ROLES OF RAF-1 KINASE IN PANCREATIC BETA-CELLS

by

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B.Sc. University of Washington, Seattle, 2002

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF

THE REQUIREMENTS FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

in

THE FACULTY OF GRADUATE STUDIES

(PHYSIOLOGY)

THE UNIVERSITY OF BRITISH COLUMBIA

(Vancouver)

August 2010

Abstract

A decrease in functional β-cell mass is key in the pathogenesis of both type 1 and type 2 diabetes and in the failure of transplanted islet grafts. Knowledge of the endogenous regulators of β-cell proliferation and survival are important for understanding the physiological regulation of β-cell mass. We have shown that physiological concentrations of the insulin hormone act directly on β-cells to promote proliferation and survival, but its mechanisms remain unclear. We hypothesized that Raf-1, a kinase upstream of both ERK1/2 and Bad, is a critical target of insulin in β-cells. To test this hypothesis, we treated primary β-cells and MIN6 β-cells with multiple insulin concentrations and examined putative downstream targets. Low concentrations of insulin rapidly activated Raf-1 and ERK1/2 in primary islets and MIN6 cells. The phosphorylation of ERK1/2 by insulin was eliminated by exposure to a Raf inhibitor or transfection with a dominant-negative Raf-1 mutant. Insulin enhanced the interaction between mitochondrial Raf-1 and Bad, promoting the inactivation of pro-apoptotic Bad. Over-expression of Raf-1 was sufficient to increase proliferation in the absence of insulin, whereas a dominant-negative Raf-1 reduced proliferation in the presence of insulin. We also tested if Raf-1 signalling plays an important role in β-cell survival both *in vitro* and *in vivo*. We utilized a Raf inhibitor and dominant-negative Raf-1 mutants to block basal Raf-1 signalling in serum free conditions *in vitro* and the Cre-lox recombination system to obtain a β-cell specific deletion of the Raf-1 gene *in vivo.* Our data show that blocking basal Raf-1 signalling *in vitro* caused apoptosis. Preliminary data indicate that β-cell specific Raf-1 knockout mice are viable, have increased fasting basal blood glucose levels and have impaired glucose tolerance compared to littermate controls, consistent with the concept that Raf-1 plays an important role in β-cell survival. Together, these findings have significant implications for the understanding of insulin signalling pathway in β-cells and the regulation of β-cell mass.

Preface

I performed and analyzed all studies reported in this thesis. I was principally involved in all aspects of the research from design, data analysis, manuscript preparation, and submission for publication of all the data discussed in this thesis. The data and concepts presented here were part of the following published articles:

1. **EU. Alejandro,** TB. Kalynyak, F. Taghizadeh, KS. Gwiazda, EK. Rawstron, KJ. Jacob and JD. Johnson. Acute Insulin Signalling in Pancreatic Beta-Cells Is Mediated by Multiple Raf-1 Dependent Pathways. *Endocrinology*, *2010* Feb 151(2): 502-12. Data shown in chapter 3.

2. JD. Johnson and **EU. Alejandro**. Control of Pancreatic Beta-cell Fate by Insulin Signaling: The Sweet Spot Hypothesis. *Cell Cycle,* 2008 May 15;7 (10):1343-7.

3. **EU. Alejandro** and JD. Johnson. Raf-1 kinase in the pancreatic beta-cell. *Cellscience*, October 16, 2008.

4. **EU. Alejandro** and JD. Johnson. Inhibition of Raf-1 alters multiple downstream pathways to induce pancreatic β-cell apoptosis. *J Biol Chem.* 2008 Jan 25;283(4):2407-17. Data shown in chapter 3 and 4.

5. J. Beith, **EU. Alejandro**, and JD. Johnson. Insulin Stimulates Primary β-cell Proliferation via Raf-1 Kinase. *Endocrinology*, 2008 May;149(5):2251-60. Data shown in chapter 3.

6. JD. Johnson, E. Bernal-Mizrachi, **EU. Alejandro**, Z. Ha, TB. Kalynyak, H. Li, JL.

Beith, J. Gross, GL. Warnock, RR. Townsend, MA. Permutt, and KS. Polonsky. Insulin protects islets from apoptosis via Pdx1 and specific changes in the human islet proteome. *Proc Natl Acad Sci*. 2006 Dec 19;103(51):19575-80. Data shown in chapter 3.

We have obtained an Animal Care Certificate (number: A07-442, Approved on October 31, 2007) from the University of British Columbia.

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List of Abbreviations

ASK-1	Apoptosis Signal-Regulating Kinase 1
Bad	BcL-2 Antagonist of Death
Bax	BcL-2 associated X protein
BcL-2	B-Cell Lymphoma 2
βIGF1RKO	β-cell Insulin-like growth factor-1 receptor knockout
βIRKO	β-cell Insulin receptor knockout
BrdU	5-Bromo-2-deoxyuridine
CHOP	C/EBP-homologous protein
CoxIV	Cytochrome c oxidase IV
CREB	cAMP response element binding
DAPI	4',6-diamidino-2-phenylindole
EGFP	Enhanced Green Fluorescent Protein
ER	Endoplasmic reticulum
ERK1/2	Extracellular Regulated Kinase1/2
FBS	Fetal bovine serum
Foxo-1	Forkhead box O1
GFP	Green Fluorescent Protein
GLP-1	Glucagon-like peptide-1
GRP78	Glucose-Regulated Protein, 78-KD
HIT-T15	Hamster insulinoma beta-cell line – clone T15
IGF-1	Insulin-like growth factor 1
INS-1	Rat insulinoma
IRS-2	Insulin Receptor Substrate 2
MIN6	Mouse insulinoma
MEK	MAPK kinase

NAADP	Nicotinic acid adenine dinucleotide phosphate
PAK1	p21-activated kinase 1
PBS	Phosphate-buffered saline
PDK-1	Phosphoinositide-dependent kinase-1
Pdx-1	Pancreatic Duodenal Homeobox-1
Pdx-1-Cre-ER™	Pancreatic Duodenal Homeobox-1-Cre recombinase Estrogen Receptor (Tamoxifen)
PI3	Phosphatidylinositol 3
PKA	Protein Kinase A
PKB/Akt	Protein Kinase B
PKC	Protein Kinase C
PP2A	Protein phosphatase 2A
Rb	Retinoblastoma
RIP-Cre	Rat-insulin promoter-Cre recombinase
TUNEL	Terminal Deoxynucleotidyl Transferase dUTP Nick End Labeling
VDAC	Voltage-dependent anion channel

Acknowledgements

We thank Drs. Elise Kohn, Christopher McIntosh, Chris Proud, Timothy Kieffer, Shernaz Bamji, and Dan Luciani for helpful discussions and advice. We thank Dr. Rong Liang for assistance with mouse husbandry, Ms. Xiaoke Hu for assistance with islet perifusions and radioimmunoassays, Dr. Michael Underhill for access to the Cellomics KineticScan instrument and Dr. Grant Churchill (University of Oxford, Oxford, UK) for the gift of Ned-19. We thank Dr. Tamas Balla (NIH, Bethesda, MD) for the Raf-1 fusion proteins, and Dr. Christopher Wright (Vanderbilt University, Nashville, TN) for the antibody against Pdx-1. We thank Dr. Manuela Baccarini (University of Vienna, Max F. Perutz Laboratories, Vienna, Austria) for the Raf-1 floxed mice. E.U.A. was supported by a National Institutes of Health National Research Service Award and a Cordula and Gunter Paetzold Fellowship at the University of British Columbia.

