 000 patients with insulin-treated diabetes. Stroke; a journal of cerebral circulation *34*, 418-421.

Laing, S.P., Swerdlow, A.J., Slater, S.D., Burden, A.C., Morris, A., Waugh, N.R., Gatling, W., Bingley, P.J., and Patterson, C.C. (2003b). Mortality from heart disease in a cohort of 23,000 patients with insulin-treated diabetes. Diabetologia *46*, 760-765.

Larsen, C.M., Faulenbach, M., Vaag, A., Volund, A., Ehses, J.A., Seifert, B., Mandrup-Poulsen, T., and Donath, M.Y. (2007a). Interleukin-1-receptor antagonist in type 2 diabetes mellitus. The New England journal of medicine *356*, 1517-1526.

Larsen, L., Tonnesen, M., Ronn, S.G., Storling, J., Jorgensen, S., Mascagni, P., Dinarello, C.A., Billestrup, N., and Mandrup-Poulsen, T. (2007b). Inhibition of histone deacetylases prevents cytokine-induced toxicity in beta cells. Diabetologia *50*, 779-789.

Lawton, P., Nelson, J., Tizard, R., and Browning, J.L. (1995). Characterization of the mouse lymphotoxin-beta gene. Journal of immunology *154*, 239-246.

Laybutt, D.R., Kaneto, H., Hasenkamp, W., Grey, S., Jonas, J.C., Sgroi, D.C., Groff, A., Ferran, C., Bonner-Weir, S., Sharma, A., and Weir, G.C. (2002). Increased expression of antioxidant and antiapoptotic genes in islets that may contribute to beta-cell survival during chronic hyperglycemia. Diabetes *51*, 413-423.

Leahy, J.L. (2005). Pathogenesis of type 2 diabetes mellitus. Arch Med Res *36*, 197-209.

Lee, J.Y., Ristow, M., Lin, X.Y., White, M.F., Magnuson, M.A., and Hennighausen, L. (2006). RIP-Cre revisited, evidence for impairments of pancreatic beta-cell function. Journal of Biological Chemistry *281*, 2649-2653.

Lee, K.U., Amano, K., and Yoon, J.W. (1988a). Evidence for initial involvement of macrophage in development of insulitis in NOD mice. Diabetes *37*, 989-991.

Lee, K.U., Kim, M.K., Amano, K., Pak, C.Y., Jaworski, M.A., Mehta, J.G., and Yoon, J.W. (1988b). Preferential infiltration of macrophages during early stages of insulitis in diabetes-prone BB rats. Diabetes *37*, 1053-1058.

Lee, S.Y., Reichlin, A., Santana, A., Sokol, K.A., Nussenzweig, M.C., and Choi, Y. (1997). TRAF2 is essential for JNK but not NF-kappa B activation and regulates lymphocyte proliferation and survival. Immunity *7*, 703-713.

Lee, T.H., Huang, Q., Oikemus, S., Shank, J., Ventura, J.J., Cusson, N., Vaillancourt, R.R., Su, B., Davis, R.J., and Kelliher, M.A. (2003). The death domain kinase RIP1 is essential for tumor necrosis factor alpha signaling to p38 mitogen-activated protein kinase. Molecular and Cellular Biology *23*, 8377-8385.

Legler, D.F., Micheau, O., Doucey, M.A., Tschopp, J., and Bron, C. (2003). Recruitment of TNF receptor 1 to lipid rafts is essential for TNFalpha-mediated NF-kappaB activation. Immunity *18*, 655-664.

Lei, K., and Davis, R.J. (2003). JNK phosphorylation of Bim-related members of the Bcl2 family induces Bax-dependent apoptosis. Proceedings of the National Academy of Sciences of the United States of America *100*, 2432-2437.

Lei, K., Nimnual, A., Zong, W.X., Kennedy, N.J., Flavell, R.A., Thompson, C.B., Bar-Sagi, D., and Davis, R.J. (2002). The Bax subfamily of Bcl2-related proteins is essential for apoptotic signal transduction by c-Jun NH(2)-terminal kinase. Molecular and Cellular Biology *22*, 4929-4942.

Lens, S.M., Vader, G., and Medema, R.H. (2006). The case for Survivin as mitotic regulator. Current opinion in cell biology *18*, 616-622.

Lentz, S.R., Tsiang, M., and Sadler, J.E. (1991). Regulation of thrombomodulin by tumor necrosis factor-alpha: comparison of transcriptional and posttranscriptional mechanisms. Blood *77*, 542-550.

Leung, J.Y., McKenzie, F.E., Uglialoro, A.M., Flores-Villanueva, P.O., Sorkin, B.C., Yunis, E.J., Hartl, D.L., and Goldfeld, A.E. (2000). Identification of phylogenetic footprints in primate tumor necrosis factor-alpha promoters. Proceedings of the National Academy of Sciences of the United States of America *97*, 6614-6618.

Li, F., and Mahato, R.I. (2008). iNOS gene silencing prevents inflammatory cytokine-induced beta-cell apoptosis. Molecular pharmaceutics *5*, 407-417.

Li, H., Kobayashi, M., Blonska, M., You, Y., and Lin, X. (2006). Ubiquitination of RIP is required for tumor necrosis factor alpha-induced NF-kappaB activation. J Biol Chem *281*, 13636-13643.

Li, P., Nijhawan, D., Budihardjo, I., Srinivasula, S.M., Ahmad, M., Alnemri, E.S., and Wang, X. (1997). Cytochrome c and dATP-dependent formation of Apaf-1/caspase-9 complex initiates an apoptotic protease cascade. Cell *91*, 479-489.

Li, X., Yang, Y., and Ashwell, J.D. (2002). TNF-RII and c-IAP1 mediate ubiquitination and degradation of TRAF2. Nature *416*, 345-347.

Li, Z.W., Chu, W., Hu, Y., Delhase, M., Deerinck, T., Ellisman, M., Johnson, R., and Karin, M. (1999). The IKKbeta subunit of IkappaB kinase (IKK) is essential for nuclear factor kappaB activation and prevention of apoptosis. The Journal of experimental medicine *189*, 1839-1845.

Liggins, C., Orlicky, D.J., Bloomquist, L.A., and Gianani, R. (2003). Developmentally regulated expression of Survivin in human pancreatic islets. Pediatric and developmental pathology : the official journal of the Society for Pediatric Pathology and the Paediatric Pathology Society *6*, 392-397.

Lin, A., and Dibling, B. (2002). The true face of JNK activation in apoptosis. Aging cell *1*, 112-116.

Liston, P., Roy, N., Tamai, K., Lefebvre, C., Baird, S., Cherton-Horvat, G., Farahani, R., McLean, M., Ikeda, J.E., MacKenzie, A., and Korneluk, R.G. (1996). Suppression of apoptosis in mammalian cells by NAIP and a related family of IAP genes. Nature *379*, 349-353.

Liu, H., Lo, C.R., and Czaja, M.J. (2002). NF-kappaB inhibition sensitizes hepatocytes to TNF-induced apoptosis through a sustained activation of JNK and c-Jun. Hepatology *35*, 772-778.

Liu, J., and Lin, A. (2005). Role of JNK activation in apoptosis: a double-edged sword. Cell research *15*, 36-42.

Liu, Y., Rabinovitch, A., Suarez-Pinzon, W., Muhkerjee, B., Brownlee, M., Edelstein, D., and Federoff, H.J. (1996a). Expression of the bcl-2 gene from a defective HSV-1 amplicon vector protects pancreatic beta-cells from apoptosis. Human gene therapy *7*, 1719-1726.

Liu, Z., Sun, C., Olejniczak, E.T., Meadows, R.P., Betz, S.F., Oost, T., Herrmann, J., Wu, J.C., and Fesik, S.W. (2000). Structural basis for binding of Smac/DIABLO to the XIAP BIR3 domain. Nature *408*, 1004-1008.

Liu, Z.G., and Han, J. (2001). Cellular responses to tumor necrosis factor. Current issues in molecular biology *3*, 79-90.

Liu, Z.G., Hsu, H., Goeddel, D.V., and Karin, M. (1996b). Dissection of TNF receptor 1 effector functions: JNK activation is not linked to apoptosis while NF-kappaB activation prevents cell death. Cell *87*, 565-576.

Liuwantara, D., Elliot, M., Smith, M.W., Yam, A.O., Walters, S.N., Marino, E., McShea, A., and Grey, S.T. (2006). Nuclear factor-kappaB regulates beta-cell death: a critical role for A20 in beta-cell protection. Diabetes *55*, 2491-2501.

Livak, K.J., and Schmittgen, T.D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods *25*, 402-408.

Lomvardas, S., and Thanos, D. (2002). Modifying gene expression programs by altering core promoter chromatin architecture. Cell *110*, 261-271.

Lopes, R.B., Gangeswaran, R., McNeish, I.A., Wang, Y., and Lemoine, N.R. (2007). Expression of the IAP protein family is dysregulated in pancreatic cancer cells and is important for resistance to chemotherapy. International journal of cancer. Journal international du cancer *120*, 2344-2352.

Lopez, J., John, S.W., Tenev, T., Rautureau, G.J., Hinds, M.G., Francalanci, F., Wilson, R., Broemer, M., Santoro, M.M., Day, C.L., and Meier, P. (2011). CARD-mediated autoinhibition of cIAP1's E3 ligase activity suppresses cell proliferation and migration. Molecular Cell *42*, 569-583.

Lopez, J., and Meier, P. (2010). To fight or die - inhibitor of apoptosis proteins at the crossroad of innate immunity and death. Current opinion in cell biology *22*, 872-881.

Lord, S.J., Rajotte, R.V., Korbutt, G.S., and Bleackley, R.C. (2003). Granzyme B: a natural born killer. Immunological reviews *193*, 31-38.

Lotspeich, W.D. (1949). The role of insulin in the metabolism of amino acids. J Biol Chem *179*, 175-180.

Lowe, C.E., Cooper, J.D., Brusko, T., Walker, N.M., Smyth, D.J., Bailey, R., Bourget, K., Plagnol, V., Field, S., Atkinson, M.*, et al.* (2007). Large-scale genetic fine mapping and genotype-phenotype associations implicate polymorphism in the IL2RA region in type 1 diabetes. Nature genetics *39*, 1074-1082.

Luo, D., Luo, Y., He, Y., Zhang, H., Zhang, R., Li, X., Dobrucki, W.L., Sinusas, A.J., Sessa, W.C., and Min, W. (2006). Differential functions of tumor necrosis factor receptor 1 and 2 signaling in ischemia-mediated arteriogenesis and angiogenesis. The American journal of pathology *169*, 1886-1898.

Luo, Y., Xu, Z., Wan, T., He, Y., Jones, D., Zhang, H., and Min, W. (2010). Endothelial-specific transgenesis of TNFR2 promotes adaptive arteriogenesis and angiogenesis. Arteriosclerosis, thrombosis, and vascular biology *30*, 1307-1314.

Mace, P.D., Smits, C., Vaux, D.L., Silke, J., and Day, C.L. (2010). Asymmetric recruitment of cIAPs by TRAF2. Journal of molecular biology *400*, 8-15.

MacEwan, D.J. (2002). TNF ligands and receptors--a matter of life and death. British journal of pharmacology *135*, 855-875.

Madge, L.A., Kluger, M.S., Orange, J.S., and May, M.J. (2008). Lymphotoxin-alpha 1 beta 2 and LIGHT induce classical and noncanonical NF-kappa B-dependent proinflammatory gene expression in vascular endothelial cells. Journal of immunology *180*, 3467-3477.

Mahoney, D.J., Cheung, H.H., Mrad, R.L., Plenchette, S., Simard, C., Enwere, E., Arora, V., Mak, T.W., Lacasse, E.C., Waring, J., and Korneluk, R.G. (2008). Both cIAP1 and cIAP2 regulate TNFalpha-mediated NF-kappaB activation. Proc Natl Acad Sci U S A *105*, 11778-11783.

Maier, L.M., and Wicker, L.S. (2005). Genetic susceptibility to type 1 diabetes. Current opinion in immunology *17*, 601-608.

Makeeva, N., Myers, J.W., and Welsh, N. (2006). Role of MKK3 and p38 MAPK in cytokine-induced death of insulin-producing cells. The Biochemical journal *393*, 129-139.

Makeeva, N., Roomans, G.M., and Welsh, N. (2007). Role of TAB1 in nitric oxide-induced p38 activation in insulin-producing cells. International journal of biological sciences *3*, 71-76.

Mandrup-Poulsen, T., Bendtzen, K., Dinarello, C.A., and Nerup, J. (1987). Human tumor necrosis factor potentiates human interleukin 1-mediated rat pancreatic beta-cell cytotoxicity. Journal of immunology *139*, 4077-4082.

Mandrup-Poulsen, T., Nerup, J., Reimers, J.I., Pociot, F., Andersen, H.U., Karlsen, A., Bjerre, U., and Bergholdt, R. (1996). Cytokines and the endocrine system. II. Roles in substrate metabolism, modulation of thyroidal and pancreatic endocrine cell functions and autoimmune endocrine diseases. European journal of endocrinology / European Federation of Endocrine Societies *134*, 21-30.

Manning, A.M., and Davis, R.J. (2003). Targeting JNK for therapeutic benefit: from junk to gold? Nature reviews. Drug discovery *2*, 554-565.

Marino, E., Tan, B., Binge, L., Mackay, C.R., and Grey, S.T. (2012). B-Cell Cross-Presentation of Autologous Antigen Precipitates Diabetes. Diabetes.

Martin, A.P., Rankin, S., Pitchford, S., Charo, I.F., Furtado, G.C., and Lira, S.A. (2008). Increased expression of CCL2 in insulin-producing cells of transgenic mice promotes mobilization of myeloid cells from the bone marrow, marked insulitis, and diabetes. Diabetes *57*, 3025-3033.

Martin, B.C., Warram, J.H., Krolewski, A.S., Bergman, R.N., Soeldner, J.S., and Kahn, C.R. (1992). Role of glucose and insulin resistance in development of type 2 diabetes mellitus: results of a 25-year follow-up study. Lancet *340*, 925-929.

Martin, S., van den Engel, N.K., Vinke, A., Heidenthal, E., Schulte, B., and Kolb, H. (2001). Dominant role of intercellular adhesion molecule-1 in the pathogenesis of autoimmune diabetes in non-obese diabetic mice. Journal of autoimmunity *17*, 109-117.

Martinon, F., and Tschopp, J. (2007). Inflammatory caspases and inflammasomes: master switches of inflammation. Cell death and differentiation *14*, 10-22.

Mastrandrea, L., Yu, J., Behrens, T., Buchlis, J., Albini, C., Fourtner, S., and Quattrin, T. (2009). Etanercept treatment in children with new-onset type 1 diabetes: pilot randomized, placebo-controlled, double-blind study. Diabetes care *32*, 1244-1249.

Mathis, D., Vence, L., and Benoist, C. (2001). beta-Cell death during progression to diabetes. Nature *414*, 792-798.

Matsumoto, S., Takita, M., Chaussabel, D., Noguchi, H., Shimoda, M., Sugimoto, K., Itoh, T., Chujo, D., Sorelle, J., Onaca, N.*, et al.* (2011). Improving Efficacy of Clinical Islet Transplantation with Iodixanol Based Islet Purification, Thymoglobulin Induction and Blockage of IL-1-beta and TNF-alpha. Cell transplantation.

Matveyenko, A.V., and Butler, P.C. (2008). Relationship between beta-cell mass and diabetes onset. Diabetes, obesity & metabolism *10 Suppl 4*, 23-31.

McCall, M., Pawlick, R., Kin, T., and Shapiro, A.M. (2012). Anakinra potentiates the protective effects of etanercept in transplantation of marginal mass human islets in immunodeficient mice. American journal of transplantation : official journal of the American Society of Transplantation and the American Society of Transplant Surgeons *12*, 322-329.

McKenzie, M.D., Dudek, N.L., Mariana, L., Chong, M.M., Trapani, J.A., Kay, T.W., and Thomas, H.E. (2006). Perforin and Fas induced by IFNgamma and TNFalpha mediate beta cell death by OT-I CTL. International immunology *18*, 837-846.

McManus, D.C., Lefebvre, C.A., Cherton-Horvat, G., St-Jean, M., Kandimalla, E.R., Agrawal, S., Morris, S.J., Durkin, J.P., and Lacasse, E.C. (2004). Loss of XIAP protein expression by RNAi and antisense approaches sensitizes cancer cells to functionally diverse chemotherapeutics. Oncogene *23*, 8105-8117.

Meglasson, M.D., and Matschinsky, F.M. (1986). Pancreatic islet glucose metabolism and regulation of insulin secretion. Diabetes/metabolism reviews *2*, 163-214.

Mehrotra, S., Languino, L.R., Raskett, C.M., Mercurio, A.M., Dohi, T., and Altieri, D.C. (2010). IAP regulation of metastasis. Cancer Cell *17*, 53-64.

Mehta, V.K., Hao, W., Brooks-Worrell, B.M., and Palmer, J.P. (1994). Low-dose interleukin 1 and tumor necrosis factor individually stimulate insulin release but in combination cause suppression. Eur J Endocrinol *130*, 208-214.

Meier, J.J., Bhushan, A., and Butler, P.C. (2006). The potential for stem cell therapy in diabetes. Pediatric research *59*, 65R-73R.

Meier, P., and Vousden, K.H. (2007). Lucifer's labyrinth--ten years of path finding in cell death. Molecular Cell *28*, 746-754.

Melendez-Ramirez, L.Y., Richards, R.J., and Cefalu, W.T. (2010). Complications of type 1 diabetes. Endocrinology and metabolism clinics of North America *39*, 625-640.

Messeguer, X., Escudero, R., Farre, D., Nunez, O., Martinez, J., and Alba, M.M. (2002). PROMO: detection of known transcription regulatory elements using species-tailored searches. Bioinformatics *18*, 333-334.

Micheau, O., Lens, S., Gaide, O., Alevizopoulos, K., and Tschopp, J. (2001). NF-kappaB signals induce the expression of c-FLIP. Molecular and Cellular Biology *21*, 5299-5305.

Micheau, O., and Tschopp, J. (2003). Induction of TNF receptor I-mediated apoptosis via two sequential signaling complexes. Cell *114*, 181-190.

Mitsiades, N., Mitsiades, C.S., Poulaki, V., Chauhan, D., Richardson, P.G., Hideshima, T., Munshi, N., Treon, S.P., and Anderson, K.C. (2002). Biologic sequelae of nuclear factor-kappaB blockade in multiple myeloma: therapeutic applications. Blood *99*, 4079-4086.

Miyazaki, J., Araki, K., Yamato, E., Ikegami, H., Asano, T., Shibasaki, Y., Oka, Y., and Yamamura, K. (1990). Establishment of a pancreatic beta cell line that retains glucose-inducible insulin secretion: special reference to expression of glucose transporter isoforms. Endocrinology *127*, 126-132.

Mori, Y., Kato, T., Kodaka, T., Kanagawa, E.M., Hori, S., and Kanagawa, O. (2008). Protection of IFN-gamma signaling-deficient NOD mice from diabetes by cyclophosphamide. International immunology *20*, 1231-1237.

Morimoto, J., Yoneyama, H., Shimada, A., Shigihara, T., Yamada, S., Oikawa, Y., Matsushima, K., Saruta, T., and Narumi, S. (2004). CXC chemokine ligand 10 neutralization suppresses the occurrence of diabetes in nonobese diabetic mice through enhanced beta cell proliferation without affecting insulitis. Journal of immunology *173*, 7017-7024.

Moriwaki, M., Itoh, N., Miyagawa, J., Yamamoto, K., Imagawa, A., Yamagata, K., Iwahashi, H., Nakajima, H., Namba, M., Nagata, S*., et al.* (1999). Fas and Fas ligand expression in inflamed islets in pancreas sections of patients with recent-onset Type I diabetes mellitus. Diabetologia *42*, 1332-1340.

Morrison, H. (1937). Contributions to the microscopic anatomy of the pancreas by Paul Langerhans (Berlin, 1869). Reprint of the German original with an English translation and an introductory essay. Bull Inst Hist Med *5*, 259-297.