Dedication

I cannot overstate my gratitude to my Ph.D. supervisor, Dr. James D. Johnson. With his enthusiasm and great efforts to explain ideas succinctly, he has helped make science both exciting and rewarding. I am indebted to my colleagues for providing a stimulating and fun environment. I am especially grateful to all members of the Johnson laboratory for being my family while in graduate school. I am fortunate to have met my Canadian girlfriends Kristin, Amy, Jen, Kamila, Marta and Corrine. Most importantly, I thank my parents, Modesto and Herminigilda Alejandro for their love and support as well as my big family for providing a loving environment. Finally, I dedicate this thesis to my husband, Mark, and daughter, Kaya – my biggest fans.

1. Introduction

1.1 Pathophysiology of type 1 and type 2 diabetes

Diabetes is a disease with major public health implications worldwide. Approximately two million Canadians (6% of the general population) live with diabetes and the prevalence among indigenous sub-populations ranks amongst the highest in the world (1, 2). The mechanisms involved in the development of both type 1 and type 2 diabetes are diverse but reduced β-cell function and survival are the major common events leading to the loss of functional β-cell mass (3). In type 1 diabetes, β-cells undergo specific autoimmune attack and loss of β-cells via apoptosis and necrosis necessitates insulin therapy to control glucose homeostasis (4-8). A promising alternative to insulin therapy is islet transplantion. However, islet function and regeneration may be hindered by β-cell death in all stages of the transplantation process (9). While type 1 diabetes is characterized by the condition of absolute insulin deficiency, type 2 diabetes is initially non-insulin dependent and classically associated with insulin resistance, abnormal insulin secretion, and/or reduced functional β-cell mass (10). Type 2 diabetes in animal models and human patients is also associated with β-cell apoptosis leading to insufficient β-cell mass and function (3, 11-13). Moreover, β-cell apoptosis has been implicated in rare inherited forms of type 2 diabetes known as maturity-onset diabetes of the young (MODY) caused by haploinsufficiency of specific transcription factors required for the development of the pancreas (14).

In the adult, the pancreatic β-cell mass adapts in response to changing metabolic demands that are either physiological, such as pregnancy, or pathophysiological, such as obesity. Obesity is the best-known risk factor for type 2 diabetes (15). However, not all obese individuals develop diabetes because their β-cells can compensate by proliferating and effectively increasing insulin secretion (16). The failure of β-cells to compensate for insulin resistance, via proliferation or increase glucose-stimulated insulin release, is a major component of impaired glucose homeostasis and diabetes (17, 18). β-Cell failure is also a consequence of the inability of pancreatic β-cells to suppress apoptosis in the face of glucolipotoxic and lipotoxic conditions (19-21) Thus, efforts to enhance proliferation and reduce apoptosis have the potential to increase functional β-cell mass, ameliorate the course of both type1 diabetes and type 2 diabetes, and improve current human islet transplantation protocols.

1.2 Mechanisms of β-cell apoptosis in type 1 and type 2 diabetes

The endocrine pancreas is actively remodeled throughout development and adult life through a balance of proliferation, differentiation, and apoptosis (22). Pancreatic β-cells are sensitive to a number of pro-apoptotic or anti-apoptotic stimuli (11, 20). In type 1 diabetes, established pro-apoptotic stimuli include proinflammatory cytokines, nitric oxide, and reactive oxygen species (11). Elevated free fatty acids, chronically high glucose, and islet amyloid are among the multiple factors that may cause β-cell destruction in type 2 diabetes (23, 24). The signalling pathways in β-cell apoptosis elicited by these stimuli are not fully understood. However, a growing body of evidence

supports a convergence in signalling pathways toward common effectors of β-cell death elicited by stimuli implicated in both type 1 and type 2 diabetes (11, 24, 25). The common apoptotic pathways implicated in both types of diabetes are induced by the activation of cysteine proteases (caspases) and the reduction of pro-survival genes such as Bcl-2 and Bcl-xL (25). Pro-apoptotic events induced by proinflammatory cytokines *in vitro* have been reported to require the activation of caspase-3, a major effector of apoptosis in β-cells (26, 27). Moreover, human islets from donors with type 2 diabetes show increased apoptosis with enhanced caspase-3 activity (13). Human islets treated with caspase-3 inhibitor are protected from apoptosis and have improved islet graft function (28). Pro-apoptotic proteins like Bax and Bad have been reported to be increased in human islets isolated from donors with diabetes and in diabetic rat islets, while levels of anti-apoptotic proteins such as Bcl-xL and Bcl-2 are reduced (29, 30). Despite numerous studies, the specific mechanisms controlling pancreatic β-cell apoptosis remain to be fully elucidated.

Since the viability of isolated islets is one of the main obstacles limiting islet transplantation success, many studies have focused on inhibiting β-cell apoptosis and other forms of cell death. Several studies have shown that over-expression of Bcl-2 and Bcl-xL proteins enhances human islet viability (31-33). Moreover, transgenic hyperexpression of Bcl-2 in animal models or stable transfection of Bcl-2 in cell-lines also exhibits protection from apoptosis (34-37). Taken together, these studies suggest that the balance of pro-apoptotic and anti-apoptotic proteins plays a key role in regulating β-cell apoptosis. The survival of the β-cell may also depend in part on the

presence of endogenous or exogenous growth factors within the pancreatic islets. *In vitro* studies show that β-cells undergo apoptosis when growth factors are depleted (38-40). On the other hand, increased growth factor signalling can rescue β-cells from apoptosis (38, 41, 42). Autocrine interactions of growth/survival factors present in the β-cell are major regulators of β-cell survival (38, 43). For example, insulin and nerve growth factor are endogenous growth factors secreted by the β-cell that enhance cell survival and proliferation (42, 44).

1.3 Insulin signalling pathway in pancreatic β-cell

Insulin is a multifunctional hormone that regulates the energy metabolism, growth, and survival of many cell types including the pancreatic β-cell (10, 45). The insulin signalling cascade is initiated by insulin binding to the insulin receptor on the surface of the cell membrane (45, 46). This in turn activates the intracellular tyrosine kinase activity of the insulin receptor causing autophosphorylation and subsequent phosphorylation of downstream adaptor proteins including the insulin receptor substrates (IRS1-4) and the SH2-containing protein (Shc)(47-49). Downstream targets of the IRS proteins can diverge into two major signalling pathways: one that involves IRS, phosphatidylinositol 3-kinase (PI3-kinase) and Akt (also known as PKB), and a second pathway that involves IRS, growth factor receptor-bound protein-2 (Grb2), and the extracellular regulated kinases (ERK1/2; also known as MAP-Kinase) (50). The initiation of the PI3-kinase pathway activates Akt, which leads to the stimulation of

several biological responses, including glucose metabolism, cell proliferation, and survival (51, 52). Independent of the PI3-kinase pathway is the association of IRS proteins with Grb2, which leads to the recruitment of the mammalian Son of Sevenless (mSOS), Ras, Raf and results in the activation of ERK1/2, which translocates to the nucleus and promotes gene transcription (47, 50). The schematic diagram of these signalling pathways is presented in Figure 1.