Moulin, M., Anderton, H., Voss, A.K., Thomas, T., Wong, W.W., Bankovacki, A., Feltham, R., Chau, D., Cook, W.D., Silke, J., and Vaux, D.L. (2012). IAPs limit activation of RIP kinases by TNF receptor 1 during development. The EMBO journal.

Mueller, C., Held, W., Imboden, M.A., and Carnaud, C. (1995). Accelerated beta-cell destruction in adoptively transferred autoimmune diabetes correlates with an increased expression of the genes coding for TNF-alpha and granzyme A in the intra-islet infiltrates. Diabetes *44*, 112-117.

Mukhopadhyay, A., Suttles, J., Stout, R.D., and Aggarwal, B.B. (2001). Genetic deletion of the tumor necrosis factor receptor p60 or p80 abrogates ligand-mediated activation of nuclear factor-kappa B and of mitogen-activated protein kinases in macrophages. J Biol Chem *276*, 31906-31912.

Nagata, M., Nakayama, H., Tanaka, T., Yoshida, R., Yoshitake, Y., Fukuma, D., Kawahara, K., Nakagawa, Y., Ota, K., Hiraki, A., and Shinohara, M. (2011). Overexpression of cIAP2 contributes to 5-FU resistance and a poor prognosis in oral squamous cell carcinoma. British Journal of Cancer *105*, 1322-1330.

Nagata, S. (1997). Apoptosis by death factor. Cell *88*, 355-365.

Nathan, D.M., Cleary, P.A., Backlund, J.Y., Genuth, S.M., Lachin, J.M., Orchard, T.J., Raskin, P., and Zinman, B. (2005). Intensive diabetes treatment and cardiovascular disease in patients with type 1 diabetes. The New England journal of medicine *353*, 2643-2653.

Nathan, D.M., Lachin, J., Cleary, P., Orchard, T., Brillon, D.J., Backlund, J.Y., O'Leary, D.H., and Genuth, S. (2003). Intensive diabetes therapy and carotid intima-media thickness in type 1 diabetes mellitus. The New England journal of medicine *348*, 2294-2303.

Nedospasov, S.A., Hirt, B., Shakhov, A.N., Dobrynin, V.N., Kawashima, E., Accolla, R.S., and Jongeneel, C.V. (1986). The genes for tumor necrosis factor (TNF-alpha) and lymphotoxin (TNF-beta) are tandemly arranged on chromosome 17 of the mouse. Nucleic acids research *14*, 7713-7725.

NEJM (1993). The effect of intensive treatment of diabetes on the development and progression of long-term complications in insulin-dependent diabetes mellitus. The Diabetes Control and Complications Trial Research Group. The New England journal of medicine *329*, 977-986.

Nerup, J., Mandrup-Poulsen, T., Helqvist, S., Andersen, H.U., Pociot, F., Reimers, J.I., Cuartero, B.G., Karlsen, A.E., Bjerre, U., and Lorenzen, T. (1994). On the pathogenesis of IDDM. Diabetologia *37 Suppl 2*, S82-89.

Nerup, J., Mandrup-Poulsen, T., Molvig, J., Helqvist, S., Wogensen, L., and Egeberg, J. (1988). Mechanisms of pancreatic beta-cell destruction in type I diabetes. Diabetes care *11 Suppl 1*, 16-23.

Nerup, J., Platz, P., Andersen, O.O., Christy, M., Lyngsoe, J., Poulsen, J.E., Ryder, L.P., Nielsen, L.S., Thomsen, M., and Svejgaard, A. (1974). HL-A antigens and diabetes mellitus. Lancet *2*, 864-866.

Nicoletti, F., Di Marco, R., Barcellini, W., Magro, G., Schorlemmer, H.U., Kurrle, R., Lunetta, M., Grasso, S., Zaccone, P., and Meroni, P. (1994). Protection from experimental autoimmune diabetes in the non-obese diabetic mouse with soluble interleukin-1 receptor. European journal of immunology *24*, 1843-1847.

Nishitoh, H., Saitoh, M., Mochida, Y., Takeda, K., Nakano, H., Rothe, M., Miyazono, K., and Ichijo, H. (1998). ASK1 is essential for JNK/SAPK activation by TRAF2. Molecular Cell *2*, 389-395.

Nistico, L., Buzzetti, R., Pritchard, L.E., Van der Auwera, B., Giovannini, C., Bosi, E., Larrad, M.T., Rios, M.S., Chow, C.C., Cockram, C.S.*, et al.* (1996). The CTLA-4 gene region of chromosome 2q33 is linked to, and associated with, type 1 diabetes. Belgian Diabetes Registry. Human molecular genetics *5*, 1075-1080.

Noguchi, H., Matsumoto, S., Onaca, N., Naziruddin, B., Jackson, A., Ikemoto, T., Shimoda, M., Fujita, Y., Chujo, D., Iwanaga, Y.*, et al.* (2009). Ductal injection of JNK inhibitors before pancreas preservation prevents islet apoptosis and improves islet graft function. Human gene therapy *20*, 73-85.

Noguchi, H., Nakai, Y., Matsumoto, S., Kawaguchi, M., Ueda, M., Okitsu, T., Iwanaga, Y., Yonekawa, Y., Nagata, H., Minami, K.*, et al.* (2005). Cell permeable peptide of JNK inhibitor prevents islet apoptosis immediately after isolation and improves islet graft function. American journal of transplantation : official journal of the American Society of Transplantation and the American Society of Transplant Surgeons *5*, 1848-1855.

Noorchashm, H., Noorchashm, N., Kern, J., Rostami, S.Y., Barker, C.F., and Naji, A. (1997). B-cells are required for the initiation of insulitis and sialitis in nonobese diabetic mice. Diabetes *46*, 941-946.

O'Brien, T.D., Butler, P.C., Westermark, P., and Johnson, K.H. (1993). Islet amyloid polypeptide: a review of its biology and potential roles in the pathogenesis of diabetes mellitus. Vet Pathol *30*, 317-332.

O'Donnell, M.A., Legarda-Addison, D., Skountzos, P., Yeh, W.C., and Ting, A.T. (2007). Ubiquitination of RIP1 regulates an NF-kappaB-independent cell-death switch in TNF signaling. Current biology : CB *17*, 418-424.

O'Reilly, L.A., Hutchings, P.R., Crocker, P.R., Simpson, E., Lund, T., Kioussis, D., Takei, F., Baird, J., and Cooke, A. (1991). Characterization of pancreatic islet cell infiltrates in NOD mice: effect of cell transfer and transgene expression. European journal of immunology *21*, 1171-1180.

Oetjen, K.A., and Duckett, C.S. (2011). Identifying the trigger of c-IAPs: structural and functional characterization of CARD-mediated modulation of ubiquitin ligase activity. Molecular Cell *42*, 553-554.

Okada, H., Bakal, C., Shahinian, A., Elia, A., Wakeham, A., Suh, W.K., Duncan, G.S., Ciofani, M., Rottapel, R., Zuniga-Pflucker, J.C., and Mak, T.W. (2004). Survivin loss in thymocytes triggers p53-mediated growth arrest and p53-independent cell death. The Journal of experimental medicine *199*, 399-410.

Orban, P.C., Chui, D., and Marth, J.D. (1992). Tissue- and site-specific DNA recombination in transgenic mice. Proceedings of the National Academy of Sciences of the United States of America *89*, 6861-6865.

Ortis, F., Pirot, P., Naamane, N., Kreins, A.Y., Rasschaert, J., Moore, F., Theatre, E., Verhaeghe, C., Magnusson, N.E., Chariot, A.*, et al.* (2008). Induction of nuclear factor-kappaB and its downstream genes by TNF-alpha and IL-1beta has a pro-apoptotic role in pancreatic beta cells. Diabetologia *51*, 1213-1225.

Oschilewski, U., Kiesel, U., and Kolb, H. (1985). Administration of silica prevents diabetes in BB-rats. Diabetes *34*, 197-199.

Ozoren, N., and El-Deiry, W.S. (2003). Cell surface Death Receptor signaling in normal and cancer cells. Seminars in cancer biology *13*, 135-147.

Pakala, S.V., Chivetta, M., Kelly, C.B., and Katz, J.D. (1999). In autoimmune diabetes the transition from benign to pernicious insulitis requires an islet cell response to tumor necrosis factor alpha. The Journal of experimental medicine *189*, 1053-1062.

Pankewycz, O.G., Guan, J.X., and Benedict, J.F. (1995). Cytokines as mediators of autoimmune diabetes and diabetic complications. Endocrine reviews *16*, 164-176.

Park, S.M., Yoon, J.B., and Lee, T.H. (2004). Receptor interacting protein is ubiquitinated by cellular inhibitor of apoptosis proteins (c-IAP1 and c-IAP2) in vitro. FEBS letters *566*, 151-156.

Paterson, A.D., Rutledge, B.N., Cleary, P.A., Lachin, J.M., and Crow, R.S. (2007). The effect of intensive diabetes treatment on resting heart rate in type 1 diabetes: the Diabetes Control and Complications Trial/Epidemiology of Diabetes Interventions and Complications study. Diabetes care *30*, 2107-2112.

Peng, H., Huang, Y., Duan, Z., Erdmann, N., Xu, D., Herek, S., and Zheng, J. (2005). Cellular IAP1 regulates TRAIL-induced apoptosis in human fetal cortical neural progenitor cells. Journal of neuroscience research *82*, 295-305.

Pennica, D., Nedwin, G.E., Hayflick, J.S., Seeburg, P.H., Derynck, R., Palladino, M.A., Kohr, W.J., Aggarwal, B.B., and Goeddel, D.V. (1984). Human tumour necrosis factor: precursor structure, expression and homology to lymphotoxin. Nature *312*, 724-729.

Perez, F., Oyarzun, A., Carrasco, E., Angel, B., Albala, C., and Santos, J.L. (2004). [Plasma levels of interleukin-1beta, interleukin-2 and interleukin-4 in recently diagnosed type 1 diabetic children and their association with beta-pancreatic autoantibodies]. Revista medica de Chile *132*, 413-420.

Perkins, N.D. (2007). Integrating cell-signalling pathways with NF-kappaB and IKK function. Nature reviews. Molecular cell biology *8*, 49-62.

Petersen, S.L., Wang, L., Yalcin-Chin, A., Li, L., Peyton, M., Minna, J., Harran, P., and Wang, X. (2007). Autocrine TNF alpha signaling renders human cancer cells susceptible to smac-mimetic-induced apoptosis. Cancer Cell *12*, 445-456.

Phillips, J.M., Parish, N.M., Raine, T., Bland, C., Sawyer, Y., De La Pena, H., and Cooke, A. (2009). Type 1 diabetes development requires both CD4+ and CD8+ T cells and

can be reversed by non-depleting antibodies targeting both T cell populations. The review of diabetic studies : RDS *6*, 97-103.

Pickersgill, L.M., and Mandrup-Poulsen, T.R. (2009). The anti-interleukin-1 in type 1 diabetes action trial--background and rationale. Diabetes/metabolism research and reviews *25*, 321-324.

Plesner, A., Liston, P., Tan, R., Korneluk, R.G., and Verchere, C.B. (2005). The X-linked inhibitor of apoptosis protein enhances survival of murine islet allografts. Diabetes *54*, 2533-2540.

Plesner, A., Soukhatcheva, G., Korneluk, R.G., and Verchere, C.B. (2010). XIAP inhibition of beta-cell apoptosis reduces the number of islets required to restore euglycemia in a syngeneic islet transplantation model. Islets *2*, 18-23.

Poitout, V., and Robertson, R.P. (2002). Minireview: Secondary beta-cell failure in type 2 diabetes--a convergence of glucotoxicity and lipotoxicity. Endocrinology *143*, 339-342.

Pomplun, D., Florian, S., Schulz, T., Pfeiffer, A.F., and Ristow, M. (2007). Alterations of pancreatic beta-cell mass and islet number due to Ins2-controlled expression of Cre recombinase: RIP-Cre revisited; part 2. Hormone and metabolic research = Hormon- und Stoffwechselforschung = Hormones et metabolisme *39*, 336-340.

Porteu, F., Brockhaus, M., Wallach, D., Engelmann, H., and Nathan, C.F. (1991). Human neutrophil elastase releases a ligand-binding fragment from the 75-kDa tumor necrosis factor (TNF) receptor. Comparison with the proteolytic activity responsible for shedding of TNF receptors from stimulated neutrophils. J Biol Chem *266*, 18846-18853.

Postic, C., Shiota, M., Niswender, K.D., Jetton, T.L., Chen, Y., Moates, J.M., Shelton, K.D., Lindner, J., Cherrington, A.D., and Magnuson, M.A. (1999). Dual roles for glucokinase in glucose homeostasis as determined by liver and pancreatic beta cell-specific gene knock-outs using Cre recombinase. J Biol Chem *274*, 305-315.

Pratley, R.E., and Gilbert, M. (2008). Targeting Incretins in Type 2 Diabetes: Role of GLP-1 Receptor Agonists and DPP-4 Inhibitors. The review of diabetic studies : RDS *5*, 73-94.

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

Prieto, J., Kaaya, E.E., Juntti-Berggren, L., Berggren, P.O., Sandler, S., Biberfeld, P., and Patarroyo, M. (1992). Induction of intercellular adhesion molecule-1 (CD54) on isolated mouse pancreatic beta cells by inflammatory cytokines. Clin Immunol Immunopathol *65*, 247-253.

Pukel, C., Baquerizo, H., and Rabinovitch, A. (1988). Destruction of rat islet cell monolayers by cytokines. Synergistic interactions of interferon-gamma, tumor necrosis factor, lymphotoxin, and interleukin 1. Diabetes *37*, 133-136.

Qin, Y., Auh, S., Blokh, L., Long, C., Gagnon, I., and Hamann, K.J. (2007). TNF-alpha induces transient resistance to Fas-induced apoptosis in eosinophilic acute myeloid leukemia cells. Cell Mol Immunol *4*, 43-52.

Qu, H.Q., Montpetit, A., Ge, B., Hudson, T.J., and Polychronakos, C. (2007). Toward further mapping of the association between the IL2RA locus and type 1 diabetes. Diabetes *56*, 1174-1176.

Raingeaud, J., Gupta, S., Rogers, J.S., Dickens, M., Han, J., Ulevitch, R.J., and Davis, R.J. (1995). Pro-inflammatory cytokines and environmental stress cause p38 mitogen-activated protein kinase activation by dual phosphorylation on tyrosine and threonine. J Biol Chem *270*, 7420-7426.

Raingeaud, J., Whitmarsh, A.J., Barrett, T., Derijard, B., and Davis, R.J. (1996). MKK3- and MKK6-regulated gene expression is mediated by the p38 mitogen-activated protein kinase signal transduction pathway. Molecular and Cellular Biology *16*, 1247-1255.

Rajan, S., Ye, J., Bai, S., Huang, F., and Guo, Y.L. (2008). NF-kappaB, but not p38 MAP kinase, is required for TNF-alpha-induced expression of cell adhesion molecules in endothelial cells. J Cell Biochem *105*, 477-486.

Redondo, M.J., Jeffrey, J., Fain, P.R., Eisenbarth, G.S., and Orban, T. (2008). Concordance for islet autoimmunity among monozygotic twins. The New England journal of medicine *359*, 2849-2850.

Redondo, M.J., Yu, L., Hawa, M., Mackenzie, T., Pyke, D.A., Eisenbarth, G.S., and Leslie, R.D. (2001). Heterogeneity of type I diabetes: analysis of monozygotic twins in Great Britain and the United States. Diabetologia *44*, 354-362.

Reinhard, C., Shamoon, B., Shyamala, V., and Williams, L.T. (1997). Tumor necrosis factor alpha-induced activation of c-jun N-terminal kinase is mediated by TRAF2. The EMBO journal *16*, 1080-1092.

Ren, J., Shi, M., Liu, R., Yang, Q.H., Johnson, T., Skarnes, W.C., and Du, C. (2005). The Birc6 (Bruce) gene regulates p53 and the mitochondrial pathway of apoptosis and is essential for mouse embryonic development. Proceedings of the National Academy of Sciences of the United States of America *102*, 565-570.

Rhode, A., Pauza, M.E., Barral, A.M., Rodrigo, E., Oldstone, M.B., von Herrath, M.G., and Christen, U. (2005). Islet-specific expression of CXCL10 causes spontaneous islet infiltration and accelerates diabetes development. J Immunol *175*, 3516-3524.

Rich, S.S. (1990). Mapping genes in diabetes. Genetic epidemiological perspective. Diabetes *39*, 1315-1319.

Richter, B.W., Mir, S.S., Eiben, L.J., Lewis, J., Reffey, S.B., Frattini, A., Tian, L., Frank, S., Youle, R.J., Nelson, D.L.*, et al.* (2001). Molecular cloning of ILP-2, a novel member of the inhibitor of apoptosis protein family. Molecular and Cellular Biology *21*, 4292-4301.

Riedl, S.J., Renatus, M., Schwarzenbacher, R., Zhou, Q., Sun, C., Fesik, S.W., Liddington, R.C., and Salvesen, G.S. (2001). Structural basis for the inhibition of caspase-3 by XIAP. Cell *104*, 791-800.

Rincon, M., and Davis, R.J. (2009). Regulation of the immune response by stress-activated protein kinases. Immunological reviews *228*, 212-224.

Robertson, R.P. (2004a). Chronic oxidative stress as a central mechanism for glucose toxicity in pancreatic islet beta cells in diabetes. J Biol Chem *279*, 42351-42354.

Robertson, R.P. (2004b). Islet transplantation as a treatment for diabetes - a work in progress. The New England journal of medicine *350*, 694-705.

Roep, B.O., Kleijwegt, F.S., van Halteren, A.G., Bonato, V., Boggi, U., Vendrame, F., Marchetti, P., and Dotta, F. Islet inflammation and CXCL10 in recent-onset type 1 diabetes. Clin Exp Immunol *159*, 338-343.

Roep, B.O., Stobbe, I., Duinkerken, G., van Rood, J.J., Lernmark, A., Keymeulen, B., Pipeleers, D., Claas, F.H., and de Vries, R.R. (1999). Auto- and alloimmune reactivity to human islet allografts transplanted into type 1 diabetic patients. Diabetes *48*, 484-490.

Ronningen, K.S., Keiding, N., and Green, A. (2001). Correlations between the incidence of childhood-onset type I diabetes in Europe and HLA genotypes. Diabetologia *44 Suppl 3*, B51-59.

Rooman, I., and Bouwens, L. (2004). Combined gastrin and epidermal growth factor treatment induces islet regeneration and restores normoglycaemia in C57Bl6/J mice treated with alloxan. Diabetologia *47*, 259-265.

Rooman, I., Lardon, J., and Bouwens, L. (2002). Gastrin stimulates beta-cell neogenesis and increases islet mass from transdifferentiated but not from normal exocrine pancreas tissue. Diabetes *51*, 686-690.

Rosenberg, L., Lipsett, M., Yoon, J.W., Prentki, M., Wang, R., Jun, H.S., Pittenger, G.L., Taylor-Fishwick, D., and Vinik, A.I. (2004). A pentadecapeptide fragment of islet

neogenesis-associated protein increases beta-cell mass and reverses diabetes in C57BL/6J mice. Annals of surgery *240*, 875-884.

Rothe, M., Pan, M.G., Henzel, W.J., Ayres, T.M., and Goeddel, D.V. (1995). The TNFR2-TRAF signaling complex contains two novel proteins related to baculoviral inhibitor of apoptosis proteins. Cell *83*, 1243-1252.

Roy, N., Deveraux, Q.L., Takahashi, R., Salvesen, G.S., and Reed, J.C. (1997). The c-IAP-1 and c-IAP-2 proteins are direct inhibitors of specific caspases. Embo J *16*, 6914-6925.

Roy, N., Mahadevan, M.S., McLean, M., Shutler, G., Yaraghi, Z., Farahani, R., Baird, S., Besner-Johnston, A., Lefebvre, C., Kang, X., and et al. (1995). The gene for neuronal apoptosis inhibitory protein is partially deleted in individuals with spinal muscular atrophy. Cell *80*, 167-178.

Rumble, J.M., and Duckett, C.S. (2008). Diverse functions within the IAP family. Journal of cell science *121*, 3505-3507.

Sah, N.K., Khan, Z., Khan, G.J., and Bisen, P.S. (2006). Structural, functional and therapeutic biology of survivin. Cancer letters *244*, 164-171.

Sakon, S., Xue, X., Takekawa, M., Sasazuki, T., Okazaki, T., Kojima, Y., Piao, J.H., Yagita, H., Okumura, K., Doi, T., and Nakano, H. (2003). NF-kappaB inhibits TNF-induced accumulation of ROS that mediate prolonged MAPK activation and necrotic cell death. The EMBO journal *22*, 3898-3909.

Sakurai, H., Suzuki, S., Kawasaki, N., Nakano, H., Okazaki, T., Chino, A., Doi, T., and Saiki, I. (2003). Tumor necrosis factor-alpha-induced IKK phosphorylation of NF-kappaB p65 on serine 536 is mediated through the TRAF2, TRAF5, and TAK1 signaling pathway. J Biol Chem *278*, 36916-36923.

Saldeen, J., Lee, J.C., and Welsh, N. (2001). Role of p38 mitogen-activated protein kinase (p38 MAPK) in cytokine-induced rat islet cell apoptosis. Biochemical pharmacology *61*, 1561-1569.

Salvesen, G.S., and Duckett, C.S. (2002). IAP proteins: blocking the road to death's door. Nat Rev Mol Cell Biol *3*, 401-410.

Samuel, T., Welsh, K., Lober, T., Togo, S.H., Zapata, J.M., and Reed, J.C. (2006). Distinct BIR domains of cIAP1 mediate binding to and ubiquitination of tumor necrosis factor receptor-associated factor 2 and second mitochondrial activator of caspases. Journal of Biological Chemistry *281*, 1080-1090.

Sandler, S., Eizirik, D.L., Svensson, C., Strandell, E., Welsh, M., and Welsh, N. (1991). Biochemical and molecular actions of interleukin-1 on pancreatic beta-cells. Autoimmunity *10*, 241-253.

Sanna, M.G., da Silva Correia, J., Ducrey, O., Lee, J., Nomoto, K., Schrantz, N., Deveraux, Q.L., and Ulevitch, R.J. (2002). IAP suppression of apoptosis involves distinct mechanisms: the TAK1/JNK1 signaling cascade and caspase inhibition. Molecular and Cellular Biology *22*, 1754-1766.

Sanna, M.G., Duckett, C.S., Richter, B.W., Thompson, C.B., and Ulevitch, R.J. (1998). Selective activation of JNK1 is necessary for the anti-apoptotic activity of hILP. Proceedings of the National Academy of Sciences of the United States of America *95*, 6015-6020.

Santoro, M.M., Samuel, T., Mitchell, T., Reed, J.C., and Stainier, D.Y. (2007). Birc2 (cIap1) regulates endothelial cell integrity and blood vessel homeostasis. Nature genetics *39*, 1397-1402.

Sarkar, S.A., Kutlu, B., Velmurugan, K., Kizaka-Kondoh, S., Lee, C.E., Wong, R., Valentine, A., Davidson, H.W., Hutton, J.C., and Pugazhenthi, S. (2009). Cytokine-mediated induction of anti-apoptotic genes that are linked to nuclear factor kappa-B (NF-kappaB) signalling in human islets and in a mouse beta cell line. Diabetologia *52*, 1092-1101.

Sarvetnick, N., Liggitt, D., Pitts, S.L., Hansen, S.E., and Stewart, T.A. (1988). Insulin-dependent diabetes mellitus induced in transgenic mice by ectopic expression of class II MHC and interferon-gamma. Cell *52*, 773-782.

Sasaki, H., Sheng, Y., Kotsuji, F., and Tsang, B.K. (2000). Down-regulation of X-linked inhibitor of apoptosis protein induces apoptosis in chemoresistant human ovarian cancer cells. Cancer Research *60*, 5659-5666.

Sauer, B. (1987). Functional expression of the cre-lox site-specific recombination system in the yeast Saccharomyces cerevisiae. Molecular and Cellular Biology *7*, 2087-2096.

Sauer, B., and Henderson, N. (1988). Site-specific DNA recombination in mammalian cells by the Cre recombinase of bacteriophage P1. Proceedings of the National Academy of Sciences of the United States of America *85*, 5166-5170.

Scherer, D.C., Brockman, J.A., Chen, Z., Maniatis, T., and Ballard, D.W. (1995). Signal-induced degradation of I kappa B alpha requires site-specific ubiquitination. Proceedings of the National Academy of Sciences of the United States of America *92*, 11259-11263.

Schile, A.J., Garcia-Fernandez, M., and Steller, H. (2008). Regulation of apoptosis by XIAP ubiquitin-ligase activity. Genes & Development *22*, 2256-2266.

Schlake, T., and Bode, J. (1994). Use of mutated FLP recognition target (FRT) sites for the exchange of expression cassettes at defined chromosomal loci. Biochemistry *33*, 12746-12751.

Schloot, N.C., Hanifi-Moghaddam, P., Goebel, C., Shatavi, S.V., Flohe, S., Kolb, H., and Rothe, H. (2002). Serum IFN-gamma and IL-10 levels are associated with disease progression in non-obese diabetic mice. Diabetes/metabolism research and reviews *18*, 64-70.

Schmittgen, T.D., and Livak, K.J. (2008). Analyzing real-time PCR data by the comparative C(T) method. Nature protocols *3*, 1101-1108.

Schmitz, M.L., and Baeuerle, P.A. (1991). The p65 subunit is responsible for the strong transcription activating potential of NF-kappa B. The EMBO journal *10*, 3805-3817.

Schoemaker, M.H., Ros, J.E., Homan, M., Trautwein, C., Liston, P., Poelstra, K., van Goor, H., Jansen, P.L., and Moshage, H. (2002). Cytokine regulation of pro- and anti-apoptotic genes in rat hepatocytes: NF-kappaB-regulated inhibitor of apoptosis protein 2 (cIAP2) prevents apoptosis. Journal of hepatology *36*, 742-750.

Schulthess, F.T., Paroni, F., Sauter, N.S., Shu, L., Ribaux, P., Haataja, L., Strieter, R.M., Oberholzer, J., King, C.C., and Maedler, K. (2009). CXCL10 impairs beta cell function and viability in diabetes through TLR4 signaling. Cell Metab *9*, 125-139.

Schulz, T.J., Glaubitz, M., Kuhlow, D., Thierbach, R., Birringer, M., Steinberg, P., Pfeiffer, A.F., and Ristow, M. (2007). Variable expression of Cre recombinase transgenes precludes reliable prediction of tissue-specific gene disruption by tail-biopsy genotyping. PLoS One *2*, e1013.

Serreze, D.V., Post, C.M., Chapman, H.D., Johnson, E.A., Lu, B., and Rothman, P.B. (2000). Interferon-gamma receptor signaling is dispensable in the development of autoimmune type 1 diabetes in NOD mice. Diabetes *49*, 2007-2011.

Shafey, D., Korneluk, R., and Holcik, M. (2006). Distinct patterns of expression of the inhibitor of apoptosis protein cIAP2 during murine embryogenesis. Apoptosis *11*, 1257-1259.

Shen, Q., Zhang, R., and Bhat, N.R. (2006). MAP kinase regulation of IP10/CXCL10 chemokine gene expression in microglial cells. Brain Res *1086*, 9-16.

Shigihara, T., Oikawa, Y., Kanazawa, Y., Okubo, Y., Narumi, S., Saruta, T., and Shimada, A. (2006). Significance of serum CXCL10/IP-10 level in type 1 diabetes. Journal of autoimmunity *26*, 66-71.

Shigihara, T., Shimada, A., Oikawa, Y., Yoneyama, H., Kanazawa, Y., Okubo, Y., Matsushima, K., Yamato, E., Miyazaki, J., Kasuga, A.*, et al.* (2005). CXCL10 DNA vaccination prevents spontaneous diabetes through enhanced beta cell proliferation in NOD mice. Journal of immunology *175*, 8401-8408.

Shin, H., Renatus, M., Eckelman, B.P., Nunes, V.A., Sampaio, C.A., and Salvesen, G.S. (2005). The BIR domain of IAP-like protein 2 is conformationally unstable: implications for caspase inhibition. The Biochemical journal *385*, 1-10.

Shiozaki, E.N., Chai, J., Rigotti, D.J., Riedl, S.J., Li, P., Srinivasula, S.M., Alnemri, E.S., Fairman, R., and Shi, Y. (2003). Mechanism of XIAP-mediated inhibition of caspase-9. Molecular Cell *11*, 519-527.

Shu, H.B., Takeuchi, M., and Goeddel, D.V. (1996). The tumor necrosis factor receptor 2 signal transducers TRAF2 and c-IAP1 are components of the tumor necrosis factor receptor 1 signaling complex. Proceedings of the National Academy of Sciences of the United States of America *93*, 13973-13978.

Silke, J., Hawkins, C.J., Ekert, P.G., Chew, J., Day, C.L., Pakusch, M., Verhagen, A.M., and Vaux, D.L. (2002). The anti-apoptotic activity of XIAP is retained upon mutation of both the caspase 3- and caspase 9-interacting sites. J Cell Biol *157*, 115-124.

Singal, D.P., and Blajchman, M.A. (1973). Histocompatibility (HL-A) antigens, lymphocytotoxic antibodies and tissue antibodies in patients with diabetes mellitus. Diabetes *22*, 429-432.

Sluss, H.K., Barrett, T., Derijard, B., and Davis, R.J. (1994). Signal transduction by tumor necrosis factor mediated by JNK protein kinases. Molecular and Cellular Biology *14*, 8376-8384.

Smith, C.A., Davis, T., Anderson, D., Solam, L., Beckmann, M.P., Jerzy, R., Dower, S.K., Cosman, D., and Goodwin, R.G. (1990). A receptor for tumor necrosis factor defines an unusual family of cellular and viral proteins. Science *248*, 1019-1023.

Smith, C.A., Farrah, T., and Goodwin, R.G. (1994). The TNF receptor superfamily of cellular and viral proteins: activation, costimulation, and death. Cell *76*, 959-962.

Smyth, D., Cooper, J.D., Collins, J.E., Heward, J.M., Franklyn, J.A., Howson, J.M., Vella, A., Nutland, S., Rance, H.E., Maier, L.*, et al.* (2004). Replication of an association between the lymphoid tyrosine phosphatase locus (LYP/PTPN22) with type 1

diabetes, and evidence for its role as a general autoimmunity locus. Diabetes *53*, 3020-3023.

Solt, L.A., Madge, L.A., and May, M.J. (2009). NEMO-binding domains of both IKKalpha and IKKbeta regulate IkappaB kinase complex assembly and classical NF-kappaB activation. J Biol Chem *284*, 27596-27608.

Somoza, N., Vargas, F., Roura-Mir, C., Vives-Pi, M., Fernandez-Figueras, M.T., Ariza, A., Gomis, R., Bragado, R., Marti, M., Jaraquemada, D., and et al. (1994). Pancreas in recent onset insulin-dependent diabetes mellitus. Changes in HLA, adhesion molecules and autoantigens, restricted T cell receptor V beta usage, and cytokine profile. Journal of immunology *153*, 1360-1377.

Song, H.Y., Regnier, C.H., Kirschning, C.J., Goeddel, D.V., and Rothe, M. (1997). Tumor necrosis factor (TNF)-mediated kinase cascades: bifurcation of nuclear factor-kappaB and c-jun N-terminal kinase (JNK/SAPK) pathways at TNF receptor-associated factor 2. Proceedings of the National Academy of Sciences of the United States of America *94*, 9792-9796.

Sorli, C.H., Zhang, H.J., Armstrong, M.B., Rajotte, R.V., Maclouf, J., and Robertson, R.P. (1998). Basal expression of cyclooxygenase-2 and nuclear factor-interleukin 6 are dominant and coordinately regulated by interleukin 1 in the pancreatic islet. Proceedings of the National Academy of Sciences of the United States of America *95*, 1788-1793.

Soumya Adhikani, M. (2009). Anti-inflammatory therapy with anakinra in newly diagnosed type 1 diabetes. (http://www.ClinicalTrials.gov). U.o.T.S.M. Center., ed.

Southern, C., Schulster, D., and Green, I.C. (1990). Inhibition of insulin secretion by interleukin-1 beta and tumour necrosis factor-alpha via an L-arginine-dependent nitric oxide generating mechanism. FEBS letters *276*, 42-44.

Spielman, R.S., Baker, L., and Zmijewski, C.M. (1980). Gene dosage and suceptibility to insulin-dependent diabetes. Annals of human genetics *44*, 135-150.

Srinivasula, S.M., and Ashwell, J.D. (2008). IAPs: what's in a name? Molecular Cell *30*, 123-135.

Stehlik, C., de Martin, R., Kumabashiri, I., Schmid, J.A., Binder, B.R., and Lipp, J. (1998). Nuclear factor (NF)-kappaB-regulated X-chromosome-linked iap gene expression protects endothelial cells from tumor necrosis factor alpha-induced apoptosis. The Journal of experimental medicine *188*, 211-216.

Stephens, L.A., Thomas, H.E., Ming, L., Grell, M., Darwiche, R., Volodin, L., and Kay, T.W. (1999). Tumor necrosis factor-alpha-activated cell death pathways in NIT-1 insulinoma cells and primary pancreatic beta cells. Endocrinology *140*, 3219-3227.

Struhl, K. (2001). Gene regulation. A paradigm for precision. Science *293*, 1054-1055.

Suarez-Pinzon, W., Sorensen, O., Bleackley, R.C., Elliott, J.F., Rajotte, R.V., and Rabinovitch, A. (1999). Beta-cell destruction in NOD mice correlates with Fas (CD95) expression on beta-cells and proinflammatory cytokine expression in islets. Diabetes *48*, 21-28.

Suarez-Pinzon, W.L., Yan, Y., Power, R., Brand, S.J., and Rabinovitch, A. (2005). Combination therapy with epidermal growth factor and gastrin increases beta-cell mass and reverses hyperglycemia in diabetic NOD mice. Diabetes *54*, 2596-2601.

Sun, C., Cai, M., Gunasekera, A.H., Meadows, R.P., Wang, H., Chen, J., Zhang, H., Wu, W., Xu, N., Ng, S.C., and Fesik, S.W. (1999). NMR structure and mutagenesis of the inhibitor-of-apoptosis protein XIAP. Nature *401*, 818-822.

Sun, C., Cai, M., Meadows, R.P., Xu, N., Gunasekera, A.H., Herrmann, J., Wu, J.C., and Fesik, S.W. (2000). NMR structure and mutagenesis of the third Bir domain of the inhibitor of apoptosis protein XIAP. J Biol Chem *275*, 33777-33781.

Sun, S.C., Ganchi, P.A., Ballard, D.W., and Greene, W.C. (1993). NF-kappa B controls expression of inhibitor I kappa B alpha: evidence for an inducible autoregulatory pathway. Science *259*, 1912-1915.

Sung, S.S., Bjorndahl, J.M., Wang, C.Y., Kao, H.T., and Fu, S.M. (1988). Production of tumor necrosis factor/cachectin by human T cell lines and peripheral blood T lymphocytes stimulated by phorbol myristate acetate and anti-CD3 antibody. The Journal of experimental medicine *167*, 937-953.

Suzuki, Y., Nakabayashi, Y., and Takahashi, R. (2001). Ubiquitin-protein ligase activity of X-linked inhibitor of apoptosis protein promotes proteasomal degradation of caspase-3 and enhances its anti-apoptotic effect in Fas-induced cell death. Proceedings of the National Academy of Sciences of the United States of America *98*, 8662-8667.

Tack, C.J., Kleijwegt, F.S., Van Riel, P.L., and Roep, B.O. (2009). Development of type 1 diabetes in a patient treated with anti-TNF-alpha therapy for active rheumatoid arthritis. Diabetologia *52*, 1442-1444.

Tada, K., Okazaki, T., Sakon, S., Kobarai, T., Kurosawa, K., Yamaoka, S., Hashimoto, H., Mak, T.W., Yagita, H., Okumura, K.*, et al.* (2001). Critical roles of TRAF2 and TRAF5 in tumor necrosis factor-induced NF-kappa B activation and protection from cell death. J Biol Chem *276*, 36530-36534.

Takahashi, R., Deveraux, Q., Tamm, I., Welsh, K., Assa-Munt, N., Salvesen, G.S., and Reed, J.C. (1998). A single BIR domain of XIAP sufficient for inhibiting caspases. J Biol Chem *273*, 7787-7790.

Tang, F., Tang, G., Xiang, J., Dai, Q., Rosner, M.R., and Lin, A. (2002). The absence of NF-kappaB-mediated inhibition of c-Jun N-terminal kinase activation contributes to tumor necrosis factor alpha-induced apoptosis. Molecular and Cellular Biology *22*, 8571-8579.

Tang, G., Minemoto, Y., Dibling, B., Purcell, N.H., Li, Z., Karin, M., and Lin, A. (2001). Inhibition of JNK activation through NF-kappaB target genes. Nature *414*, 313-317.

Tartaglia, L.A., Weber, R.F., Figari, I.S., Reynolds, C., Palladino, M.A., Jr., and Goeddel, D.V. (1991). The two different receptors for tumor necrosis factor mediate distinct cellular responses. Proceedings of the National Academy of Sciences of the United States of America *88*, 9292-9296.

Thiefes, A., Wolter, S., Mushinski, J.F., Hoffmann, E., Dittrich-Breiholz, O., Graue, N., Dorrie, A., Schneider, H., Wirth, D., Luckow, B.*, et al.* (2005). Simultaneous blockade of NFkappaB, JNK, and p38 MAPK by a kinase-inactive mutant of the protein kinase TAK1 sensitizes cells to apoptosis and affects a distinct spectrum of tumor necrosis factor [corrected] target genes. J Biol Chem *280*, 27728-27741.

Thomas, H.E., Angstetra, E., Fernandes, R.V., Mariana, L., Irawaty, W., Jamieson, E.L., Dudek, N.L., and Kay, T.W. (2006). Perturbations in nuclear factor-kappaB or c-Jun N-terminal kinase pathways in pancreatic beta cells confer susceptibility to cytokine-induced cell death. Immunol Cell Biol *84*, 20-27.

Thomas, H.E., Irawaty, W., Darwiche, R., Brodnicki, T.C., Santamaria, P., Allison, J., and Kay, T.W. (2004). IL-1 receptor deficiency slows progression to diabetes in the NOD mouse. Diabetes *53*, 113-121.

Thomas, H.E., Parker, J.L., Schreiber, R.D., and Kay, T.W. (1998). IFN-gamma action on pancreatic beta cells causes class I MHC upregulation but not diabetes. J Clin Invest *102*, 1249-1257.

Thompson, W.L., and Van Eldik, L.J. (2009). Inflammatory cytokines stimulate the chemokines CCL2/MCP-1 and CCL7/MCP-3 through NFkB and MAPK dependent pathways in rat astrocytes [corrected]. Brain research *1287*, 47-57.

Thomson, G., Robinson, W.P., Kuhner, M.K., Joe, S., MacDonald, M.J., Gottschall, J.L., Barbosa, J., Rich, S.S., Bertrams, J., Baur, M.P., and et al. (1988). Genetic heterogeneity, modes of inheritance, and risk estimates for a joint study of Caucasians with insulin-dependent diabetes mellitus. American journal of human genetics *43*, 799-816.

Ting, A.T., Pimentel-Muinos, F.X., and Seed, B. (1996). RIP mediates tumor necrosis factor receptor 1 activation of NF-kappaB but not Fas/APO-1-initiated apoptosis. The EMBO journal *15*, 6189-6196.

Todaro, G.J., and Green, H. (1963). Quantitative studies of the growth of mouse embryo cells in culture and their development into established lines. J Cell Biol *17*, 299-313.

Tournier, C., Hess, P., Yang, D.D., Xu, J., Turner, T.K., Nimnual, A., Bar-Sagi, D., Jones, S.N., Flavell, R.A., and Davis, R.J. (2000). Requirement of JNK for stress-induced activation of the cytochrome c-mediated death pathway. Science *288*, 870-874.

Trapp, T., Korhonen, L., Besselmann, M., Martinez, R., Mercer, E.A., and Lindholm, D. (2003). Transgenic mice overexpressing XIAP in neurons show better outcome after transient cerebral ischemia. Molecular and cellular neurosciences *23*, 302-313.