To date, the majority of studies on β-cell survival pathways have focused primarily on Akt, its upstream regulators and downstream targets (53). Studies using cultured insulinoma cells and in Akt over-expression systems had suggested that insulin promotes its pro-survival effect via IRS2/PI3-kinase/Akt (51, 53). Indeed, the expression of constitutively active Akt protected the INS-1 rat β-cell line from free fatty acid-induced apoptosis (54). Moreover, transgenic expression of constitutively active Akt in β-cells was also protective against streptozotocin-induced diabetes and shows increased β-cell size and total islet mass (51, 55). Interestingly, transgenic mice expressing a dominant-negative/kinase-dead form of Akt under the control of the insulin promoter were shown to have normal β-cell mass and did not display an increase in islet apoptosis (52). Moreover, Aikin *et al* reported that in freshly isolated human islets insulin caused an increase in Akt phosphorylation despite amplified β-cell apoptosis (56). Thus, Akt activity alone may not be able to completely prevent primary β-cell apoptosis. The above experiments also suggest that Akt is sufficient to prevent apoptosis but not necessary to keep β-cells alive. Indeed, several other pro-survival kinases are known to be important in the β-cell. For example, protein kinase A (PKA) is also a critical

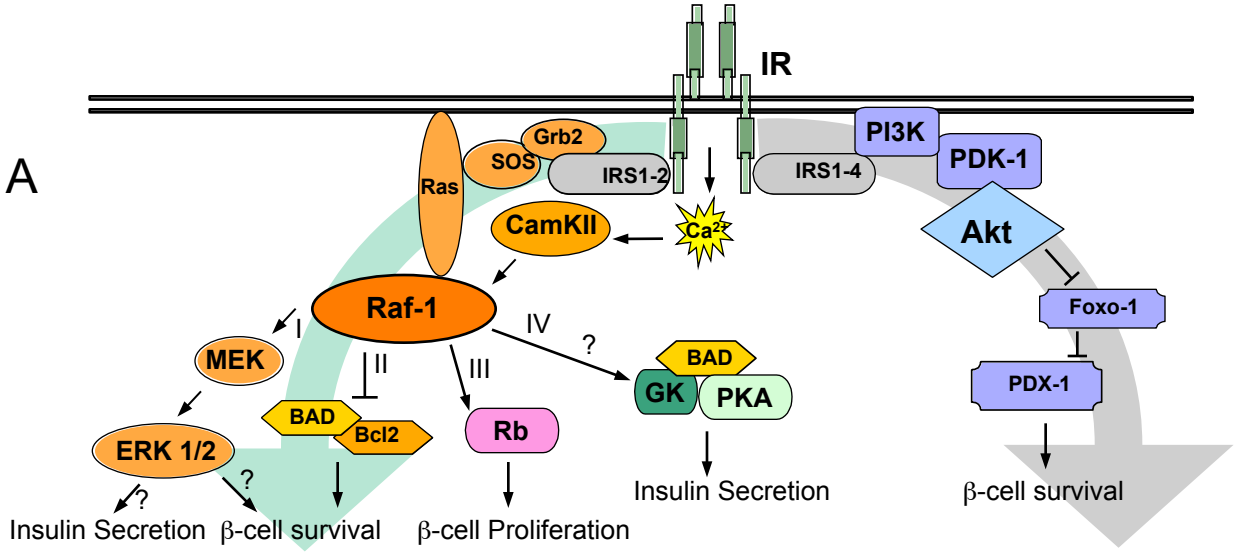
regulator of β-cell survival. PKA activates cAMP-response element-binding protein (CREB) via ERK1/2 (57, 58), which in turn controls the expression of important pro-survival genes such as Bcl-2 and IRS-2 (59-61). Together, these data support the concept that multiple signalling kinases may be important for β-cell survival.

The insulin/Ras/Raf/ERK1/2 signalling pathway has been well studied in many cell types (62), but remains under-studied in the β-cell. Most members of this pathway are found in human and mouse islets as well as in β-cell lines, including the Raf family of cytoplasmic serine/threonine kinases. All three Raf kinase family members (A-Raf, B-Raf, and C-Raf/Raf-1) are expressed in human and rodent islets, as well as in β-cell lines (63-66). They all share a common structure but differ in their tissue expression profile, regulation, and ability to potently activate the MEK/ERK1/2 cascade (62). Another common property of Raf kinases is their ability to interact with other proteins on cellular membranes. Early studies described Raf-1 kinase recruitment to the plasma membrane by Ras, a guanine-nucleotide-binding protein (67-69). Immediately after Raf-1 activation in the plasma membrane, the kinase associates with and activates cytoplasmic MEK, an upstream kinase activator of ERK1/2 (70-72). Raf-1 kinase is ubiquitously expressed and highly regulated at the post-translational level by phosphorylation, oligomerization, interaction with adaptor/scaffolding proteins, and by its sub-cellular localization (67, 73-75). Raf-1 kinase is localized in the cytoplasm, mitochondria, and nucleus in primary islet β-cells and MIN6 cells (see subsequent chapters below). Among the Raf family members, Raf-1 is the isoform reported to interact with the outer mitochondrial membrane (76), further supporting the idea that

Raf-1 has multiple functions in promoting cell survival.

Although Raf-1/ERK1/2 is best known as a pro-survival kinase in many cell types, the role of ERK1/2 in pancreatic β-cell survival remains controversial. Some studies support an anti-apoptotic role for ERK1/2, while others suggest a requirement for ERK1/2 in various forms of β-cell apoptosis (11, 40, 66, 77, 78). For example, several groups have reported that acute treatment with high glucose stimulates PKA/ERK1/2 signalling to positively regulate β-cell growth and survival (11, 58, 66, 79). However, sustained ERK1/2 activation has been shown to be required in chronic high-glucose or the cytokine interleukin-1β-induced β-cell apoptosis (77, 78, 80). It is important to point out, however, that these findings implicating ERK1/2 activation as a requirement for β-cell apoptosis were obtained by pharmacological approaches *in vitro* and prolonged incubation of up to four days. They should be confirmed by molecular approaches and shorter treatment incubations. A noteworthy observation is that ERK1/2 activation induced apoptosis in other cell types, including neuronal cells (81, 82). Together, these findings clearly suggest that ERK1/2 signalling activates both the pro-survival and pro-apoptotic conditions but the cellular outcome may depend on the timing and duration of ERK1/2 activation. The mechanisms of a putative dual role for ERK1/2 remain to be investigated. Nevertheless, these studies highlight the importance of understanding both the spatial and temporal effects of ERK1/2 activation in mammalian cells (83).

Figure 1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IR
Grb2
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ERK 1/2
BAD
Bcl2
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Insulin Secretion
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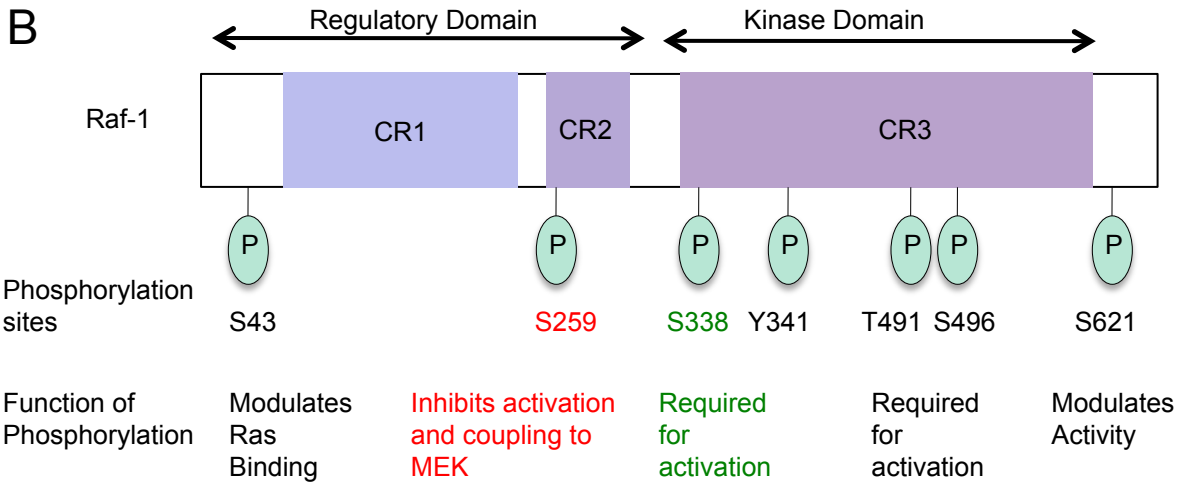