Ueda, H., Howson, J.M., Esposito, L., Heward, J., Snook, H., Chamberlain, G., Rainbow, D.B., Hunter, K.M., Smith, A.N., Di Genova, G.*, et al.* (2003). Association of the T-cell regulatory gene CTLA4 with susceptibility to autoimmune disease. Nature *423*, 506-511.

Uglialoro, A.M., Turbay, D., Pesavento, P.A., Delgado, J.C., McKenzie, F.E., Gribben, J.G., Hartl, D., Yunis, E.J., and Goldfeld, A.E. (1998). Identification of three new single nucleotide polymorphisms in the human tumor necrosis factor-alpha gene promoter. Tissue antigens *52*, 359-367.

Uno, S., Imagawa, A., Okita, K., Sayama, K., Moriwaki, M., Iwahashi, H., Yamagata, K., Tamura, S., Matsuzawa, Y., Hanafusa, T.*, et al.* (2007). Macrophages and dendritic cells infiltrating islets with or without beta cells produce tumour necrosis factor-alpha in patients with recent-onset type 1 diabetes. Diabetologia *50*, 596-601.

Uno, S., Imagawa, A., Saisho, K., Okita, K., Iwahashi, H., Hanafusa, T., and Shimomura, I. Expression of chemokines, CXC chemokine ligand 10 (CXCL10) and CXCR3 in the inflamed islets of patients with recent-onset autoimmune type 1 diabetes. Endocr J.

Uren, A.G., Coulson, E.J., and Vaux, D.L. (1998). Conservation of baculovirus inhibitor of apoptosis repeat proteins (BIRPs) in viruses, nematodes, vertebrates and yeasts. Trends in biochemical sciences *23*, 159-162.

Uren, A.G., Pakusch, M., Hawkins, C.J., Puls, K.L., and Vaux, D.L. (1996). Cloning and expression of apoptosis inhibitory protein homologs that function to inhibit apoptosis and/or bind tumor necrosis factor receptor-associated factors. Proceedings of the National Academy of Sciences of the United States of America *93*, 4974-4978.

Vallabhapurapu, S., Matsuzawa, A., Zhang, W., Tseng, P.H., Keats, J.J., Wang, H., Vignali, D.A., Bergsagel, P.L., and Karin, M. (2008). Nonredundant and complementary functions of TRAF2 and TRAF3 in a ubiquitination cascade that activates NIK-dependent alternative NF-kappaB signaling. Nature Immunology *9*, 1364-1370.

van Belle, T.L., Coppieters, K.T., and von Herrath, M.G. (2011). Type 1 diabetes: etiology, immunology, and therapeutic strategies. Physiological Reviews *91*, 79-118.

van der Windt, D.J., Bottino, R., Casu, A., Campanile, N., Smetanka, C., He, J., Murase, N., Hara, H., Ball, S., Loveland, B.E., *et al.* (2009). Long-term controlled normoglycemia in diabetic non-human primates after transplantation with hCD46 transgenic porcine islets. American journal of transplantation : official journal of the American Society of Transplantation and the American Society of Transplant Surgeons *9*, 2716-2726.

Varfolomeev, E., Blankenship, J.W., Wayson, S.M., Fedorova, A.V., Kayagaki, N., Garg, P., Zobel, K., Dynek, J.N., Elliott, L.O., Wallweber, H.J., *et al.* (2007). IAP antagonists induce autoubiquitination of c-IAPs, NF-kappaB activation, and TNFalpha-dependent apoptosis. Cell *131*, 669-681.

Varfolomeev, E., Goncharov, T., Fedorova, A.V., Dynek, J.N., Zobel, K., Deshayes, K., Fairbrother, W.J., and Vucic, D. (2008). c-IAP1 and c-IAP2 are critical mediators of tumor necrosis factor alpha (TNFalpha)-induced NF-kappaB activation. J Biol Chem *283*, 24295-24299.

Varfolomeev, E., Goncharov, T., Maecker, H., Zobel, K., Komuves, L.G., Deshayes, K., and Vucic, D. (2012). Cellular inhibitors of apoptosis are global regulators of NF-kappaB and MAPK activation by members of the TNF family of receptors. Science signaling *5*, ra22.

Varfolomeev, E., and Vucic, D. (2008). (Un)expected roles of c-IAPs in apoptotic and NFkappaB signaling pathways. Cell Cycle *7*, 1511-1521.

Varfolomeev, E.E., and Ashkenazi, A. (2004). Tumor necrosis factor: an apoptosis JuNKie? Cell *116*, 491-497.

Varona-Santos, J.L., Pileggi, A., Molano, R.D., Sanabria, N.Y., Ijaz, A., Atsushi, M., Ichii, H., Pastori, R.L., Inverardi, L., Ricordi, C., and Fornoni, A. (2008). c-Jun N-terminal kinase 1 is deleterious to the function and survival of murine pancreatic islets. Diabetologia *51*, 2271-2280.

Vaux, D.L., and Silke, J. (2005). IAPs, RINGs and ubiquitylation. Nat Rev Mol Cell Biol *6*, 287-297.

Vella, A., Cooper, J.D., Lowe, C.E., Walker, N., Nutland, S., Widmer, B., Jones, R., Ring, S.M., McArdle, W., Pembrey, M.E*., et al.* (2005). Localization of a type 1 diabetes locus in the IL2RA/CD25 region by use of tag single-nucleotide polymorphisms. American journal of human genetics *76*, 773-779.

Verhagen, A.M., Coulson, E.J., and Vaux, D.L. (2001). Inhibitor of apoptosis proteins and their relatives: IAPs and other BIRPs. Genome Biol *2*, REVIEWS3009.

Vince, J.E., Pantaki, D., Feltham, R., Mace, P.D., Cordier, S.M., Schmukle, A.C., Davidson, A.J., Callus, B.A., Wong, W.W., Gentle, I.E*., et al.* (2009). TRAF2 must bind to cellular inhibitors of apoptosis for tumor necrosis factor (tnf) to efficiently activate nf-{kappa}b and to prevent tnf-induced apoptosis. J Biol Chem *284*, 35906-35915.

Vince, J.E., Wong, W.W., Khan, N., Feltham, R., Chau, D., Ahmed, A.U., Benetatos, C.A., Chunduru, S.K., Condon, S.M., McKinlay, M*., et al.* (2007). IAP antagonists target cIAP1 to induce TNFalpha-dependent apoptosis. Cell *131*, 682-693.

Vincenti, M.P., Coon, C.I., Lee, O., and Brinckerhoff, C.E. (1994). Regulation of collagenase gene expression by IL-1 beta requires transcriptional and post-transcriptional mechanisms. Nucleic acids research *22*, 4818-4827.

Voorbij, H.A., Jeucken, P.H., Kabel, P.J., De Haan, M., and Drexhage, H.A. (1989). Dendritic cells and scavenger macrophages in pancreatic islets of prediabetic BB rats. Diabetes *38*, 1623-1629.

Vucic, D. (2008). Targeting IAP (inhibitor of apoptosis) proteins for therapeutic intervention in tumors. Current cancer drug targets *8*, 110-117.

Vucic, D., Stennicke, H.R., Pisabarro, M.T., Salvesen, G.S., and Dixit, V.M. (2000). ML-IAP, a novel inhibitor of apoptosis that is preferentially expressed in human melanomas. Curr Biol *10*, 1359-1366.

Wachlin, G., Augstein, P., Schroder, D., Kuttler, B., Kloting, I., Heinke, P., and Schmidt, S. (2003). IL-1beta, IFN-gamma and TNF-alpha increase vulnerability of pancreatic beta cells to autoimmune destruction. J Autoimmun *20*, 303-312.

Walter, U., Franzke, A., Sarukhan, A., Zober, C., von Boehmer, H., Buer, J., and Lechner, O. (2000). Monitoring gene expression of TNFR family members by beta-cells during development of autoimmune diabetes. European journal of immunology *30*, 1224-1232.

Wang, B., Andre, I., Gonzalez, A., Katz, J.D., Aguet, M., Benoist, C., and Mathis, D. (1997). Interferon-gamma impacts at multiple points during the progression of autoimmune diabetes. Proceedings of the National Academy of Sciences of the United States of America *94*, 13844-13849.

Wang, C.H., Tsao, Y.P., Chen, H.J., Chen, H.L., Wang, H.W., and Chen, S.L. (2000). Transcriptional repression of p21((Waf1/Cip1/Sdi1)) gene by c-jun through Sp1 site. Biochemical and Biophysical Research Communications *270*, 303-310.

Wang, C.Y., Mayo, M.W., Korneluk, R.G., Goeddel, D.V., and Baldwin, A.S. (1998). NF-kappa B antiapoptosis: Induction of TRAF1 and TRAF2 and c-IAP1 and c-IAP2 to suppress caspase-8 activation. Science *281*, 1680-1683.

Wang, H., Gambosova, K., Cooper, Z.A., Holloway, M.P., Kassai, A., Izquierdo, D., Cleveland, K., Boney, C.M., and Altura, R.A. (2010). EGF regulates survivin stability through the Raf-1/ERK pathway in insulin-secreting pancreatic beta-cells. BMC molecular biology *11*, 66.

Wang, L., Du, F.H., and Wang, X.D. (2008). TNF-alpha induces two distinct caspase-8 activation pathways. Cell *133*, 693-703.

Wang, Y., Suominen, J.S., Parvinen, M., Rivero-Muller, A., Kiiveri, S., Heikinheimo, M., Robbins, I., and Toppari, J. (2005). The regulated expression of c-IAP1 and c-IAP2 during the rat seminiferous epithelial cycle plays a role in the protection of germ cells from Fas-mediated apoptosis. Molecular and cellular endocrinology *245*, 111-120.

Warnakulasuriyarachchi, D., Cerquozzi, S., Cheung, H.H., and Holcik, M. (2004). Translational induction of the inhibitor of apoptosis protein HIAP2 during endoplasmic reticulum stress attenuates cell death and is mediated via an inducible internal ribosome entry site element. J Biol Chem *279*, 17148-17157.

Watihayati, M.S., Fatemeh, H., Marini, M., Atif, A.B., Zahiruddin, W.M., Sasongko, T.H., Tang, T.H., Zabidi-Hussin, Z.A., Nishio, H., and Zilfalil, B.A. (2009). Combination of SMN2 copy number and NAIP deletion predicts disease severity in spinal muscular atrophy. Brain & development *31*, 42-45.

Wei, Y., Fan, T., and Yu, M. (2008). Inhibitor of apoptosis proteins and apoptosis. Acta biochimica et biophysica Sinica *40*, 278-288.

Weir, G.C., and Bonner-Weir, S. (1990). Islets of Langerhans: the puzzle of intraislet interactions and their relevance to diabetes. J Clin Invest *85*, 983-987.

Weiss, T., Grell, M., Siemienski, K., Muhlenbeck, F., Durkop, H., Pfizenmaier, K., Scheurich, P., and Wajant, H. (1998). TNFR80-dependent enhancement of TNFR60-induced cell death is mediated by TNFR-associated factor 2 and is specific for TNFR60. Journal of immunology *161*, 3136-3142.

Wertz, I.E., O'Rourke, K.M., Zhou, H., Eby, M., Aravind, L., Seshagiri, S., Wu, P., Wiesmann, C., Baker, R., Boone, D.L.*, et al.* (2004). De-ubiquitination and ubiquitin ligase domains of A20 downregulate NF-kappaB signalling. Nature *430*, 694-699.

Weyer, C., Bogardus, C., Mott, D.M., and Pratley, R.E. (1999). The natural history of insulin secretory dysfunction and insulin resistance in the pathogenesis of type 2 diabetes mellitus. J Clin Invest *104*, 787-794.

Wilcox, G. (2005). Insulin and insulin resistance. The Clinical biochemist. Reviews / Australian Association of Clinical Biochemists *26*, 19-39.

Wilkinson, J.C., Cepero, E., Boise, L.H., and Duckett, C.S. (2004). Upstream regulatory role for XIAP in receptor-mediated apoptosis. Molecular and Cellular Biology *24*, 7003-7014.

Willcox, A., Richardson, S.J., Bone, A.J., Foulis, A.K., and Morgan, N.G. (2009). Analysis of islet inflammation in human type 1 diabetes. Clinical and experimental immunology *155*, 173-181.

Wilmanski, J.M., Petnicki-Ocwieja, T., and Kobayashi, K.S. (2008). NLR proteins: integral members of innate immunity and mediators of inflammatory diseases. Journal of leukocyte biology *83*, 13-30.

Witkowski, P., Zakai, S.B., Rana, A., Sledzinski, Z., and Hardy, M.A. (2006). Pancreatic islet transplantation, what has been achieved since Edmonton break-through. Annals of transplantation : quarterly of the Polish Transplantation Society *11*, 5-13; discussion 32-43.

Wolter, S., Doerrie, A., Weber, A., Schneider, H., Hoffmann, E., von der Ohe, J., Bakiri, L., Wagner, E.F., Resch, K., and Kracht, M. (2008). c-Jun controls histone modifications, NF-kappaB recruitment, and RNA polymerase II function to activate the ccl2 gene. Molecular and Cellular Biology *28*, 4407-4423.

Wong, W.W., Gentle, I.E., Nachbur, U., Anderton, H., Vaux, D.L., and Silke, J. (2010). RIPK1 is not essential for TNFR1-induced activation of NF-kappaB. Cell death and differentiation *17*, 482-487.

Wu, C.J., Conze, D.B., Li, X., Ying, S.X., Hanover, J.A., and Ashwell, J.D. (2005). TNF-alpha induced c-IAP1/TRAF2 complex translocation to a Ubc6-containing compartment and TRAF2 ubiquitination. The EMBO journal *24*, 1886-1898.

Wu, H., Panakanti, R., Li, F., and Mahato, R.I. (2010). XIAP Gene Expression Protects beta-Cells and Human Islets from Apoptotic Cell Death. Molecular pharmaceutics.

Xia, Y., Pauza, M.E., Feng, L., and Lo, D. (1997). RelB regulation of chemokine expression modulates local inflammation. The American journal of pathology *151*, 375-387.

Xiao, G., Fong, A., and Sun, S.C. (2004). Induction of p100 processing by NF-kappaB-inducing kinase involves docking IkappaB kinase alpha (IKKalpha) to p100 and IKKalpha-mediated phosphorylation. J Biol Chem *279*, 30099-30105.

Xu, L., Zhu, J., Hu, X., Zhu, H., Kim, H.T., LaBaer, J., Goldberg, A., and Yuan, J. (2007). c-IAP1 cooperates with Myc by acting as a ubiquitin ligase for Mad1. Molecular Cell *28*, 914-922.

Yagi, N., Yokono, K., Amano, K., Nagata, M., Tsukamoto, K., Hasegawa, Y., Yoneda, R., Okamoto, N., Moriyama, H., Miki, M., and et al. (1995). Expression of intercellular adhesion molecule 1 on pancreatic beta-cells accelerates beta-cell destruction by cytotoxic T-cells in murine autoimmune diabetes. Diabetes *44*, 744-752.

Yang, C.H., Murti, A., Pfeffer, S.R., Basu, L., Kim, J.G., and Pfeffer, L.M. (2000a). IFNalpha/beta promotes cell survival by activating NF-kappa B. Proceedings of the National Academy of Sciences of the United States of America *97*, 13631-13636.

Yang, C.M., Luo, S.F., Hsieh, H.L., Chi, P.L., Lin, C.C., Wu, C.C., and Hsiao, L.D. Interleukin-1beta induces ICAM-1 expression enhancing leukocyte adhesion in human rheumatoid arthritis synovial fibroblasts: involvement of ERK, JNK, AP-1, and NF-kappaB. J Cell Physiol *224*, 516-526.

Yang, X.D., Tisch, R., Singer, S.M., Cao, Z.A., Liblau, R.S., Schreiber, R.D., and McDevitt, H.O. (1994). Effect of tumor necrosis factor alpha on insulin-dependent diabetes mellitus in NOD mice. I. The early development of autoimmunity and the diabetogenic process. The Journal of experimental medicine *180*, 995-1004.

Yang, Y., Fang, S., Jensen, J.P., Weissman, A.M., and Ashwell, J.D. (2000b). Ubiquitin protein ligase activity of IAPs and their degradation in proteasomes in response to apoptotic stimuli. Science *288*, 874-877.

Yeh, W.C., Shahinian, A., Speiser, D., Kraunus, J., Billia, F., Wakeham, A., de la Pompa, J.L., Ferrick, D., Hum, B., Iscove, N.*, et al.* (1997). Early lethality, functional NF-kappaB activation, and increased sensitivity to TNF-induced cell death in TRAF2-deficient mice. Immunity *7*, 715-725.

Yeruva, S., Ramadori, G., and Raddatz, D. (2008). NF-kappaB-dependent synergistic regulation of CXCL10 gene expression by IL-1beta and IFN-gamma in human intestinal epithelial cell lines. Int J Colorectal Dis *23*, 305-317.

Yoon, J.W., and Jun, H.S. (2005). Autoimmune destruction of pancreatic beta cells. American journal of therapeutics *12*, 580-591.

You, M., Ku, P.T., Hrdlickova, R., and Bose, H.R., Jr. (1997). ch-IAP1, a member of the inhibitor-of-apoptosis protein family, is a mediator of the antiapoptotic activity of the v-Rel oncoprotein. Molecular and Cellular Biology *17*, 7328-7341.

Yuasa, T., Ohno, S., Kehrl, J.H., and Kyriakis, J.M. (1998). Tumor necrosis factor signaling to stress-activated protein kinase (SAPK)/Jun NH2-terminal kinase (JNK) and p38. Germinal center kinase couples TRAF2 to mitogen-activated protein kinase/ERK kinase kinase 1 and SAPK while receptor interacting protein associates with a mitogen-activated protein kinase kinase kinase upstream of MKK6 and p38. J Biol Chem *273*, 22681-22692.

Zandi, E., Rothwarf, D.M., Delhase, M., Hayakawa, M., and Karin, M. (1997). The IkappaB kinase complex (IKK) contains two kinase subunits, IKKalpha and IKKbeta, necessary for IkappaB phosphorylation and NF-kappaB activation. Cell *91*, 243-252.

Zarnegar, B., Yamazaki, S., He, J.Q., and Cheng, G. (2008a). Control of canonical NF-kappaB activation through the NIK-IKK complex pathway. Proc Natl Acad Sci U S A *105*, 3503-3508.

Zarnegar, B.J., Wang, Y.Y., Mahoney, D.J., Dempsey, P.W., Cheung, H.H., He, J., Shiba, T., Yang, X.L., Yeh, W.C., Mak, T.W.*, et al.* (2008b). Noncanonical NF-kappa B activation requires coordinated assembly of a regulatory complex of the adaptors cIAP1, cIAP2, TRAF2 and TRAF3 and the kinase NIK. Nature Immunology *9*, 1371-1378.

Zhang, L., Blackwell, K., Thomas, G.S., Sun, S., Yeh, W.C., and Habelhah, H. (2009). TRAF2 suppresses basal IKK activity in resting cells and TNFalpha can activate IKK in TRAF2 and TRAF5 double knockout cells. J Mol Biol *389*, 495-510.

Zhang, S., and Kim, K.H. (1995). TNF-alpha inhibits glucose-induced insulin secretion in a pancreatic beta-cell line (INS-1). FEBS letters *377*, 237-239.

Zhang, S., Lin, Z.N., Yang, C.F., Shi, X., Ong, C.N., and Shen, H.M. (2004). Suppressed NF-kappaB and sustained JNK activation contribute to the sensitization effect of parthenolide to TNF-alpha-induced apoptosis in human cancer cells. Carcinogenesis *25*, 2191-2199.

Zhao, X., Laver, T., Hong, S.W., Twitty, G.B., Jr., Devos, A., Devos, M., Benveniste, E.N., and Nozell, S.E. (2011a). An NF-kappaB p65-cIAP2 link is necessary for mediating resistance to TNF-alpha induced cell death in gliomas. Journal of neuro-oncology *102*, 367-381.