B
Regulatory Domain
Kinase Domain
Raf-1
CR1
CR2
CR3
P
Phosphorylation sites
S43
S259
S338
Y341
T491
S496
S621
Function of Phosphorylation
Modulates Ras Binding
Inhibits activation and coupling to MEK
Required for activation
Required for activation
Modulates Activity

Figure 1. **Schematic diagram of the insulin signalling pathway and phosphorylation sites of Raf-1.** (A) Simplified model of anti-apoptotic insulin signalling in the pancreatic β-cell. Insulin receptors and/or IGF-1 receptors activate a series of common signalling events that can be broadly divided into two arms: the PI3K/Akt/Pdx-1/Foxo-1 and the Ras/Raf/ERK1/2 signalling pathways. Akt is activated through a PI3-kinase-dependent pathway, and prevents β-cell apoptosis in part through Pdx-1, Foxo-1, and Bcl-2 family proteins. Raf-1 kinase is situated at a key intersection of that MAP-kinase arm of insulin signalling. Raf-1 is positively regulated both by Ras-dependent events and Ca^{2+}/ CaMKII signalling and inhibited by Akt cross-talk. Activation of Raf-1 can lead to: (I) activation of MEK/ERK1/2 pathway, (II) the Bcl-2-mediated translocation of Raf-1 to mitochondrial where Bad is inhibited, (III) interaction with Retinoblastoma (Rb) protein to promote cell proliferation or (IV) regulation of Protein Kinase A (PKA), Bad and Glucokinase complex. Many signalling molecules have been omitted for clarity. (B) Structure and phosphorylation sites of Raf-1 (modified version from Kolch et al, *Expert Rev Mol Med,* 2002Apr 25;4(8):1-18.)

1.4 Insulin regulates β-cell survival and proliferation

A growing body of evidence suggests that insulin itself is a key regulator of islet apoptosis and β-cell mass *in vitro* (42) and *in vivo* (84-86). The insulin receptor, insulin receptor substrates IRS-1-4, and their downstream targets PI3K/Pdk-1/Akt, Pdx-1, and ERK1/2 are present and positively regulate β-cell survival and proliferation (58, 87-89). It was initially reported that β-cell–specific insulin receptor knockout (βIRKO) mice had reduced insulin content and a decrease in glucose-stimulated insulin secretion (90). As such, the βIRKO mice manifest progressive glucose intolerance leading to overt diabetes (90). However, reassessment of these mice showed that many of these defects could be accounted for by a significant reduction in β-cell mass, due to increased β-cell apoptosis and decreased β-cell proliferation (84). Interestingly, the βIRKO mice, but not mice lacking β-cell IGF-1 receptors (βIGFRKO), have increased apoptosis and reduced β-cell mass (85, 86). Okada *et al* examined the relative significance of insulin versus IGF-1 receptors in compensatory islet growth induced by obesity (86). In these studies, βIRKO and βIGFRKO mice were fed with high-fat diets and both groups developed insulin resistance. However, only the βIRKO, not the βIGFRKO, mice that exhibited poor islet growth, suggesting that insulin rather than IGF-1 plays a more critical role in obesity-induced β-cell expansion, a known physiological stimulus of β-cell growth. Together, these gene knockout studies show strong evidence that insulin regulates survival and proliferation of adult β-cells.

1.5 Raf-1 kinase activation in other cell types

The regulation of Raf-1 kinase is a complex process and the exact mechanisms controlling this activation remain unclear. It is thought that the initial event is Ras-dependent recruitment of inactive Raf-1 from the cytosol to the plasma membrane, followed by the interaction between the Ras-binding domain (RBD) of Raf-1 (residues 51-131) with the switch 1 region of activated Ras-GTP (69, 91). A second Ras binding domain, the Cysteine-Rich Domain (CRD, residues 130-184) is also involved in Raf-1 activation, although it is not required for membrane recruitment (92). Once Raf-1 has been localized to the plasma membrane, a sequence of dephosphorylation, phosphorylation, phospholipid binding and interaction with 14-3-3 proteins and possibly dimerization must occur to facilitate full Raf-1 activation. One of the key phosphorylation sites regulating Raf-1 activation is located at serine 259, which appears to be the main target for inhibitory phosphorylation of full-length Raf-1. The mutation of serine 259 to either alanine or aspartic acid renders Raf-1 largely resistant to both PKA (93) and Akt inhibition (94). Phosphatase 2A (PP2A) has been shown to dephosphorylate serine 259, which is required for Raf-1 activation. For example, okadaic acid, an inhibitor of PP2A, causes hyperphosphorylation on serine 259 and interferes with Raf-1 activation (95). Moreover, treatment of cells with general inhibitors of PP2A results in the accumulation of inactive 14-3-3-Raf-1 complexes at the plasma membrane (96). Thus, serine 259 plays a pivotal role in Raf-1 activation as well as in the interaction with activators and substrates.

The activating phosphorylation sites of Raf-1 (located on serine 338, tyrosine 341, threonine 491, and serine 494) are located in the catalytic domain of the kinase (97-99). Twenty years ago, a low concentration of insulin (100 pM) was shown to increase serine phosphorylation and subsequent activity of Raf-1 within minutes in HeLa, NIH 3T3, and Chinese hamster ovary cells, suggesting that insulin can directly activate Raf-1 through interaction with its own receptor (73) and not via IGF-1 or IGF-2 receptors, which would both require higher insulin concentrations (74). However, it is also plausible that insulin activates an intermediate protein serine/threonine Raf-1 kinase kinase such as Protein Kinase C (PKC). Indeed, PKC has been shown to phosphorylate Raf-1 (100, 101). However, insulin still stimulated Raf-1 phosphorylation normally in H35 cells with 93% of cytosolic reduction of PKC activity by phorbol 12-myristate 13-acetate (74), suggesting an insulin-induced Raf-1 activation can be independent of PKC. Another potential mechanism would involve an insulin-generated second messenger like calcium (Ca^{2+}) that indirectly activates the Raf-1 kinase. Our group has shown that insulin generates Ca^{2+} signals in primary islets and in MIN6 cells (102, 103). In fibroblast and L6 skeletal muscle cells, insulin has been demonstrated to elicit Ca^{2+} signals that lead to the activation of calmodulin-dependent protein kinase II (CaMKII) and Raf-1/ERK1/2 activation (104, 105). It is therefore of interest to test whether insulin-generated Ca^{2+} signals can modulate Raf-1 activation via CaMKII. p21 activated kinase (PAK) and Src are also reported to phosphorylate the activating sites of Raf-1 in COS-7 cells (97-99). Interestingly, insulin has been reported to activate PAK in muscle (106) and in intestinal cells (107), but this remains to be tested in pancreatic β-cells.