Zhao, Y., Conze, D.B., Hanover, J.A., and Ashwell, J.D. (2007). Tumor necrosis factor receptor 2 signaling induces selective c-IAP1-dependent ASK1 ubiquitination and terminates mitogen-activated protein kinase signaling. J Biol Chem *282*, 7777-7782.

Zhao, Y., Krishnamurthy, B., Mollah, Z.U., Kay, T.W., and Thomas, H.E. (2011b). NF-kappaB in type 1 diabetes. Inflammation & allergy drug targets *10*, 208-217.

Zheng, C., Kabaleeswaran, V., Wang, Y., Cheng, G., and Wu, H. (2010). Crystal structures of the TRAF2: cIAP2 and the TRAF1: TRAF2: cIAP2 complexes: affinity, specificity, and regulation. Molecular Cell *38*, 101-113.

Zhou, H., Du, M.Q., and Dixit, V.M. (2005). Constitutive NF-kappaB activation by the t(11;18)(q21;q21) product in MALT lymphoma is linked to deregulated ubiquitin ligase activity. Cancer Cell *7*, 425-431.

Zhou, J., Yuen, N.K., Zhan, Q., Velazquez, E.F., Murphy, G.F., Giobbie-Hurder, A., and Hodi, F.S. (2012). Immunity to the melanoma inhibitor of apoptosis protein (ML-IAP; livin) in patients with malignant melanoma. Cancer immunology, immunotherapy : CII *61*, 655-665.

Zhu, X.D., and Sadowski, P.D. (1995). Cleavage-dependent ligation by the FLP recombinase. Characterization of a mutant FLP protein with an alteration in a catalytic amino acid. J Biol Chem *270*, 23044-23054.

Zou, H., Li, Y., Liu, X., and Wang, X. (1999). An APAF-1.cytochrome c multimeric complex is a functional apoptosome that activates procaspase-9. J Biol Chem *274*, 11549-11556.

Diabetologia
DOI 10.1007/s00125-012-2784-x

ARTICLE

Baculoviral inhibitors of apoptosis repeat containing (BIRC) proteins fine-tune TNF-induced nuclear factor κB and c-Jun N-terminal kinase signalling in mouse pancreatic beta cells

B. M. Tan · N. W. Zammit · A. O. Yam · R. Slattery · S. N. Walters · E. Malle · S. T. Grey

Received: 9 July 2012 / Accepted: 19 October 2012

Abstract
Aims/hypothesis For beta cells, contact with TNF-α triggers signalling cascades that converge on pathways important for cell survival and inflammation, specifically nuclear factor κB (NF-κB), c-Jun N-terminal kinase (JNK) and p38 mitogen-activated protein kinase pathways. Here, we investigated the function of baculoviral inhibitors of apoptosis repeat containing (BIRC) proteins in regulating TNF signalling cascades.
Methods TNF regulation of Birc genes was studied by mRNA expression and promoter analysis. Birc gene control of cell signalling was studied in beta cell lines, and in islets from *Birc2*$^{-/-}$ and *Birc3*$^{-/-}$ mice, and from *Birc3*$^{-/-}$*Birc2*Δ beta cell mice that selectively lack *Birc2* and *Birc3* (double knockout [DKO]). Islet function was tested by intraperitoneal glucose tolerance test and transplantation.
Results TNF-α selectively induced *Birc3* in beta cells, which in turn was sufficient to drive and potentiate NF-κB reporter activity. Conversely, *Birc3*$^{-/-}$ islets exhibited delayed TNF-α-induced IκBα degradation with reduced expression of *Ccl2* and *Cxcl10*. DKO islets showed a further delay in IκBα degradation kinetics. Surprisingly, DKO islets exhibited stimulus-independent and TNF-dependent hyperexpression of TNF target genes *A20* (also known as *Tnfaip3*), *Icam1*, *Ccl2* and *Cxcl10*. DKO islets showed hyperphosphorylation of the JNK-substrate, c-Jun, while a JNK-antagonist prevented increases of *Icam1*, *Ccl2* and *Cxcl10* expression. Proteosome blockade of MIN6 cells phenocopied DKO islets. DKO islets showed more rapid loss of glucose homeostasis when challenged with the inflammatory insult of transplantation.
Conclusions/interpretation BIRC3 provides a feed-forward loop, which, with BIRC2, is required to moderate the normal speed of NF-κB activation. Paradoxically, BIRC2 and BIRC3 act as a molecular brake to rein in activation of the JNK signalling pathway. Thus BIRC2 and BIRC3 fine-tune NF-κB and JNK signalling to ensure transcriptional responses are appropriately matched to extracellular inputs. This control is critical for the beta cell's stress response.

Keywords Beta cell · BIRC · Diabetes · Gene · Inflammation · Islet · JNK · NF-κB · TNF

Abbreviations

ASK1	Apoptosis signal-regulating kinase 1
BIRC	Baculoviral inhibitors of apoptosis repeat containing proteins
DKO	*Birc3*$^{-/-}$*Birc2*Δ beta cell mice that selectively lack *Birc2* and *Birc3*
eGFP	Enhanced green fluorescent protein
eGFP-*Birc3*	pIRES2-eGFP-*Birc3*
IAP	Inhibitors of apoptosis
IκBα	Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha
JNK	c-Jun N-terminal kinase
NF-κB	Nuclear factor of kappa light polypeptide gene enhancer in B cells
NIK	NF-κB-inducing kinase
p38	p38 mitogen-activated protein kinases

Electronic supplementary material The online version of this article (doi:10.1007/s00125-012-2784-x) contains peer-reviewed but unedited supplementary material, which is available to authorised users.

B. M. Tan · N. W. Zammit · A. O. Yam · S. N. Walters · E. Malle · S. T. Grey (✉)
Gene Therapy and Autoimmunity Group, Immunology Program, Garvan Institute of Medical Research, 384 Victoria Street, Darlinghurst, NSW 2010, Australia
e-mail: s.grey@garvan.org.au

R. Slattery
Department of Immunology, Faculty of Medicine, Nursing and Health Sciences, Monash University, The Alfred Hospital, Melbourne, VIC, Australia

PDTC	Pyrrolidine dithiocarbamate
pIRES2	Plasmid containing an internal ribosome entry site two
RIPK1	Receptor-interacting protein kinase 1
TNFR	TNF receptor
TRAF	TNFR-associated factor
XIAP	X-linked inhibitor of apoptosis protein

Introduction

TNF-α is a pleiotropic cytokine that is involved in the pathogenesis of autoimmune type 1 diabetes and influences cell proliferation, inflammation and cell death. TNF-α mediates its actions through two distinct receptors, TNF receptor (TNFR) 1/p55 and TNFR2/p75, both of which are expressed on virtually all cell types [1]. TNFR1 is predominantly responsible for TNF signalling in most cell types [1], mediated by the sequential formation of two complexes upon TNFR1 ligation [2]. Complex I forms with the recruitment of TNFR1-associated death domain protein, which subsequently recruits TNFR-associated factor (TRAF) 2, TRAF5 and receptor-interacting protein kinase 1 (RIPK1) [2]. Within complex I, RIPK1 is essential for activating the nuclear factor of kappa light polypeptide gene enhancer in B cells (NF-κB) pathway [3], while TRAF2 is required for activation of the c-Jun N-terminal kinase (JNK) pathway [4]. These signalling cascades are imperative for the control of genes involved in immune response, inflammation and cell survival [1]. In addition, a secondary complex, spatially and temporally distinct from complex I, comprises Fas-associated death domain protein and caspase 8, which triggers a pro-apoptotic cascade [2]. Normally, TNF-α-stimulated cells are protected from the pro-apoptotic force of complex II by the expression of NF-κB-induced genes, including *A20* (also known as *Tnfaip3*) and *Cflip* (also known as *Cflar*), both of which are regulated via complex I. *A20* and *Cflip* prevent activation of caspase-8, possibly at the level of complex II [5, 6]. Thus, TNF-α triggers a co-ordinated and complex cellular program regulating cell proliferation, inflammation and cell death.

The contribution of TNF-α to type 1 diabetes pathogenesis is multifaceted. A characteristic feature of diabetes is the infiltration of the pancreatic islets with T cells, with TNFRI-null islets being protected from destruction by T lymphocytes [7]. TNF-α is an early cytokine detected within the immune infiltrate surrounding islets [8]. In the context of type 1 diabetes, TNF-α exerts detrimental effects, including heightened expression of inflammatory genes in pancreatic beta cells [9], the impairment of insulin secretion [10] and the triggering of beta cell apoptosis [5]. TNF-α's ability to induce and perpetuate diabetes has been clearly demonstrated in 3-week-old NOD mice, which upon administration of exogenous TNF-α had accelerated onset of diabetes and increased disease frequency [11]. In contrast, diabetes progression can be ameliorated by anti-TNF-α monoclonal antibody treatment [11]. Despite the importance of TNF-α in the pathogenesis of type 1 diabetes, the exact molecular machinery governing the TNF signalling cascade in islets and pancreatic beta cells remains poorly understood. An improved understanding of TNF signalling could lead to novel diagnostics or therapies for patients with type 1 diabetes.

Inhibitors of apoptosis (IAP) or baculoviral IAP repeat containing proteins (BIRC) are implicated in the regulation of downstream TNF signalling networks [12]. A total of eight BIRC proteins has been identified, namely: neuronal AIP (BIRC1), cellular IAP1 (BIRC2), cellular IAP2 (BIRC3), X-linked inhibitor of apoptosis protein (XIAP/BIRC4), survivin (TIAP/BIRC5), baculoviral IAP repeat-containing ubiquitin-conjugating enzyme (BRUCE/Apollon/BIRC6), Melanoma-IAP (ML-IAP/Livin/kidney-IAP/BIRC7) and ILP-2 (Testis Specific-IAP)/BIRC8). A key cellular function of BIRC proteins is the regulation of apoptosis [12]. Indeed, XIAP can prevent apoptosis in a number of cell types, including islets [13–16] by preventing direct IAP binding protein with low pI (DIABLO)-mediated cleavage of caspase 9 [17]. While BIRC proteins were thought to primarily act as regulators of apoptosis, this function has not been established for all its members; it is now recognised that BIRC proteins are also involved in the regulation of diverse cellular functions, including the regulation of signalling and inflammation [12]. Further to this, BIRC2 and BIRC3, together with TRAF2, have been identified as key components of the TNF signalling pathway that are necessary for TNF-mediated NF-κB activation [18]. With reference to beta cells, some studies have shown that beta cells express *Birc3*, and that *Birc3* expression is regulated by the TNF-α-mediated NF-κB pathway [5, 19]; however, its binding partner, *Birc2*, has not been examined. This suggested to us that in beta cells, BIRC3 may participate in a TNF signalling feedback loop, an idea that has not been tested to date. Here we examined the requirements for BIRC3 and BIRC2 in TNF signalling in beta cells.

Methods

Mouse strains BALB/c and C57BL/6 mice were from Australian BioResource (Mossvale, NSW, Australia). *Birc2*$^{-/-}$ and *Birc3*$^{-/-}$ mice were a kind gift of D. Vaux (Cell Signalling and Cell Death division, Walter and Eliza Hall Institute, Parkville, VIC, Australia). *Birc3*$^{-/-}$ mice containing *Birc2*$^{loxP/lox\text{-}P}$ were crossed with RIP-*Cre* mice (*Cre* driven by the rat insulin promoter) (Jax Mice, Bar Harbour, ME, USA) to generate beta cell-specific double knockout *Birc3*$^{-/-}$*Birc2*Δ beta cell mice that selectively lack *Birc2* and *Birc3* (double knockout [DKO]). Procedures complied with the Australian Code of Practice for Care and Use of Animals for Scientific Purposes.

Cytokines Mouse islets were isolated as described [20]. Either 70–100 islets or 1×10^6 MIN6 beta cells [21] were stimulated with 200 U/ml of TNF-α for 4 h (RNA analysis) and 8 h (promoter analysis) (R&D Systems, Minneapolis, MN, USA). β-TC_3 cells were used for some transfection studies [22]. In some cases, cells were pretreated for 1 h with 1 μmol/l actinomycin D, 50 μmol/l SP600125, 1 μmol/l MG-132 and 50 μmol/l pyrrolidine dithiocarbamate (PDTC) (all from Sigma-Aldrich, St Louis, MO, USA), and with 20 μmol/l SB203580 (Cell Signalling Technology, Beverly, MA, USA), prior to cytokine stimulation. Western blotting was performed using standard protocols with IκBα antibody (9242L), phospho-c-Jun (Ser73, 9164S) (Cell Signalling Technology), β-actin (Clone AC-15; A5441; Sigma-Aldrich) and horseradish peroxidase-conjugated antibodies (Pierce, Rockford, IL, USA), and chemiluminescence (GE Healthcare, Uppsala, Sweden).

Quantitative RT-PCR Total RNA and cDNA were generated from beta cell lines and mouse islets using standard techniques previously described by this research group [5]. Relative gene expression is the ratio to the housekeeping gene, Cyclophilin A (*Ppia*); fold-changes were analysed using the $2^{-\Delta\Delta C_t}$ method. Primers used for PCR are provided in electronic supplementary material (ESM) Table 1.

Cloning of Birc3 and promoter The *Birc3* coding region was cloned from cytokine-stimulated mouse islets, using the primers shown in ESM Table 2, with a PCR system (Expand High Fidelity PCR; Roche, Indianapolis, IN, USA), and subcloned into a pIRES2-eGFP vector (Clontech Laboratories, Mountain View, CA, USA). Putative transcription factor binding sites in the *Birc3* proximal promoter were identified using PROMO3.0 [23]. The mouse *Birc3* 5′ untranslated region was amplified from genomic DNA using the primers shown in ESM Table 3 with KOD Hot Start DNA Polymerase (Merck, Darmstadt, Germany) and subcloned into a pGL3 basic vector (Promega, Sydney, NSW, Australia).

Transient transfections Transfection of MIN6 and β-TC_3 cells was performed using techniques and expression plasmids encoding NF-κB.Luc (Promega), *RelA*/*p65*, A20.Luc, and plasmid containing beta galactosidase under the control of the Rous Sarcoma virus (pRSV-β-galactosidase) as previously described by us [5]. Luciferase values (Luciferase Assay System; Promega) were normalised to β-galactosidase activity

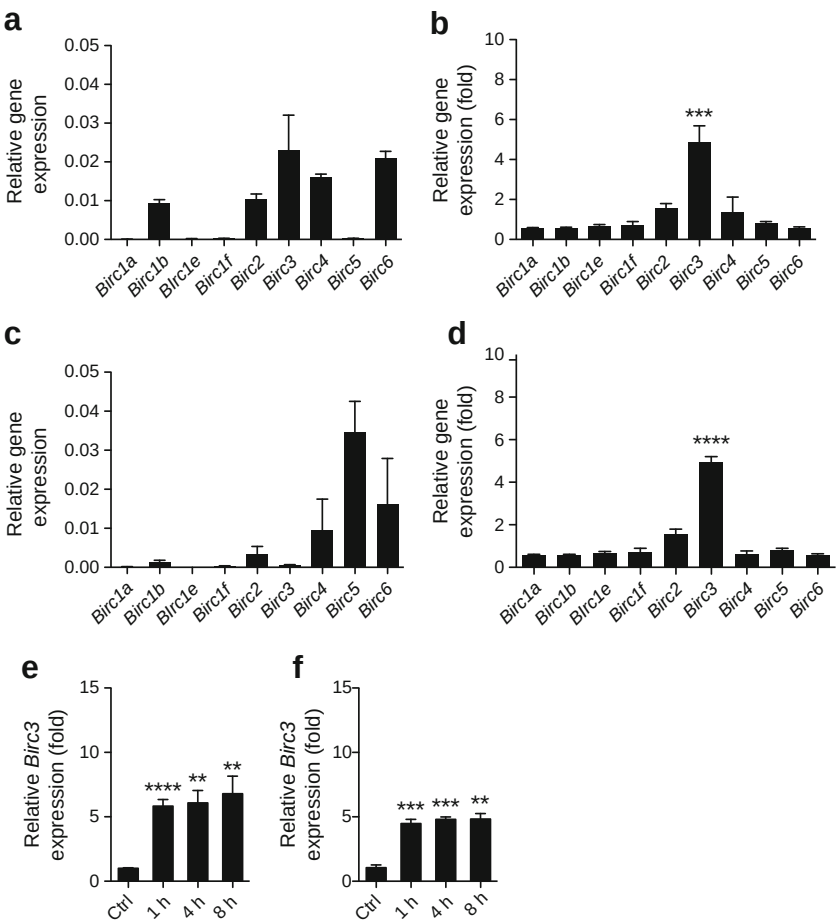


Fig. 1 *Birc3* is an early immediate-response gene. (**a**) Expression of Birc genes, as indicated, in primary mouse islets that were left untreated or (**b**) were stimulated with TNF-α for 4 h, and in (**c**) MIN6 cells left untreated or (**d**) stimulated with TNF-α for 4 h. (**e**) Expression of *Birc3* in primary islets and (**f**) in MIN6 cells stimulated with TNF-α for 1 to 8 h. Data represent mean ± SEM of gene expression relative to *Ppia* from at least three independent experiments. Statistical comparisons were by ANOVA with pair-wise multiple comparisons made using an unpaired *t* test or single unpaired *t* test between two groups. $^{**}p\leq0.01$, $^{***}p\leq0.001$ and $^{****}p\leq0.0001$. *Birc1a*, also known as *Naip1*; *Birc1b*, also known as *Naip2*; *Birc1e*, also known as *Naip5*; *Birc1f*, also known as *Naip6*; *Birc4*, also known as *Xiap*