1.6 Regulation of adult pancreatic β-cell proliferation: possible role of Raf-1

A critical aspect of β-cell mass maintenance, especially in young mice and young humans is the ability of existing β-cells to replicate. Whereas they were once thought to be permanently post-mitotic, it is now better appreciated that mature β-cells can replicate, albeit at an extremely low rate (108-110). However, the mitogenic roles of specific kinases and growth factor signalling pathways, including Raf-1, remain under-studied in the β-cell. The first study to implicate Raf-1 in β-cell proliferation investigated the role of an endogenous Raf-1 inhibitor, Raf Kinase Inhibitory Protein (RKIP), which is expressed in insulin producing cells in the islet (63). Over-expression of human RKIP in the SV-40 transformed hamster HIT-T15 β-cell line inhibited proliferation measured by FACS analysis (63). RKIP, which specifically disrupts Raf-1/MEK/ERK1/2 signalling via physical interaction with Raf-1, significantly decreased the percentage of cells in G2 and S phase (63). Whether a specific Raf-1 inhibitor or a dominant-negative mutant Raf-1 would inhibit primary β-cell proliferation has not been tested. Whether Raf-1 mediates effects of other β-cell growth factors is also not clear. Trümper *et al* (64) suggested that the proliferative effect of glucose and glucagon-like peptide-1 (GLP-1) in human β-cells is not primarily mediated by Raf-1 and instead implicated B-Raf. However, ruling out the role of Raf-1 in this pathway is difficult given the cooperative hetero-dimerization of Raf-1 and B-Raf. Thus, Raf-1 may be an important co-factor for the activation of B-Raf (111). There is a long and growing list of both exogenous and endogenous growth factors that promote β-cell proliferation *in vivo* and *in vitro*, including insulin, hepatocyte

growth factor, GLP-1/exendin-4, and IGF-1, and many of these growth factors activate the MEK/ERK1/2 signalling pathway (39, 112). In the context of β-cell proliferation, the mechanisms downstream of ERK1/2 that have been implicated are S6 kinase, Elk, Myc and CREB (113-116). It will be of interest to further delineate the mechanisms and functions of the Raf-1/MEK/ERK1/2 signalling cascade in β-cell proliferation.

Aside from its established role via ERK1/2 in proliferation, Raf-1 has been reported to directly associate with cell-cycle components in the nucleus of various human cancer cell lines (117-119). This mechanism involves Raf-1 translocation to the nucleus and interaction with Retinoblastoma (Rb) protein (117, 118), a key 'pocket protein' regulator of G1/S phase in the cell cycle. When Rb is active, it is dephosphorylated and binds to the E2F family of cell cycle regulatory genes, which leads to transcriptional repression of downstream genes and causes cell cycle arrest. Conversely, when Rb is phosphorylated, it becomes inactive and releases E2Fs, removing the transcriptional brake of critical cell cycle genes, and thus allowing the cell cycle to progress. Functioning as a gatekeeper of the cell cycle, Rb is tightly regulated by cyclin-dependent kinases (cdk)-4 and -6. To date, the role of Rb in β-cell cell cycle remains elusive. Over-expression of cyclin D1/cdk-4, which removes the brake imposed by Rb on β-cell cell cycle, increased rat and human β-cell proliferation (120). However, β-cell specific deletion of Rb had a non-significant effect in β-cell proliferation, β-cell mass and function *in vivo* (121), suggesting an undefined mechanism that may have compensated for the lack of Rb. Indeed, deletion of both Rb and its homolog p130 blocked β-cell proliferation (122). The differences in protein expression of cell cycle proteins among

primary islets and various β-cell lines (123) may also dictate the outcome of β-cell fate. Thus, a greater knowledge of the β-cell cell cycle is necessary to understand basic β-cell biology and mechanisms of β-cell proliferation.

1.7 β-cell ER-stress in diabetes: possible role of Raf-1

Pancreatic β-cell death can be initiated by a number of stresses (124). One of these stress-induced apoptosis mechanisms is a chronic activation of the unfolded protein response, which is triggered by endoplasmic reticulum (ER) stress conditions resulting from the accumulation of misfolded proteins in the ER. Pancreatic β-cells are particularly prone to ER stress due to the high demands of insulin protein biosynthesis and secretion (125). Both diabetic patients and type 2 diabetic rodent models show an increase in ER stress, suggesting that this event may play a central role in the initiation and progression of β-cell dysfunction and death in type 2 diabetes (126-129). Although the role of Raf-1 in ER stress-related cell death is not clear, current studies suggest that Raf-1 regulates ER stress in various cell types. For example, deactivation of MEK, an immediate downstream target of Raf-1, promoted ER stress in human melanoma cells (130). Raf-1 has also been reported to reduce ER stress-induced apoptosis in human lung cancer H460 cells (131). It has recently been suggested that Raf-1 might be associated with GRP78/BiP, a pro-survival Ca^{2+} binding chaperone and one of the key regulators of unfolded protein response signalling. GRP78 is generally thought to be an ER-residing chaperone, but extra-ER GRP78 localization has been observed in multiple

cell types (131-134). For example, unfolded protein response-induced GRP78 mitochondrial localization was reported in 9L rat brain tumour cells treated with the Ca^{2+} ionophore A23187 or the ER Ca^{2+}-ATPase inhibitor thapsigargin, both of which disrupt ER Ca^{2+} homeostasis and elicit the unfolded protein response (134). Interestingly, GRP78 is thought to bind and stabilize Raf-1 in mitochondria, rather than ER, where it enhances Raf-1 activation via serine 338 (131). Raf-1 serine 338 mutant blocked GRP78's pro-survival properties, suggesting the requirement of Raf-1 for GRP78 protection in ER-stress-induced apoptosis (131).

The unfolded protein response is a highly conserved cellular response that involves the up-regulation of ER-resident chaperones, such as GRP78 to facilitate the correct folding of secretory and transmembrane proteins. However, excessive and prolonged unfolded protein response can lead to the induction of CHOP, a marker of chronic ER stress and a critical transcription factor mediating ER stress-induced apoptosis in many cell types including the pancreatic β-cell (125, 135-137). CHOP gene ablation in diabetes mouse models (e.g. Akita mice) delayed ER stress–mediated diabetes and enhanced β-cell function and survival (125, 138). Our group and others have employed the detection of CHOP protein levels as an index of β-cell ER-stress (129, 139).

1.8 Pro-survival effects of Raf-1 at the mitochondria

A body of literature from many cell models suggests that Raf-1 has pro-survival effects independent of ERK1/2 activity (76, 140, 141). Global Raf-1 knockout mice die around mid-gestation due to deregulated apoptosis in several tissues (140). Unexpectedly, the activity and levels of both MEK and ERK1/2 in these mice were normal. This suggested that the other two Raf isoforms may have compensated for the lack of Raf-1 in the MEK/ERK1/2 module, or that Raf-1 may have promoted survival independent of ERK1/2. Evidence has suggested a novel ERK1/2-independent mechanism involving Bcl-2-mediated targeting of Raf-1 to the mitochondria (142-144). Once at the mitochondria, Raf-1 phosphorylates Bad on serine 112 and causes the inactivation and sequesterization of Bad in the cytoplasm by a 14-3-3 scaffolding protein, thereby promoting cell survival (142-144). Moreover, Bad phosphorylation at serine 112 is reported to cause its poly-ubiquitylation and degradation (145). Galmiche *et al* (76) reported that among the three Rafs, only Raf-1 efficiently bound to purified mitochondria, further supporting the notion that Raf-1 possesses a unique pro-survival mechanism independent of ERK1/2 signalling (see Figure 1A; pathway I and II). Thus, the molecular mechanisms and consequences of Raf-1 localization to the mitochondria are important issues to address. Wang *et al* (142, 143) reported that the BH4 domain of Bcl-2 is a critical region that binds to the catalytic domain of Raf-1 in Sf9 insect cells. Bcl-2 family proteins have four conserved Bcl-2 homology domains: BH1, BH2, BH3, and BH4. All anti-apoptotic members (including Bcl-2 and Bcl-xL) contain the BH4 domain, while the pro-apoptotic members lack BH4, with the exception of Bcl-xS.