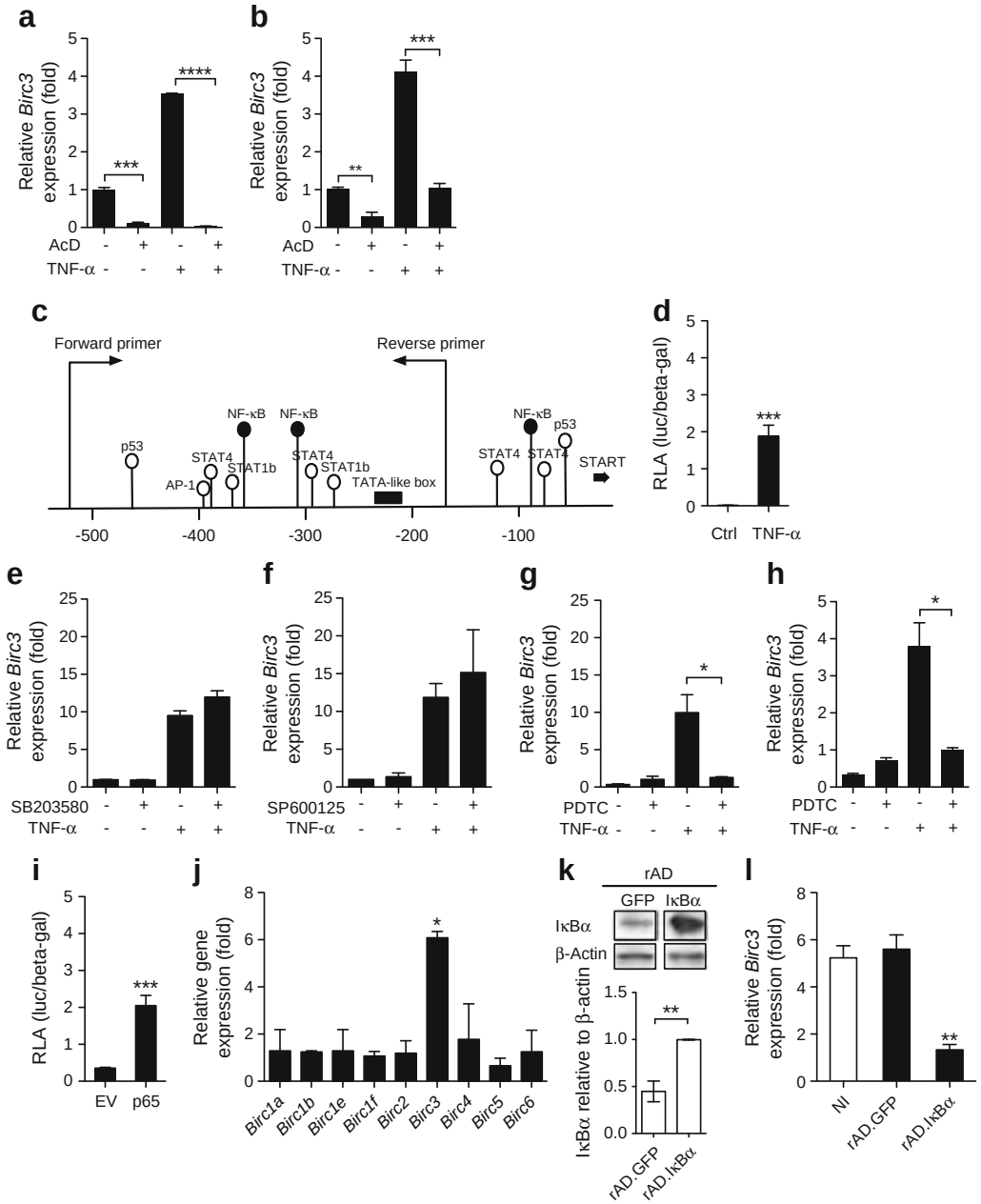


Fig. 2 *Birc3* is regulated by de novo transcription. *Birc3* expression in primary islets (**a**) or MIN6 cells (**b**) that were left untreated or were pretreated for 1 h with actinomycin D (AcD), with or without TNF-α stimulation for 4 h. (**c**) Diagram of putative transcription elements in the *Birc3* proximal promoter ~500 bp upstream of the transcription start site (START), identified using PROMO 3.0 software. AP-1, activator protein 1; STAT, signal transducer and activator of transcription. (**d**) Induction of the *Birc3* reporter in MIN6 cells by TNF-α exposure for 8 h. RLA, relative luciferase activity. (**e**) Expression of *Birc3* in MIN6 cells that were treated with or without TNF-α for 4 h, and were either left untreated or had been pretreated for 1 h with SB203580, (**f**) SP600125 or (**g**) PDTC. (**h**) Expression of *Birc3* in primary islets that were left untreated or were pretreated for 1 h with PDTC, with or without TNF-α stimulation for 4 h. (**i**) Induction of the *Birc3* reporter in MIN6 cells by *RelA*/*p65*. EV, control vector. (**j**) Induction by *RelA*/*p65* of endogenous Birc genes mRNA, as indicated, in β-TC_3 cells. *Birc1a*, also known as *Naip1*; *Birc1b*, also known as *Naip2*; *Birc1e*, also known as *Naip5*; *Birc1f*, also known as *Naip6*; *Birc4*, also known as *Xiap*. (**k**) Western blot analysis of IκBα levels in MIN6 cells transduced with rAD.GFP or rAD.IκBα. β-Actin was used as a loading control. Representative blots are shown. IκBα (39 kDa) was quantified by densitometry and expressed relative to β-actin. (**l**) Induction of *Birc3* by MIN6 cells left non-infected (NI), or transduced with rAD.GFP or rAD.IκBα prior to TNF-α stimulation for 4 h. (**a**, **b**, **e**–**h**, **j**, **l**) Data represent mean ± SEM of gene expression relative to *Ppia* from three independent experiments or (**d**, **k**) mean ± SEM from three independent experiments. Statistical comparisons were made by single unpaired *t* test between two groups. *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$ and ****$p \leq 0.0001$

(Galacto-Star; Applied Biosystems, Bedford, MA, USA) for relative luciferase activity.

Adenovirus transduction MIN6 cells (1×10^6) were infected for 1.5 h with recombinant adenovirus expressing GFP (rAd.GFP) or rAd.IκBα [24] at a multiplicity of infection of 100:1 in serum-free medium before adding FCS-enriched medium to achieve a 10% (vol./vol.) FCS concentration. Cells were incubated overnight and the medium was replaced prior to stimulation with TNF-α for 4 h.

In vivo studies Intraperitoneal glucose tolerance tests were performed on 12-week-old male mice administered 2 g/kg glucose following a 16 h fast. For islet transplantation, islets from donor (H-2^b) mice were transplanted into recipient mice (H-2^k) that had been rendered diabetic with 200 mg/kg streptozotocin (Sigma-Aldrich) as previously described [25].

Statistics Statistical analyses were performed using GraphPad Prism 5 software (GraphPad Software, San Diego, CA, USA).

Results

Birc3 is an early immediate-response gene regulated by de novo transcription In the steady state, *Birc3* was highly expressed (Fig. 1a) and was selectively increased by TNF-α in primary islets ($p \leq 0.0001$) (Fig. 1b) and MIN6 cells (Fig. 1c, d). Once induced, high levels of *Birc3* mRNA were maintained in primary islets ($p \leq 0.01$ and $p \leq 0.0001$) (Fig. 1e) and MIN6 cells ($p \leq 0.01$ and $p \leq 0.001$) (Fig. 1f).

To determine the mechanism of *Birc3* induction, primary islets and MIN6 cells were pretreated with the transcription inhibitor, actinomycin D, prior to TNF-α stimulation. Pretreatment with an optimal dose of actinomycin D (1 μmol/l) resulted in >70% suppression of TNF-α-induced *Birc3* expression in islets ($p \leq 0.0001$) (Fig. 2a) and MIN6 cells ($p \leq 0.001$) (Fig. 2b). Thus *Birc3* is an inflammation-regulated, early immediate-response gene regulated via de novo gene transcription.

NF-κB controls TNF-induced Birc3 expression The 5′ untranslated region of the *Birc3* proximal promoter contained three putative consensus NF-κB binding sites and a TATA-like box with the sequence TTTAAA' (Fig. 2c). Potential putative activator protein 1, signal transducers and activators of transcription 1 and protein 53 (p53) binding sites were also identified. This region was cloned to generate the *Birc3* reporter, which showed an approximately twofold increase upon TNF-α stimulation ($p \leq 0.01$) (Fig. 2d). These results indicate that the *Birc3* proximal promoter contains cytokine responsive elements that direct *Birc3* gene expression.

To determine the molecular mechanisms driving *Birc3* transcription, MIN6 cells were left untreated, or were pretreated with SP600125, SB203580 or PDTC to selectively target the JNK, p38 mitogen-activated protein kinases (p38) or NF-κB pathways respectively, prior to TNF-α stimulation. *Birc3* mRNA levels were measured by quantitative RT-PCR. Neither SB203580 (Fig. 2e) nor SP600125 (Fig. 2f), but only PDTC pre-treatment inhibited TNF-α-induced *Birc3* expression, e.g. by ~90% for MIN6 cells ($p \leq 0.05$) (Fig. 2g) and ~75% for islets ($p \leq 0.05$) (Fig. 2h). These data show that NF-κB is necessary for TNF-α-induced *Birc3* expression.

NF-κB is sufficient to drive de novo Birc3 expression When MIN6 cells were co-transfected with *Birc3* reporter and a *RelA*/*p65* expression vector, *Birc3* reporter activity was increased by twofold ($p \leq 0.001$) (Fig. 2i). To determine whether *RelA*/*p65* was sufficient to drive endogenous *Birc3* gene expression, β-TC_3 cells were transfected with the *RelA*/*p65* expression vector and Birc family gene expression determined by quantitative RT-PCR. In this case, *Birc3* was selectively induced (~sixfold,

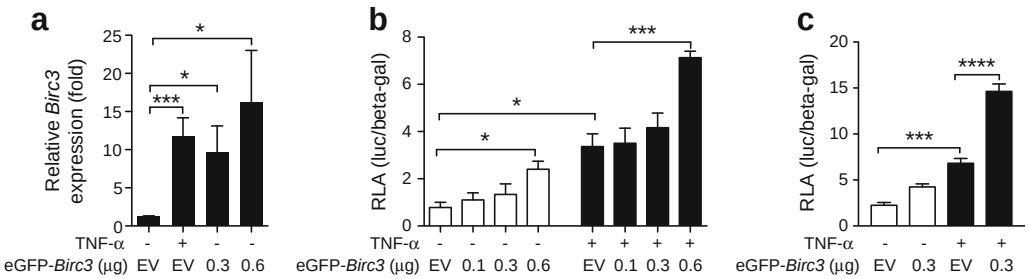
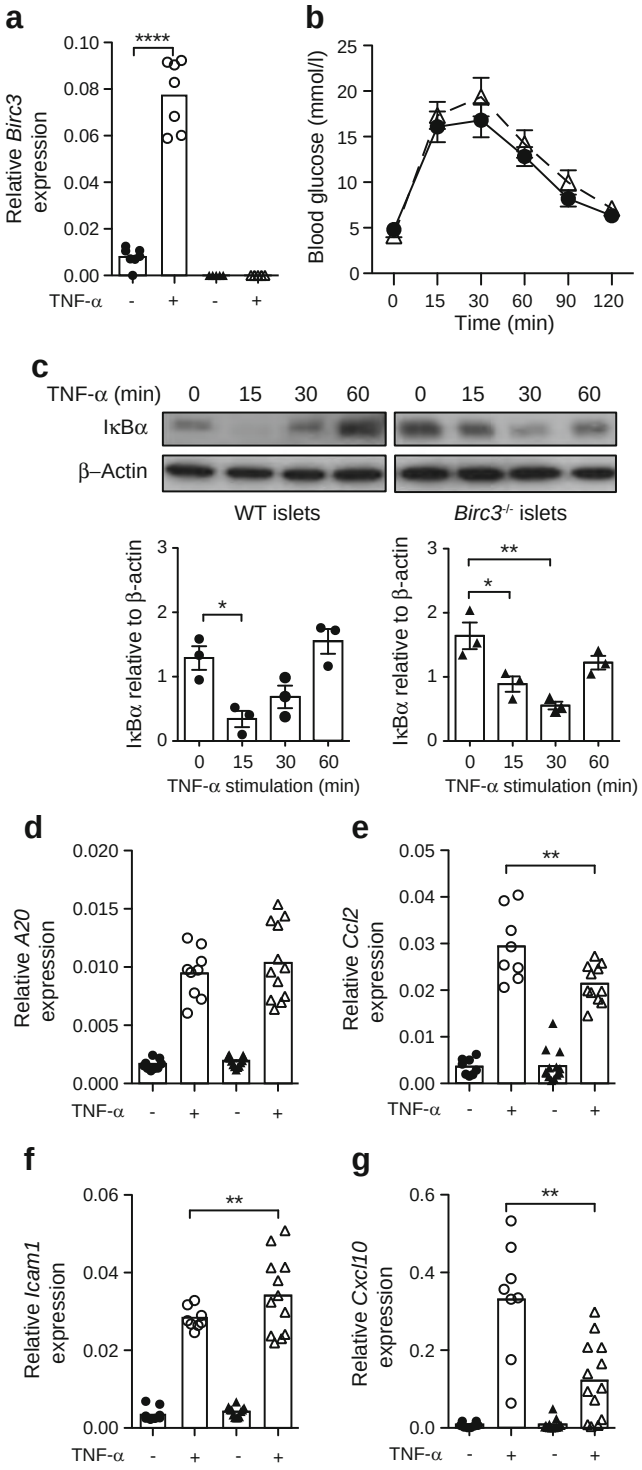


Fig. 3 Increased *Birc3* regulates NF-κB signalling. (**a**) Endogenous expression of *Birc3* in MIN6 cells transfected either with 0.6 μg control vector (EV) for 24 h, in the presence or absence of TNF-α for 4 h, or with 0.3 μg–0.6 μg eGFP-*Birc3*. Data represent mean ± SEM of gene expression relative to *Ppia* from six independent experiments. (**b**) Induction of the NF-κB reporter in MIN6 cells transfected with either 0.6 μg EV or 0.1 to 0.6 μg eGFP-*Birc3*, in the absence or presence of TNF-α for 8 h. Data represent mean ± SEM from three independent experiments. (**c**) Induction of *A20* reporter in β-TC_3 cells transfected with either 0.3 μg EV or 0.3 μg eGFP-*Birc3*, in the absence or presence of TNF-α for 8 h. Data represent mean ± SEM from four independent experiments. Statistical comparisons were made by single unpaired *t* test between two groups or by ANOVA with pairwise multiple comparisons made using an unpaired *t* test. *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$ and ****$p \leq 0.0001$



◀ **Fig. 4** A redundant role for *Birc3* in NF-κB signalling. (**a**) Expression of *Birc3* in wild type (WT) primary islets in the absence (black circles) or presence (white circles) of TNF-α for 4 h, and in *Birc3*$^{-/-}$ islets under the same conditions (absence, black triangles; presence, white triangles). Values from individual experiments are shown ($n \geq 5$ per group). Bars represent mean ± SEM of gene expression relative to *Ppia*. (**b**) Blood glucose concentrations during intraperitoneal glucose tolerance tests on 12-week-old male wild-type (black circles, solid line) and *Birc3*$^{-/-}$ (white triangles, dotted line) mice. Data represent mean ± SEM from four mice per group. (**c**) Western blot analysis of NF-κB activation measured by IκBα degradation in wild-type (WT) and *Birc3*$^{-/-}$ islets that were either left untreated or were stimulated with TNF-α at the indicated times. β-Actin was used as a loading control. Representative blots are shown. IκBα (39 kDa) was quantified by densitometry and expressed relative to β-actin. Data represent mean ± SEM from three independent experiments. (**d**) Expression of *A20*, (**e**) *Ccl2*, (**f**) *Icam1* and (**g**) *Cxcl10* in wild-type islets in the absence (black circles) or presence (white circles) of TNF-α for 4 h, and in *Birc3*$^{-/-}$ islets under the same conditions (absence, black triangles; presence, white triangles). Values from individual experiments are shown ($n \geq 11$ per group). Bars represent mean ± SEM of gene expression relative to *Ppia*. Statistical comparisons were made by single unpaired *t* test between two groups. *$p \leq 0.05$, **$p \leq 0.01$ and ****$p \leq 0.0001$

$p \leq 0.05$) (Fig. 2j). Moreover, overabundance of IκBα in MIN6 cells (Fig. 2k) resulted in a 77% suppression of TNF-α-induced *Birc3* expression ($p \leq 0.01$) (Fig. 2l). Thus, NF-κB is necessary and sufficient to drive de novo transcription of *Birc3* mRNA.

Increased Birc3 regulates NF-κB signalling Our results imply a model whereby TNF-α activates NF-κB, which subsequently induces increased *Birc3* expression. To elucidate the function of increased *Birc3* in pancreatic beta cells, MIN6 cells were transfected with the pIRES2-eGFP control vector or 0.3 to 0.6 μg pIRES2-eGFP-*Birc3* (eGFP-*Birc3*). eGFP-*Birc3*-transfected MIN6 cells expressed *Birc3* in a range similar to TNF-α-stimulated MIN6 cells (i.e. 12-fold, $p \leq 0.001$) (Fig. 3a). Subsequently, MIN6 cells were transfected with a luciferase reporter containing two tandem NF-κB responsive elements (NF-κB.Luc), plus either 0.1 to 0.6 μg eGFP-*Birc3*, or 0.6 μg control vector. In MIN6 cells, eGFP-*Birc3*-expressing cells showed a dose-dependent increase in NF-κB reporter activity (Fig. 3b), while a combination of TNF-α stimulation and 0.6 μg eGFP-*Birc3* showed a further increase of NF-κB reporter activity, from 3.4-fold ($p \leq 0.05$) with TNF-α alone to sevenfold with eGFP-*Birc3* ($p \leq 0.01$).

To elucidate how *Birc3* modulates a native NF-κB promoter, the effect of *Birc3* activity on the NF-κB-driven *A20* reporter [5] was examined. β-TC_3 cells were transfected with 2 μg *A20*.Luc and either 0.25 μg pIRES2-eGFP control vector or eGFP-*Birc3*. *A20* reporter activity increased from sevenfold ($p \leq 0.001$) for TNF-α alone to 9.5-fold for TNF-α and eGFP-*Birc3* (Fig. 3c). These data demonstrate that increased *Birc3* expression is sufficient to drive NF-κB activity and enhance TNF-α-induced NF-κB signalling.

A redundant role for Birc3 in NF-κB signalling The effect of *Birc3* loss-of-function on beta cell TNF-α-mediated NF-κB signalling was examined in *Birc3*$^{-/-}$ mice (Fig. 4a). *Birc3* is not necessary for beta cell function as indicated by the normal glucose tolerance of *Birc3*$^{-/-}$ mice (Fig. 4b). We analysed TNF-α-induced NF-κB signalling in wild-type or *Birc3*$^{-/-}$ islets via western blot analysis of IκBα levels [26]. Compared with wild-type islets, TNF-α-stimulated

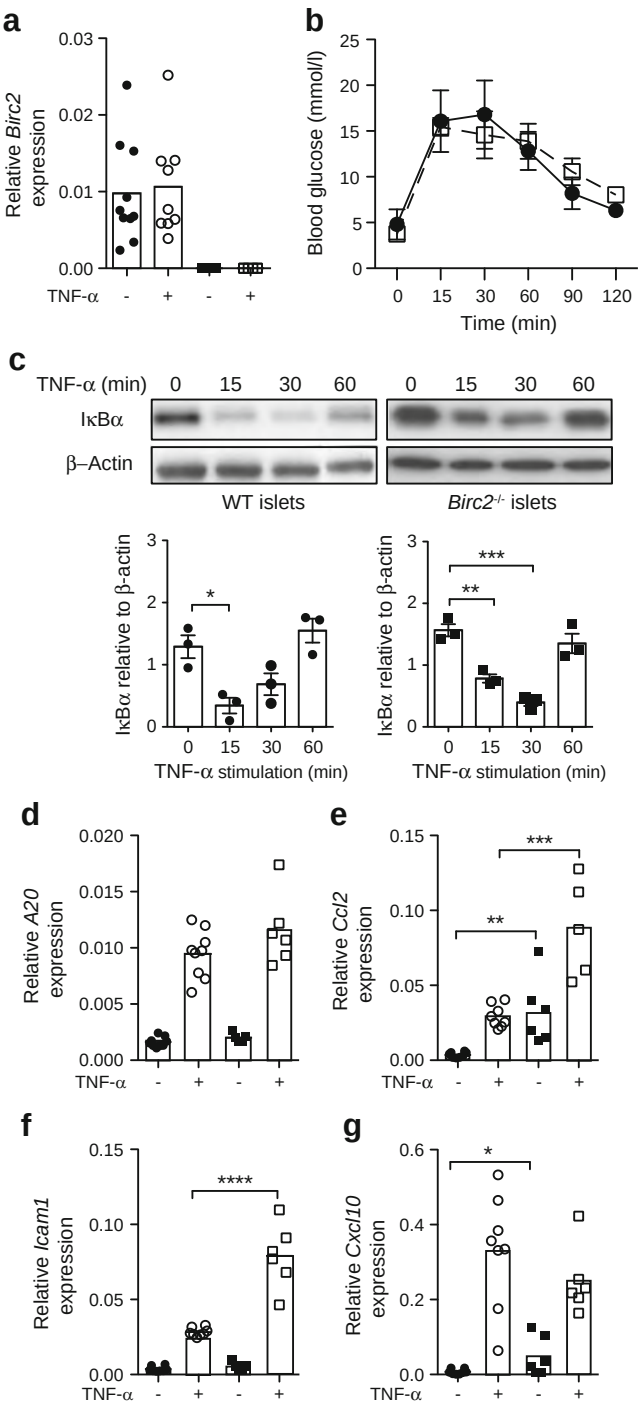


Fig. 5 *Birc2* provides negative control of TNF-pathways in beta cells. (**a**) Expression of *Birc2* in wild-type islets in the absence (black circles) or presence (white circles) of TNF-α for 4 h, and in *Birc2*$^{-/-}$ islets under the same conditions (absence, black squares; presence, white squares). Values from each experiment are shown ($n \geq 6$ per group). Bars represent mean ± SEM of gene expression relative to *Ppia*. (**b**) Blood glucose concentrations during intraperitoneal glucose tolerance tests on 12-week-old male wild-type (black circles, solid line) and *Birc2*$^{-/-}$ (white squares, dotted line) mice. Data represent mean ± SEM from four mice per group. (**c**) Western blot analysis of NF-κB activation measured by IκBα degradation in wild-type (WT) and *Birc2*$^{-/-}$ islets that were left untreated or were stimulated with TNF-α at the indicated times. β-Actin was used as a loading control. Representative blots are shown. IκBα (39 kDa) was quantified by densitometry and expressed relative to β-actin. Data represent mean ± SEM from three independent experiments. (**d**) Expression of *A20*, (**e**) *Ccl2*, (**f**) *Icam1* and (**g**) *Cxcl10* in wild-type islets in the absence (black circles) or presence (white circles) of TNF-α for 4 h, and in *Birc2*$^{-/-}$ islets under the same conditions (absence, black squares; presence, white squares). Values from individual experiments are shown ($n \geq 5$ per group). Bars represent mean ± SEM of gene expression relative to *Ppia*. Statistical comparisons were made by single unpaired *t* test between two groups. *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$ and ****$p \leq 0.0001$

Birc3$^{-/-}$ islets showed delayed IκBα degradation, but a normal return to baseline levels at 60 min (Fig. 4c). Analysis of NF-κB-regulated and TNF-α-induced genes in *Birc3*$^{-/-}$ islets revealed an abnormal expression pattern. While normal *A20* induction (Fig. 4d) was observed, expression of *Ccl2* ($p \leq 0.01$) (Fig. 4e) and *Cxcl10* ($p \leq 0.01$) (Fig. 4g) was reduced, with *Icam1* levels being modestly increased ($p \leq 0.01$) (Fig. 4f). These data indicate that BIRC3 is necessary to fine-tune cellular responses to TNF-α.

Birc2 provides negative control of TNF pathways in beta cells BIRC2 can compensate for BIRC3 [3, 27], therefore we examined NF-κB signalling in single *Birc2*$^{-/-}$ islets (Fig. 5a). *Birc2*$^{-/-}$ mice had normal glucose homeostasis (Fig. 5b). Regarding NF-κB signalling, *Birc2*$^{-/-}$ islets had delayed kinetics of TNF-α-stimulated IκBα degradation (Fig. 5c). Noticeably, in the absence of a pro-inflammatory stimulus, *Birc2*$^{-/-}$ islets expressed high levels of *Ccl2* (ninefold greater, $p \leq 0.01$) (Fig. 5e) and *Cxcl10* (sevenfold greater, $p \leq 0.05$) (Fig. 5g) compared with wild-type islets. Furthermore, while TNF-α-stimulated *Birc2*$^{-/-}$ islets expressed normal induced levels of *A20* (Fig. 5d), induction of *Ccl2* (threefold, $p \leq 0.001$) and *Icam1* (threefold, $p \leq 0.0001$) mRNA was significantly higher than for wild-type islets (Fig. 5e, f). These findings suggest that *Birc2* functions as a negative control factor, dampening expression of TNF target genes in islet cells.

Dysregulated gene expression in the absence of Birc2 and Birc3 As BIRC2 and BIRC3 can compensate for each other to allow NF-κB signalling [3, 27], we generated DKO mice. DKO islets exhibited loss of BIRC2 and BIRC3 (Fig. 6a, b), but normal glucose homeostasis, indicating that these two proteins are dispensable for physiological beta cell function (Fig. 6c). Analysis of TNF-α-induced IκBα degradation revealed that NF-κB signalling kinetics were significantly delayed in the absence of BIRC2 and BIRC3 (Fig. 6d). Moreover, DKO islets showed significant disturbances in the regulation of TNF target genes, expressing significantly higher stimulus-independent levels of *A20* (fourfold, $p \leq 0.0001$), *Ccl2* (eightfold, $p \leq 0.001$), *Icam1* (22-fold, $p \leq 0.0001$)

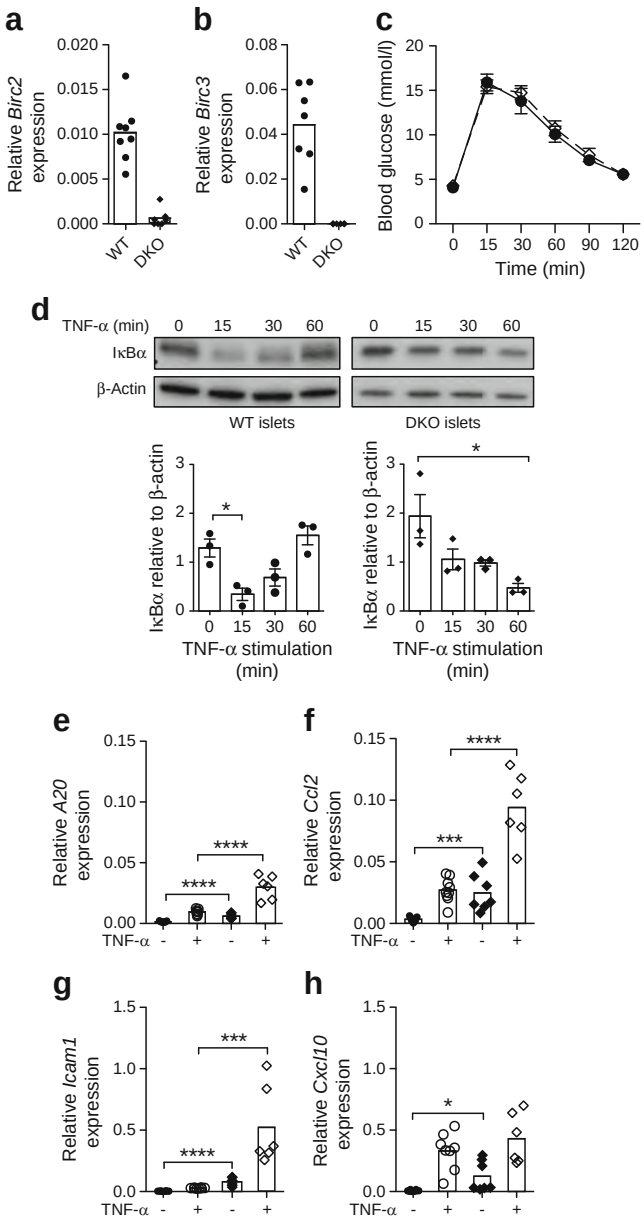


Fig. 6 Dysregulated gene expression in the absence of *Birc2* and *Birc3*. (**a**) Expression of *Birc2* and (**b**) *Birc3* in wild-type islets (black circles) and DKO islets (black diamonds). Values from individual experiment are shown ($n \geq 7$ per group). Bars represent mean ± SEM of gene expression relative to *Ppia*. (**c**) Blood glucose concentrations during intraperitoneal glucose tolerance tests on 12-week-old male wild-type (black circles, solid line) and DKO (white diamonds, dotted line) mice. Data represent mean ± SEM from at least seven mice per group. (**d**) Western blot analysis of NF-κB activation measured by IκBα degradation in wild-type (WT) and DKO islets that were left untreated or were stimulated with TNF-α at the indicated times. β-Actin was used as a loading control. Representative blots are shown. IκBα (39 kDa) was quantified by densitometry and expressed relative to β-actin. Data represent mean ± SEM from three independent experiments. (**e**) Expression of *A20*, (**f**) *Ccl2*, (**g**) *Icam1* and (**h**) *Cxcl10* in wild-type islets in the absence (black circles) or presence (white circles) of TNF-α for 4 h, and in DKO islets under the same conditions (absence, black diamonds; presence, white diamonds). Values from individual experiments are shown ($n \geq 6$ per group). Bars represent mean ± SEM of gene expression relative to *Ppia*. Statistical comparisons were made by single unpaired *t* test between two groups. *$p \leq 0.05$, ***$p \leq 0.001$ and ****$p \leq 0.0001$

and *Cxcl10* (17-fold, $p \leq 0.05$) compared with resting wild-type islets (Fig. 6e–h). Analysis of TNF-α-induced responses showed hyperinduction of *A20* (threefold, $p \leq 0.0001$), *Ccl2* (fourfold, $p \leq 0.0001$) and *Icam1* (18-fold, $p \leq 0.001$), but not of *Cxcl10* mRNA compared with wild-type islets (Fig. 6e–h). Therefore, *Birc2*$^{-/-}$- and *Birc3*$^{-/-}$-deficient beta cells show aberrant NF-kB signalling and dysregulated control of TNF target genes.

Birc2 and Birc3 fine-tune inflammatory gene expression in islet cells We examined the relative contribution of BIRC2 and BIRC3 to the phenotypes observed for DKO islets. We found that in the absence of a TNF-stimulus, *A20* ($p \leq 0.0001$) (Fig. 7a) and *Icam1* ($p \leq 0.0001$) (Fig. 7c) expression was increased in DKO islets, but not in *Birc2*$^{-/-}$ or *Birc3*$^{-/-}$ islets, indicating that neither BIRC2 nor BIRC3 alone can exert molecular control over *A20* and *Icam1* expression. In contrast, molecular control of *Ccl2* expression was strictly controlled by BIRC2, as the phenotype of *Birc2*$^{-/-}$ islets was identical to DKO islets ($p \leq 0.001$ and $p \leq 0.05$) (Fig. 7b). Like *Ccl2*, control of *Cxcl10* expression appears to be more strongly dependent on the presence of BIRC2 ($p \leq 0.05$ and $p \leq 0.05$) (Fig. 7d).

BIRC proteins regulate gene transcription through JNK When DKO islets were treated with actinomycin D, the stimulus-independent expression of *A20* (13-fold, $p \leq 0.0001$) and *Cxcl10* (tenfold, $p \leq 0.01$), but not of *Ccl2* or *Icam1*, was suppressed (Fig. 7e–h), indicating that basal *A20* and *Cxcl10* mRNA levels are regulated by active transcription in DKO islets.

We hypothesised that the increased stimulus-independent transcription rates of *A20* and *Cxcl10* in DKO islets might be due to loss of control of the normal regulatory paths that control TNF-α-induced gene expression. We first sought to identify the possible pathways involved in normal TNF-α-induced gene expression. To do this, MIN6 cells were left untreated, or were pretreated with PDTC, SP600125 or SB203580 to inhibit NF-κB, JNK or p38 respectively, prior to TNF-α stimulation for 4 h. Our findings show that, in MIN6 cells, TNF-α-stimulated *A20* was regulated by NF-κB, but not by the JNK or p38 pathways (Fig. 8a), whereas TNF-α-induced *Ccl2*, *Icam1* and *Cxcl10* expression was regulated by the NF-κB and the JNK pathways (Fig. 8b–d).

We next tested the idea that the increased stimulus-independent gene expression in DKO islets might be

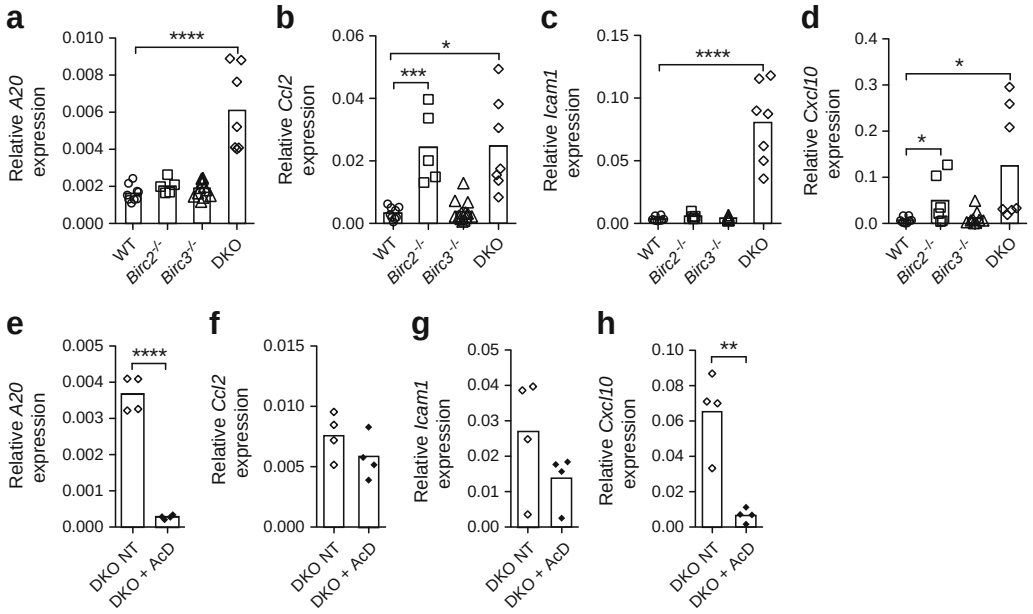


Fig. 7 *Birc2* and *Birc3* fine-tune inflammatory gene expression in islet cells. (**a**) Basal levels of *A20*, (**b**) *Ccl2*, (**c**) *Icam1* and (**d**) *Cxcl10* mRNA from wild-type (WT) (white circles), *Birc2*$^{-/-}$ (white squares), *Birc3*$^{-/-}$ (white triangles) or DKO (white diamonds) islets. Data sourced from Figs. 4, 5 and 6. Values from individual experiments are shown ($n \geq 5$ per group). (**e**) Basal levels of *A20*, (**f**) *Ccl2*, (**g**) *Icam1* and (**h**) *Cxcl10* from DKO islets either left untreated (NT) (white diamonds) or treated for 8 h with actinomycin D (AcD) (black diamonds). Values from individual experiments are shown ($n=4$ per group). Statistical comparisons were made by single unpaired *t* test between two groups. Bars represent mean ± SEM of gene expression relative to *Ppia*. *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$ and ****$p \leq 0.0001$

due to hyperactivation of those same pathways involved in the regulation of normal TNF-α-induced gene expression. To test this, DKO islets were treated with inhibitors and gene expression was analysed by quantitative RT-PCR. Notably, the stimulus-independent expression of *Ccl2*, *Icam1* and *Cxcl10* was sensitive to SP600125, but not to SB203580 or PDTC treatment (Fig. 8e–h). Moreover, examination of JNK activation by analysis of c-Jun phosphorylation showed hyperphosphorylation of c-Jun in DKO islets compared with wild-type islets (Fig. 8i). These findings indicate that loss of *Birc2* and *Birc3* dysregulates fine control over the JNK pathway, such that beta cells are hypersensitive to the triggering of JNK signalling, resulting in the loss of fine control over gene transcription.

Proteosome inhibition mimics BIRC2 and BIRC3 deficiency BIRC proteins function as ubiquitin modifying enzymes that target proteins for proteosomal degradation [12]. We tested the idea that, in the absence of BIRC proteins, the accumulation of signalling components normally targeted for degradation, perhaps regulatory kinases, could trigger activation of signalling pathways without appropriate external inputs. To do this, we treated MIN6 cells with the proteosome inhibitor, MG-132, to examine whether loss of proteosome activity could replicate the DKO islet phenotype. Compared with control MIN6 cells, MG-132-treated cells exhibited increased expression of *Ccl2* (50%, $p \leq 0.05$), *Icam1* (50%, $p \leq 0.001$) and *Cxcl10* (80%, $p \leq 0.05$), but not of *A20* (Fig. 9a–d). Moreover, based on analysis of c-Jun phosphorylation, MG-132-treated MIN6 cells also showed increased JNK pathway activation (Fig. 9e). Thus blockade of the proteosome resulted in dysregulated JNK pathway activation in a stimulus-independent manner, with hyperexpression of *Ccl2*, *Icam1* and *Cxcl10* inflammatory genes.

DKO islets have impaired function after transplantation JNK is acutely activated in islet cells during the process of islet isolation [28] and antagonisation of JNK signalling is beneficial for islet survival after isolation [29]. Therefore, we predicted that DKO islets would fair worse in vivo following transplantation. To test this idea, wild-type or DKO islets were transplanted into diabetic allogeneic recipients. Analysis of blood glucose levels showed that wild-type and DKO islets provided good metabolic control immediately after transplantation (Fig. 10). As early graft function can be affected by loss of islet mass due to cell death [30, 31], this suggests that DKO islets were not hypersensitive to death stimuli. However, by post-operative day 6, the function of DKO islets began to deteriorate, a process that worsened with time. In contrast, wild-type islets showed good metabolic control until at least post-operative day 18. The earlier

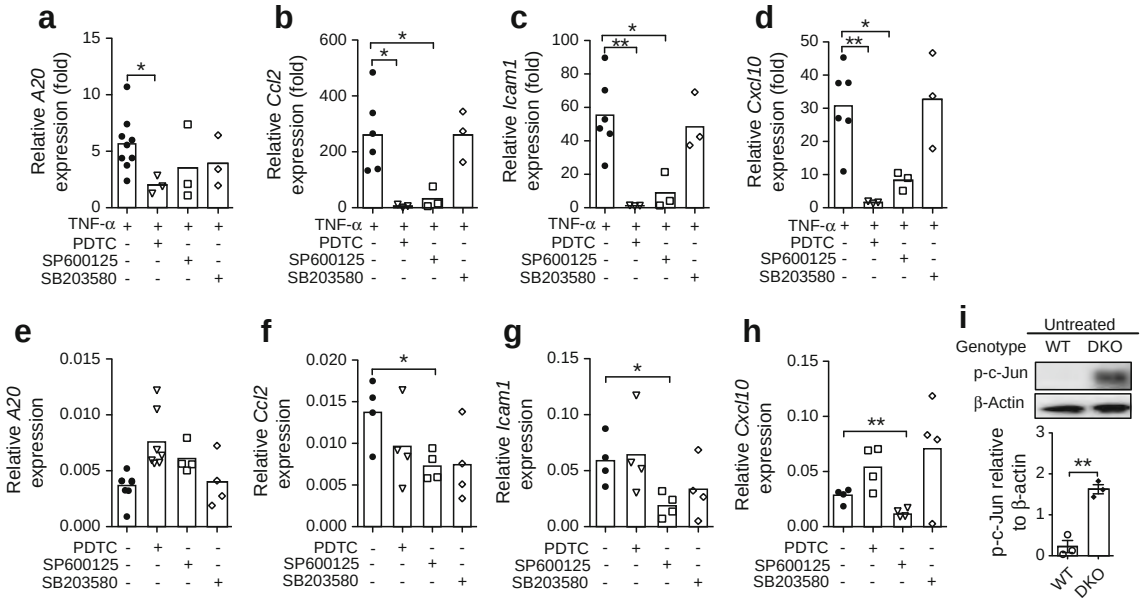


Fig. 8 *Birc2* and *Birc3* fine-tune TNF signalling in islet cells. (**a**) Expression of *A20*, (**b**) *Ccl2*, (**c**) *Icam1* and (**d**) *Cxcl10* in MIN6 cells left untreated (black circles) or pretreated for 1 h with PDTC (white inverted triangles), SP600125 (white squares) or SB203580 (white diamonds) prior to TNF-α stimulation for 4 h. Values from individual experiments are shown ($n \geq 3$ per group). (**e**) Expression of *A20*, (**f**) *Ccl2*, (**g**) *Icam1* and (**h**) *Cxcl10* in DKO islets left untreated (black circles) or treated for 8 h with PDTC (white inverted triangles), SP600125 (white squares) or SB203580 (white diamonds). Values from individual experiments are shown ($n=4$ per group). **a**–**h** Bars represent mean ± SEM of gene expression relative to *Ppia*. (**i**) Western blot analysis of JNK activation by c-Jun phosphorylation in resting wild-type (WT) and DKO islets. β-Actin was used as a loading control. Representative blots are shown. Phosphorylated c-Jun (48 kDa) was quantified by densitometry and expressed relative to β-actin. Data represent mean ± SEM from three independent experiments. Statistical comparisons were made by single unpaired *t* test between two groups. *$p \leq 0.05$ and **$p \leq 0.01$

loss of DKO islet function could reflect loss of control of inflammatory signalling.