Interestingly, an interaction between Raf-1 and Bcl-xS has also been described in Sf9 insect cells (143). The consequence of this novel protein-protein interaction is unknown, but merits confirmation in mammalian cells. It is possible that Raf-1 may prevent Bcl-xS from inducing cell death. It is also interesting to determine whether Bcl-xL targets Raf-1 to mitochondria, especially in situations where Bcl-xL is preferentially expressed over Bcl-2. Indeed, Bcl-2 and Raf-1 do not have a mutual requirement in suppressing apoptosis (146). Critical players in the pro-survival actions of Raf-1 described to date, such as Bcl-2 and Bad, are expressed in pancreatic β-cells (147). The localization of Raf-1 in β-cells has not been described. It will also be critical to determine the downstream targets of Raf-1 after its mitochondrial translocation. The inactivation of Bad may provide only one potential mechanism to modulate cell viability.

The unique function of Raf-1 on the mitochondria has ignited great interest in the field of apoptosis research. Many studies have been conducted to understand the outcome of Raf-1 mitochondrial translocation and have uncovered non-ERK1/2 mediated pro-survival targets (142, 143). For example, one study implicated Raf-1 as a negative regulator of the mitochondrial voltage-dependent anion channel (VDAC), which controls mitochondrial permeability, and thereby prevents cytochrome c release and caspase activation in growth factor-starved cells (148). Raf-1 has also been reported to promote cell survival by inhibiting Apoptosis Signal regulating Kinase-1 (ASK-1)(141). ASK-1 is upstream of JNK and p38 and it participates in the cellular stress response (149). ASK-1 promotes extrinsic apoptosis induced by death receptors activated by the cytokines Fas and TNF-α, both of which are implicated in β-cell apoptosis (150-152).

Another addition to the growing list of Raf-1 targets is mammalian sterile 20–like kinase (MST2), which is found to be increased in Raf-1 deficient cells (153). None of these proteins have been studied extensively in the β-cell. Therefore, it will be of interest to understand the roles they play in β-cell survival and function. Raf-1 kinase is a target of numerous growth factors and positioned in the centre of a complex signalling network. It will be fascinating to understand the molecular mechanisms of how Raf-1 regulates cell survival, and how these multiple pathways are integrated.

1.9 Raf-1 on insulin secretion and general β-cell function

In addition to regulating β-cell survival and proliferation, it is also possible that Raf-1 might be involved in the physiological regulation of insulin secretion. Numerous kinases, including Akt, have been shown to regulate glucose-stimulated insulin release (52). Longuet *et al* (154, 155) suggested that ERK1/2 controls insulin secretion by phosphorylating synapsin I, a protein that is involved in insulin exocytosis by liberating insulin granules from the actin cytoskeleton.

Because Raf-1 interacts with Bcl-2 protein family members Bcl-2 and Bad, it is possible that Raf-1 may regulate their newly uncovered non-apoptotic functions at the mitochondria (see Figure 1A; Pathway IV) (37, 156). Danial *et al* (156) discovered an unforeseen role for pro-apoptotic Bad protein in mitochondrial glucose metabolism. Specifically, the BH3 death domain of Bad is necessary for mitochondrial oxidation of

glucose by forming a complex with glucokinase and PKA. Bad knockout mice have impaired glucose-stimulated insulin secretion and fail to increase their β-cell mass in response to high-fat feeding (147). Interestingly, βIRKO mice exhibited reduced complexing of Bad to glucokinase and PKA, and that is associated with their attenuated β-cell function (157). Raf-1 kinase regulates the phosphorylation state of Bad at serine 112, which may in turn control Bad's cellular localization and roles in β-cell survival and insulin secretion.

1.10 Statement of objectives

The main objective of the present study is to investigate the mechanisms by which insulin acts on β-cells to promote survival. We examined whether Raf-1 is present and functional in β-cells and determined whether this kinase plays a role in β-cell survival. We evaluated whether insulin activates Raf-1 and examined downstream targets of Raf-1, including both ERK1/2 activation and mitochondrial Bad inactivation. We further tested the hypothesis that Raf-1 may play an important role in β-cell survival *in vitro* and function *in vivo*. To test this hypothesis, we utilized the Cre-lox system under control of the insulin promoter to obtain a pancreatic β-cell specific ablation of Raf-1 kinase ($RIPCre^{+/-}$:$Raf\text{-}1^{flox/flox}$) and their littermate controls ($RIPCre^{+/-}$:$Raf\text{-}1^{wt/wt}$) and performed glucose tolerance and insulin tolerance tests. Furthermore, we tested if islets isolated from $RIPCre^{+/-}$:$Raf\text{-}1^{flox/flox}$ mice have impaired response to both glucose and KCl. These studies are the first to implicate Raf-1 in pancreatic β-cell survival and function and

provide a greater understanding of the mechanisms of insulin signalling.

2. Materials and Methods

2.1 Reagents

The following inhibitors were obtained from Calbiochem (La Jolla, CA): HNMPA-AM (hydroxy-2-naphthalenylmethylphosphonic acid), a Raf inhibitor, GW5074 (5-iodo-3-[(3,5-dibromo-4-hydroxyphenyl)methylene]-2-indolinone), two different MEK inhibitors, PD98059 (2′-amino-3′-methoxyflavone) and UO126 (1,4-diamino-2,3-dicyano-1,4-*bis*(2-aminophenylthio)butadiene), the PI3-kinase inhibitor LY294002 (2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one), as well as two Akt inhibitors, Akt Inhibitor VII (TATAkti*)* and Akt Inhibitor VIII (1,3-dihydro-1-(1-((4-(6-phenyl-1H-imidazo[4,5-g]quinoxalin-7-yl)phenyl)methyl)-4-piperidinyl)-2H-benzimidazol-2-one (Akti-1/2). Other reagents were from Sigma (St. Louis, MO). Unless otherwise indicated, concentrations of specific inhibitors were expected to be maximally effective based on their known IC_{50} values. For GW5074, we used concentrations within the values reported to block the kinase in intact cells (5-100μM)(144, 158). Raf-1 fusion proteins were provided by Dr. Tamas Balla (NIH, Bethesda, MD). The NAADP-receptor inhibitor Ned-19 (159) was a gift from Dr. Grant Churchill (University of Oxford, Oxford, UK).

2.2 Generation of conditional deletion of Raf-1 in β-cells in vivo

Mice with global Raf-1 deletion are not viable (140). Thus, we utilized the Cre-lox system to obtain a pancreatic β-cell specific ablation of floxed Raf-1 alleles (Dr. Manuela Baccarini, University of Vienna, Max F. Perutz Laboratories, Vienna, Austria). We obtained β-cell specific Raf-1KO ($RIPCre^{+/-}$/$Raf\text{-}1^{flox/flox}$) mice and littermate controls ($RIPCre^{+/-}$:$Raf\text{-}1^{wt/wt}$) by breeding male $RIPCre^{+/-}$:$Raf\text{-}1^{flox/wt}$ and female $RIPCre^{-/-}$:$Raf\text{-}1^{flox/wt}$ mice. $RIPCre^{+/-}$:$Raf\text{-}1^{flox/flox}$ have a Cre-mediated deletion of exon 3, a key step that removes critical information required for Raf-1 function and results in a frameshift, thereby generating a premature stop codon. In the Raf-1 locus, exon 3 encodes almost the entire Ras Binding Domain. RIP-Cre mediated deletion of exon 3 generates a transcript with a premature stop codon at the splice junction between exons 2 and 4. Litters were weaned at 21 days and DNA samples were collected for genotyping. DNA purification was achieved by using Gentra Puregene Mouse Tail Kit (Qiagen, Valencia, CA). The primer sequences are in Table 1.

2.3 MIN6-cell line culture

MIN6 (mouse insulinoma) cells were obtained from Dr. Timothy Kieffer's laboratory (Life Sciences Centre, University of British Columbia) under Material Transfer Agreement from Dr. Jun-ichi Miyazaki (Osaka, Japan). MIN6 cells were originally derived from transgenic mouse insulinomas as previously described (160). MIN6 cells

were cultured in DMEM (Gibco/Invitrogen, Burlington, ON) containing 25 mM glucose, 10% fetal bovine serum (Gibco/Invitrogen) and 100 IU/ml penicillin, 100 µg/ml streptomycin (Gibco/Invitrogen). MIN6 cells were passaged every 2-3 days using 0.5% Trypsin-EDTA (Gibco/Invitrogen) to maintain an overall confluency of 70-80%. For Western blot assays, MIN6 cells were seeded in 6 or 12 well culture plates (Nunc, Rochester, NY) 12 hours, washed and serum deprived for 6 hours before treatment to ensure a final treatment confluency of 80%. For immunofluorescent assays, MIN6 were seeded onto coverslips and allowed to adhere overnight.

2.4 Human islet isolation

Human islets were obtained from the Michael Smith Foundation for Health Research Centre for Human Islet Transplant and Beta-Cell Regeneration (Vancouver, BC, Canada). Islets were isolated from both male and female donors ranging from 40-70 years of age with no history of diabetes. Human islets were cultured in 11.1 mM glucose RPMI 1640 media (Gibco/Invitrogen) supplemented with 100 IU/ml penicillin, 100 µg/ml streptomycin (Gibco/Invitrogen) and 10% fetal bovine serum (Gibco/Invitrogen). Cells were cultured at 37^{o}C and 5% CO_2 in 35x10 mm Nunc suspension dishes (Nunc) until treatment.

2.5 Mouse islet isolation

Primary mouse islets were isolated from three month-old C57Bl6/J mice (Jax, Bar Harbour, MA), unless otherwise indicated, using collagenase and filtration as described previously (161) approved by the University of British Columbia Animal Care Committee (see Appendix) as follows. Briefly, mice were sacrificed using a CO_2 chamber and cervical dislocation. Mouse pancreata were exposed and the bile duct ligated. The pancreas was then inflated via the pancreatic duct with 2-5 mL collagenase (0.375 mg/mL, Sigma Type XI) in 1X Hanks solution (137 mM NaCl, 5.4 mM KCl, 4.2 mM NaH_2PO_4, 4.1 KH_2PO_4, 10 mM HEPES, 1 mM $MgCl_2$, and 5 mM glucose). The pancreas was then removed and placed in a 50 mL conical tube (BD Falcon, Franklin Lakes, NJ) containing 5 mL collagenase in 1X Hanks solution. The pancreas was then incubated for 13 minutes in a 37°C water bath after which ten vigorous shakes took place to homogenize the pancreas in solution after 6 and 13 minutes of incubation. The solution was then washed with 20 mL Hanks solution containing 1 mM $CaCl_2$. The mixture was centrifuged at 1200 rpm for 30 seconds and the supernatant was aspirated. This step was repeated twice, replacing 1X Hanks solution with Roswell Park Memorial Institute (RPMI) 1640 (Gibco/Invitrogen) on the last resuspension. The new solution was filtered using a pre-wet 70 µm nylon filter (BD Biosciences) and the captured islets were transferred into a 35x10 mm Nunc suspension dish (Nunc) containing complete RPMI 1640. The islets were then hand picked and cultured overnight at 37^oC 95% O_2 and 5% CO_2 in RPMI 1640 media with 11.1 mM glucose. Media were supplemented with 100

IU/ml penicillin, 100 µg/ml streptomycin (Gibco/Invitrogen). Fetal bovine serum (10% v/v) was added when indicated.

2.6 Islet cell dispersion

Islet dispersion was carried out by four consecutive washes with Ca^{2+}/Mg^{2+}-free MEM (Mediatech, Herndon, VA) followed by a gentle repetitive pipetting in the presence of a trypsin-EDTA solution (Gibco/Invitrogen) diluted 1:9 in MEM. Afterwards, the cells were washed with Ca^{2+}/Mg^{2+}-free MEM, and then seeded onto glass coverslips in completed RPMI 1640 media (Gibco/Invitrogen). Following dispersion, the cells were allowed to adhere to the coverslips for 3 hours in serum free 5 mM glucose before the addition of 10% serum free RPMI. The cells were allowed to adhere further by culturing overnight at 37^{o}C and 5% CO_2 until treatment.

2.7 Gene expression detection

Total RNA was isolated from mouse primary islets and MIN6 cells using the Qiagen RNeasy kit (Mississauga, ON). cDNA was reverse-transcribed using Superscript III from Invitrogen (Burlington, ON). PCR amplification (in the linear range) was carried out using 1 cycle of 94°C for 5 min, 30 cycles of 94°C for 30 sec, 55°C for 30 sec, 72°C

for 45 sec, and 1 cycle of 72°C for 7 min. Primers (see Table 3) were purchased from Integrated DNA Technologies (Coralville, IA).

2.8 Protein detection by immunoblot and mitochondria isolation

MIN6 cells, human islets or mouse islets were washed twice after treatments with ice-cold PBS before adding cell lysis buffer with protease inhibitor cocktail (Cell Signalling Technology, Danvers, MA). Whole cell lysates were either freeze-thawed twice or sonicated and protein concentrations were determined by using the BCA protein assay (Pierce Biotechnology, Rockford, IL). Protein lysates (5-40μg) were subjected to PAGE electrophoresis and transferred to polyvinylidene fluoride membranes. Membranes were then blocked with I-block solution (Tropix, Bedford, MA), washed and probed with primary antibodies. Primary antibodies against phosphorylated Raf-1 (serine 259 and serine 338), ERK1/2, phosphorylated ERK1/2 (Threonine 202/Tyrosine 204), Bad, phosphorylated Bad (serine 112), Bax, Bcl-2, phosphorylated ASK-1 (Threonine 845), ASK-1, phosphorylated Foxo-1, Foxo-1 and cleaved caspase-3 were from Cell Signalling Technology. Antibodies against β-actin, CoxIV, and Raf-1 were from Novus Biologicals (Littleton, CO), Abcam (Cambridge, MA) and BD Biosciences (Franklin Lakes, NJ), respectively. Primary rabbit monoclonal antibody to CHOP/Gadd 153 was from Santa Cruz (Santa Cruz, CA). Antibodies against Pdx-1 were provided by Dr. Christopher Wright (Vanderbilt University, Nashville, TN) and subsequently purchased from Chemicon (Temecula, CA). Bridge-1 antibody was

obtained from BD Biosciences. Secondary antibodies were purchased from Cell Signalling Technology. Immunodetection was performed with ECL Western Blotting Detection Reagents from Amersham (Buckinghamshire, UK). Densitometric analysis was performed using Photoshop (Adobe Systems Inc, San Jose, CA). The brightness and contrast of blot images were moderately adjusted using Photoshop after recommended and scientifically acceptable procedures (162). To protect the integrity of research, no information was obscured or eliminated from the original data. Mitochondria isolation kit (MITOISO1) was from Sigma.