Discussion

Our studies present novel insights into the molecular control of TNF signalling in primary pancreatic beta cells and the role of BIRC proteins in this process. Surprisingly, both single *Birc2*$^{-/-}$ and DKO islets, as well as MIN6 cells treated with proteosome inhibitors, exhibited dysregulated expression of select TNF target genes. The augmented stimulus-independent expression of *Ccl2*, *Cxcl10* and *Icam1* mRNA observed for DKO islets was substantially reduced by antagonising JNK signalling. Also, DKO islets showed stimulus-independent hyperphosphorylation of c-Jun. We were also able to demonstrate a role for the JNK pathway in the normal de novo regulation of these same genes in

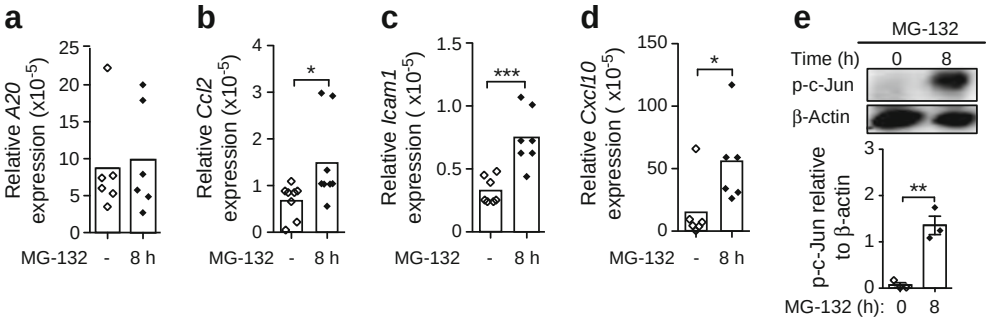


Fig. 9 Proteosome inhibition mimics BIRC2 and BIRC3 deficiency. Expression of *A20* (**a**), *Ccl2* (**b**), *Icam1* (**c**) and *Cxcl10* (**d**) in MIN6 cells left untreated (white diamonds) or treated for 8 h with MG-132 (black diamonds). Values from individual experiments are shown ($n \geq 5$ per group). Bars represent mean ± SEM of gene expression relative to *Ppia*. (**e**) Western blot analysis of JNK activation by c-Jun phosphorylation in MIN6 cells with or without MG-132 treatment for 8 h. β-Actin was used as a loading control. Representative blots are shown. Phosphorylated c-Jun (48 kDa) was quantified by densitometry and expressed relative to β-actin. Data represent mean ± SEM from three independent experiments. Statistical comparisons were made by single unpaired *t* test between two groups. *$p \leq 0.05$, **$p \leq 0.01$ and ***$p \leq 0.001$

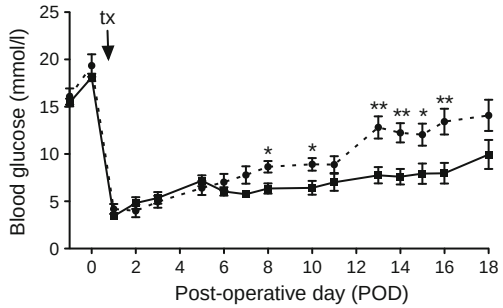


Fig. 10 DKO islets have impaired function after transplantation. Blood glucose values over time of diabetic CBA mice receiving allogeneic wild-type (solid line) or DKO islets (dotted line); tx, transplant day. Values are mean ± SEM ($n \geq 7$ per group). Statistical comparisons were made by single unpaired *t* test between two groups. *$p \leq 0.05$ and **$p \leq 0.01$

islets and beta cells. These data could indicate that BIRC2 and BIRC3 negatively regulate the JNK pathway and subsequent JNK/activator protein-1-regulated gene expression in islet cells.

Interestingly, for B lymphocytes, loss of BIRC2 also leads to prolonged TNF-mediated JNK signalling [32]. BIRC2 and BIRC3 are recruited to the TNFR1 complex via interaction with TRAF2 [18, 33]. TRAF2 is necessary for TNF-α-mediated JNK signalling, but is redundant in the NF-κB or p38 pathways [4, 34, 35]. BIRC2 modulates the duration of TNF-α-induced JNK signalling by targeting TRAF2 and the mitogen-activated protein kinase kinase kinase protein, apoptosis signal-regulating kinase 1 (ASK1), for proteosomal degradation [32, 36]. Our data indicate that this pathway may operate in beta cells. Indeed, treatment of MIN6 cells with the proteosome inhibitor, MG-132, phenocopied DKO islets, lending support to this concept. Hence, one possibility is that, in the absence of BIRC2 and/or BIRC3, TRAF2 and ASK1 accumulate, subsequently triggering hyperactivation of c-Jun, with dysregulated expression of downstream target genes. Hyperactivation of c-Jun might also affect the duration of signalling, contributing to the enhanced gene expression observed for DKO islets following TNF-ligation.

The stimulus-independent expression of *Cxcl10* and *A20* observed in DKO islets showed different sensitivities to JNK inhibition; hyperexpression of *A20* was not sensitive to JNK inhibition. In islets, *A20* is an NF-κB-regulated gene [5] that forms a feedback loop to modulate NF-κB activation [20, 37] and control inflammation [38]. Some data show that BIRC antagonists promote degradation of BIRC proteins, which can subsequently trigger stimulus-independent NF-κB signalling. This may be mediated by increased availability of RIPK1, allowing RIPK1–TNFR1 interactions to trigger NF-κB activation [39], subsequently driving expression of NF-κB target genes [40]. Another possibility is regulation of the non-canonical NF-κB component, v-rel reticuloendotheliosis viral oncogene homolog B (RelB). Indeed, $Relb^{-/-}$ fibroblasts exhibit increased NF-κB activity with stimulus-independent expression of NF-κB target genes [41]. Moreover, the non-canonical pathway is regulated at the level of NF-κB-inducing kinase (NIK) [42]. $Birc2^{-/-}$ mouse embryonic fibroblasts cells (MEFs) and $Birc2^{-/-}Birc3^{-/-}$ cancer cells accumulate high NIK levels, and NIK in turn phosphorylates IκB kinase (IKK)α, inducing activation of the non-canonical NF-κB pathway [39, 40]. Hence BIRC2 and BIRC3 provide negative regulatory control over non-canonical NF-κB signalling.

With regard to NF-κB, DKO islets, interestingly, exhibit altered kinetics of IκBα degradation, which presumably reflects a necessary role for BIRC proteins in controlling the speed of NF-κB signalling in beta cells. NF-κB activation is sensitive to proteosome inhibition [43, 44], and BIRC proteins, via their Really Interesting New Gene (RING) domains, can target substrates for proteosomal degradation through ubiquitin editing [3]. One BIRC substrate is RIPK1, which recruits the transforming growth factor (TGF)-beta activated kinase 1 (TAK1)–TGF-beta activated kinase 1/mitogen-activated protein kinase kinase kinase 7 (MAP3K7) binding protein 2 (TAB2)–TAB3 and IKKα–IKKβ–NF-κB essential modulator (NEMO) complexes, triggering TAK1-dependent phosphorylation of IKKβ [45]. The activated NF-κB signalosome [46] subsequently phosphorylates IκBα, initiating the necessary steps for NF-κB translocation [47]. Thus, by controlling the availability of RIPK1 through ubiquitin editing [39], BIRC proteins have the capacity to fine-tune the speed of NF-κB signalling.

Our data provide new insights into the mechanisms by which BIRC proteins control TNF signalling. TNF-α stimulation induces NF-κB activation, which drives early immediate expression of *Birc3* in beta cells. The increased levels of BIRC3 may be required to fine-tune TNF-induced inflammatory signalling for full expression of TNF-α-induced genes like *Ccl2* and *Cxcl10*, while suppressing others, including *Icam1*. Thus, increased BIRC3, in combination with BIRC2, acts simultaneously as a positive factor for NF-κB signalling, but also as a molecular brake that provides modulatory control over the JNK signalling axis. BIRC proteins prevent activation of this pathway in the absence of an overt extracellular signal normally provided by TNF. Thus under inflammatory conditions, pancreatic islets rapidly induce *Birc3* to fine-tune NF-κB and JNK signalling pathways to ensure beta cell transcriptional responses are appropriately matched to extracellular inputs. Maintaining a balanced response may be critical for beta cell function under conditions of cellular stress. Thus, for example, beta cell failure in the context of type 2 diabetes is associated with exacerbated *CXCL10* expression [48], while activation of the JNK pathway impairs islet survival and function [49, 50]. Moreover, as shown here in the context of islet transplantation, the absence of BIRC proteins impairs islet grafts, resulting in a more rapid loss of glucose homeostasis.

Acknowledgements The authors thank J. Cantley and R. Laybutt, (Diabetes and Obesity Research Program, Garvan Institute, Darlinghurst, NSW, Australia) for their insightful critique and helpful discussion. We also acknowledge the technical expertise of staff from the Australian Bioresources and Biological Testing Facility, Garvan Institute, Australia. We thank D. Vaux and H. Carter (Cell Signalling and Cell Death Division, Walter and Eliza Hall, Parkville, VIC, Australia) for providing the *Birc2*$^{-/-}$ and *Birc3*$^{-/-}$ mice.

Funding This project was supported by National Health and Medical Research (NHMRC) grants 427695 to S.T. Grey. B.M. Tan was supported by an Australian Postgraduate Award. S.T. Grey is an Australian Research Council Future Fellow and Honorary NHMRC Senior Research Fellow.

Duality of interest The authors declare that there is no duality of interest associated with this manuscript.

Contribution statement BMT, NWZ, AOY, RS, SNW, EM and STG made substantial contributions to the study conception and design, the acquisition of data, and to the analysis and interpretation of data. They were all involved in drafting the article and or revising it critically for important intellectual content. All authors made final approval of the version to be published. STG directed the research and is the guarantor of the study.

References

1. Varfolomeev EE, Ashkenazi A (2004) Tumor necrosis factor: an apoptosis JuNKie? Cell 116:491–497
2. Micheau O, Tschopp J (2003) Induction of TNF receptor I-mediated apoptosis via two sequential signaling complexes. Cell 114:181–190
3. Mahoney DJ, Cheung HH, Mrad RL et al (2008) Both cIAP1 and cIAP2 regulate TNFalpha-mediated NF-kappaB activation. Proc Natl Acad Sci U S A 105:11778–11783
4. Habelhah H, Takahashi S, Cho SG, Kadoya T, Watanabe T, Ronai Z (2004) Ubiquitination and translocation of TRAF2 is required for activation of JNK but not of p38 or NF-kappaB. EMBO J 23:322–332
5. Liuwantara D, Elliot M, Smith MW et al (2006) Nuclear factor-kappaB regulates beta-cell death: a critical role for A20 in beta-cell protection. Diabetes 55:2491–2501
6. Cottet S, Dupraz P, Hamburger F, Dolci W, Jaquet M, Thorens B (2002) cFLIP protein prevents tumor necrosis factor-alpha-mediated induction of caspase-8-dependent apoptosis in insulin-secreting betaTc-Tet cells. Diabetes 51:1805–1814
7. Pakala SV, Chivetta M, Kelly CB, Katz JD (1999) In autoimmune diabetes the transition from benign to pernicious insulitis requires an islet cell response to tumor necrosis factor alpha. Exp Med 189:1053–1062
8. Dahlen E, Dawe K, Ohlsson L, Hedlund G (1998) Dendritic cells and macrophages are the first and major producers of TNF-alpha in pancreatic islets in the nonobese diabetic mouse. J Immunol 160:3585–3593
9. Ortis F, Pirot P, Naamane N et al (2008) Induction of nuclear factor-kappaB and its downstream genes by TNF-alpha and IL-1beta has a pro-apoptotic role in pancreatic beta cells. Diabetologia 51:1213–1225
10. Zhang S, Kim KH (1995) TNF-alpha inhibits glucose-induced insulin secretion in a pancreatic beta-cell line (INS-1). FEBS Lett 377:237–239
11. Yang XD, Tisch R, Singer SM et al (1994) Effect of tumor necrosis factor alpha on insulin-dependent diabetes mellitus in NOD mice. I. The early development of autoimmunity and the diabetogenic process. J Exp Med 180:995–1004
12. Gyrd-Hansen M, Meier P (2010) IAPs: from caspase inhibitors to modulators of NF-kappaB, inflammation and cancer. Nat Rev Cancer 10:561–574
13. Emamaullee JA, Rajotte RV, Liston P et al (2005) XIAP overexpression in human islets prevents early posttransplant apoptosis and reduces the islet mass needed to treat diabetes. Diabetes 54:2541–2548
14. Plesner A, Liston P, Tan R, Korneluk RG, Verchere CB (2005) The X-linked inhibitor of apoptosis protein enhances survival of murine islet allografts. Diabetes 54:2533–2540
15. Ou D, Wang X, Metzger DL et al (2005) Synergistic inhibition of tumor necrosis factor-related apoptosis-inducing ligand-induced apoptosis in human pancreatic beta cells by Bcl-2 and X-linked inhibitor of apoptosis. Hum Immunol 66:274–284
16. Plesner A, Soukhatcheva G, Korneluk RG, Verchere CB (2010) XIAP inhibition of beta-cell apoptosis reduces the number of islets required to restore euglycemia in a syngeneic islet transplantation model. Islets 2:18–23
17. Shiozaki EN, Chai J, Rigotti DJ et al (2003) Mechanism of XIAP-mediated inhibition of caspase-9. Molecular Cell 11:519–527
18. Rothe M, Pan MG, Henzel WJ, Ayres TM, Goeddel DV (1995) The TNFR2-TRAF signaling complex contains two novel proteins related to baculoviral inhibitor of apoptosis proteins. Cell 83:1243–1252
19. Sarkar SA, Kutlu B, Velmurugan K et al (2009) Cytokine-mediated induction of anti-apoptotic genes that are linked to nuclear factor kappa-B (NF-kappaB) signalling in human islets and in a mouse beta cell line. Diabetologia 52:1092–1101
20. Grey ST, Arvelo MB, Hasenkamp W, Bach FH, Ferran C (1999) A20 inhibits cytokine-induced apoptosis and nuclear factor kappaB-dependent gene activation in islets. J Exp Med 190:1135–1146
21. Miyazaki J, Araki K, Yamato E et al (1990) Establishment of a pancreatic beta cell line that retains glucose-inducible insulin secretion: special reference to expression of glucose transporter isoforms. Endocrinology 127:126–132
22. D'Ambra R, Surana M, Efrat S, Starr RG, Fleischer N (1990) Regulation of insulin secretion from beta-cell lines derived from transgenic mice insulinomas resembles that of normal beta-cells. Endocrinology 126:2815–2822
23. Farre D, Roset R, Huerta M et al (2003) Identification of patterns in biological sequences at the ALGGEN server: PROMO and MALGEN. Nucleic Acids Res 31:3651–3653
24. Wrighton CJ, Hofer-Warbinek R, Moll T, Eytner R, Bach FH, de Martin R (1996) Inhibition of endothelial cell activation by adenovirus-mediated expression of I kappa B alpha, an inhibitor of the transcription factor NF-kappa B. J Exp Med 183:1013–1022
25. Walters S, Webster KE, Sutherland A et al (2009) Increased CD4+Foxp3+ T cells in BAFF-transgenic mice suppress T cell effector responses. J Immunol 182:793–801
26. Beg AA, Finco TS, Nantermet PV, Baldwin AS Jr (1993) Tumor necrosis factor and interleukin-1 lead to phosphorylation and loss of I kappa B alpha: a mechanism for NF-kappa B activation. Mol Cell Biol 13:3301–3310
27. Varfolomeev E, Goncharov T, Fedorova AV et al (2008) c-IAP1 and c-IAP2 are critical mediators of tumor necrosis factor alpha (TNFalpha)-induced NF-kappaB activation. J Biol Chem 283:24295–24299
28. Abdelli S, Abderrahmani A, Hering BJ, Beckmann JS, Bonny C (2007) The c-Jun N-terminal kinase JNK participates in cytokine- and isolation stress-induced rat pancreatic islet apoptosis. Diabetologia 50:1660–1669

29. Noguchi H, Nakai Y, Matsumoto S et al (2005) Cell permeable peptide of JNK inhibitor prevents islet apoptosis immediately after isolation and improves islet graft function. Am J Transplant 5:1848–1855
30. Biarnes M, Montolio M, Nacher V, Raurell M, Soler J, Montanya E (2002) Beta-cell death and mass in syngeneically transplanted islets exposed to short- and long-term hyperglycemia. Diabetes 51:66–72
31. Grey ST, Longo C, Shukri T et al (2003) Genetic engineering of a suboptimal islet graft with A20 preserves beta cell mass and function. J Immunol 170:6250–6256
32. Zhao Y, Conze DB, Hanover JA, Ashwell JD (2007) Tumor necrosis factor receptor 2 signaling induces selective c-IAP1-dependent ASK1 ubiquitination and terminates mitogen-activated protein kinase signaling. J Biol Chem 282:7777–7782
33. Shu HB, Takeuchi M, Goeddel DV (1996) The tumor necrosis factor receptor 2 signal transducers TRAF2 and c-IAP1 are components of the tumor necrosis factor receptor 1 signaling complex. Proc Natl Acad Sci U S A 93:13973–13978
34. Yeh WC, Shahinian A, Speiser D et al (1997) Early lethality, functional NF-kappaB activation, and increased sensitivity to TNF-induced cell death in TRAF2-deficient mice. Immunity 7:715–725
35. Lee SY, Reichlin A, Santana A, Sokol KA, Nussenzweig MC, Choi Y (1997) TRAF2 is essential for JNK but not NF-kappa B activation and regulates lymphocyte proliferation and survival. Immunity 7:703–713
36. Samuel T, Welsh K, Lober T, Togo SH, Zapata JM, Reed JC (2006) Distinct BIR domains of cIAP1 mediate binding to and ubiquitination of tumor necrosis factor receptor-associated factor 2 and second mitochondrial activator of caspases. J Biol Chem 281:1080–1090
37. Wertz IE, O'Rourke KM, Zhou H et al (2004) De-ubiquitination and ubiquitin ligase domains of A20 downregulate NF-kappaB signalling. Nature 430:694–699
38. Lee EG, Boone DL, Chai S et al (2000) Failure to regulate TNF-induced NF-kappaB and cell death responses in A20-deficient mice. Science 289:2350–2354
39. Vince JE, Wong WW, Khan N et al (2007) IAP antagonists target cIAP1 to induce TNFalpha-dependent apoptosis. Cell 131:682–693
40. Varfolomeev E, Blankenship JW, Wayson SM et al (2007) IAP antagonists induce autoubiquitination of c-IAPs, NF-kappaB activation, and TNFalpha-dependent apoptosis. Cell 131:669–681
41. Xia Y, Pauza ME, Feng L, Lo D (1997) RelB regulation of chemokine expression modulates local inflammation. Am J Pathol 151:375–387
42. Zarnegar BJ, Wang YY, Mahoney DJ et al (2008) Noncanonical NF-kappa B activation requires coordinated assembly of a regulatory complex of the adaptors cIAP1, cIAP2, TRAF2 and TRAF3 and the kinase NIK. Nat Immunol 9:1371–1378
43. Mathes E, O'Dea EL, Hoffmann A, Ghosh G (2008) NF-kappaB dictates the degradation pathway of IkappaBalpha. EMBO J 27:1357–1367
44. Chan JY, Biden TJ, Laybutt DR (2012) Cross-talk between the unfolded protein response and nuclear factor-kappaB signalling pathways regulates cytokine-mediated beta cell death in MIN6 cells and isolated mouse islets. Diabetologia 55:2999–3009
45. Bertrand MJM, Milutinovic S, Dickson KM et al (2008) cIAP1 and cIAP2 facilitate cancer cell survival by functioning as E3 ligases that promote RIP1 ubiquitination. Molecular Cell 30:689–700
46. Kelliher MA, Grimm S, Ishida Y, Kuo F, Stanger BZ, Leder P (1998) The death domain kinase RIP mediates the TNF-induced NF-kappaB signal. Immunity 8:297–303
47. Henkel T, Machleidt T, Alkalay I, Kronke M, Ben-Neriah Y, Baeuerle PA (1993) Rapid proteolysis of I kappa B-alpha is necessary for activation of transcription factor NF-kappa B. Nature 365:182–185
48. Schulthess FT, Paroni F, Sauter NS et al (2009) CXCL10 impairs beta cell function and viability in diabetes through TLR4 signaling. Cell Metab 9:125–139
49. Fornoni A, Pileggi A, Molano RD et al (2008) Inhibition of c-jun N terminal kinase (JNK) improves functional beta cell mass in human islets and leads to AKT and glycogen synthase kinase-3 (GSK-3) phosphorylation. Diabetologia 51:298–308
50. Varona-Santos JL, Pileggi A, Molano RD et al (2008) c-Jun N-terminal kinase 1 is deleterious to the function and survival of murine pancreatic islets. Diabetologia 51:2271–2280