2.9 Co-immunoprecipitation

Co-immunoprecipitation of Pdx-1 and Bridge-1 was performed on lysates from primary mouse islets treated with insulin as follows. Approximately 1,000 islets were cultured in 5 mM glucose serum-free RPMI medium 1640 (with 0.1% BSA) with or without insulin. Cells were placed in lysis buffer (Cell Signalling Technology), and 100 μg of total protein was incubated with Pdx-1 antibody overnight (a no-antibody negative control also was included). Protein A/G beads (Pierce Biotechnology) were added for 3 h. The immunoprecipitation beads were washed five times with lysis buffer, including protease inhibitors, and the resulting immunoprecipitate was then run on a SDS gel and probed with anti-rat Bridge-1 antibody overnight. Twenty micrograms of total lysate (from control cells) was run on the gel as a positive control. In preliminary experiments, we used the MIN6 β-cell line to test for interactions after co-immunoprecipitation with

both available Pdx-1 antibodies and the Bridge-1 antibody. The greater and most specific coimmunoprecipitation was observed with a polyclonal Pdx-1 antibody (results not shown), generously provided by Christopher Wright (Vanderbilt University, Nashville, TN).

Co-immunoprecipitations of Raf-1 and Bcl-2 family proteins (Bcl-2, Bad and Bcl-xL antibodies from Cell Signalling Technology) were performed on MIN6 cells lysates. Briefly, 600 μg of total protein was incubated with antibodies overnight, after which 50 μl of protein A/G beads (Santa Cruz Biotechnology) were incubated for 3 hours in PBS with protease inhibitors cocktail at 4^{o}C. The slurry of antibody, beads and lysates were spun down for 30 seconds, 8000 rpm using Ultrafree-MC filters (Millipore, Billerica, MA). The immunoprecipitation beads were then washed 3 times with PBS with protease inhibitors, and the immunoprecipitate was then electrophoresed on a SDS gel and probed with anti-Raf-1 antibody overnight. Raf-1 antibodies were from BD Biosciences and Epitomics (Burlingame, CA), and detected using protein A conjugated with HRP.

2.10 Immunofluorescence imaging

Immunofluorescence analysis of endogenous Raf-1 in dispersed islet β-cells and MIN6-cells was performed as described (163) using a Zeiss 200M inverted microscope equipped with a 100x (1.45 numerical aperture) objective. Briefly, cells were fixed in

fresh 4% paraformaldehyde for 10 minutes, permeabilized using 0.1% Triton-X100 for 10 minutes and blocked using 10% normal goat serum. Raf-1 was detected using a rabbit polyclonal primary antibody (Cell Signalling Technology) and goat secondary antibody conjugated to FITC (Jackson Immunoresearch, West Grove, PA). Primary antibodies to insulin (Linco Research, St. Charles, MO) were detected with goat secondary antibodies conjugated to Texas Red (Jackson Immunoresearch). To analyse Raf-1 fusions proteins cellular localization, 1 μg DNA of Raf-1-GFP, Raf-1^{51-131}-GFP, Raf-1^{51-220}-GFP, kindly provided by Dr. Tamás Balla (Endocrinology and Reproduction Research Branch, NIH), and 1 μg DNA of mitochondria-targeted dsRed vector, kindly provided by Dr. Heidi M. McBride (University of Ottawa), were co-transfected into MIN6-cells using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) for 24 hours before adding 0.1 μg/mL of Hoechst 33342 DNA dye (Molecular Probes/Invitrogen) and performing live cell imaging. Pearson's Correlation (PC) between Raf-1-GFP proteins and DsRed-Mito or Hoechst (nucleus) was calculated using SlideBook 4.2 software (Intelligent Imaging Innovations, Denver, CO).

2.11 Analysis of proliferating cells in MIN6 cells

MIN6 cells were transfected with full-length or truncated, dominant-negative Raf-1-GFP fusion proteins under the control of the CMV promoter (164). We have established that these Raf-1 mutant proteins decrease phosphorylated ERK1/2 in MIN6 β-cells as expected. EGFP cDNA, provided by Dr. Chris Proud (University of British

Columbia) was transfected as a control. Twenty-four hours after transfection, MIN6 cells were treated with appropriate media (10% serum, serum free, 0.2 nM or 200 nM insulin) for six hours. BrdU was added 2 hour prior to fixing the cells for staining. GFP-positive cells were studied 30 hours after transfection.

2.12 Analysis of programmed cell death using real-time imaging

An environmentally controlled chamber, high-throughput imaging system (KineticScan, Cellomics Inc., Pittsburgh, PA) was used to measure cell death in MIN6 cells and primary mouse dispersed β-cells treated with various inhibitors. Cell death was counted in real-time by imaging propidium iodide incorporation into nuclear DNA. This dye only crosses the plasma membrane of dying cells. Primary mouse islets were dispersed (~5,000-10,000 cells/well) 24 hours after isolation and allowed to attach in a 96 well Viewplate (Packard, Meriden, CT), centrifuged for 30 seconds at 200 rpm, and left to grow overnight before adding treatments. MIN6 cells at approximately 75% confluency (~10,000-15,000 cells/well) were seeded in a 96 well Viewplate and left to grow overnight and washed before adding treatment media. Treatments were prepared in serum-free media containing 2.5 ng/μl of propidium iodide with or without GW5074, U0126, PD98059, LY294002, Akti-1/2, TAT-Akt-In, and HNMPA. Data analysis was performed using a macro written by Dr. Dan Luciani in Microsoft Excel. Three independent cultures were analyzed per imaging run and each experimental run was performed on at least three different days.

2.13 Analysis of programmed cell death using TUNEL and DNA Ladders

The terminal deoxynucleotidyl transferase biotin-dUTP nick end labeling (TUNEL) technique was also employed to measure programmed cell death in human or mouse dispersed β-cells and MIN6 cells. Transformed MIN6 cells were seeded onto glass coverslips at 75% confluency and approximately 20 human or mouse islets were dispersed per coverslip. Cells were incubated overnight before being treated for 24 hours. After incubation with the appropriate treatments, cells were washed 3x with ice-cold phosphate-buffered-saline (PBS) before being fixed with fresh 4% paraformaldehyde at room temperature for 10 minutes, then permeabilized for 10 minutes with 0.1% Triton-X100 and blocked with DAKO Protein Block Serum-Free (Dako, Carpinteria, CA) for 30 minutes. Cells were mounted in Vectashield Medium with DAPI (Vector Labs, Burlingame, CA). Islet apoptosis was also measured using a previously described variation of a PCR-enhanced DNA laddering protocol modified for use in small numbers of islets (23, 42).

2.14 Purification of cells over-expressing Raf-1 fusion proteins by FACS

Raf-1 fusion proteins (Raf-1-GFP, Raf-1^{51-131}-GFP, and Raf-1^{51-220}-GFP were transiently over-expressed in MIN6 cells under the control of the CMV promoter. Raf-1^{51-131}-GFP, and Raf-1^{51-220}-GFP displayed a dominant-negative (DN) effect on ERK1/2 activity as previously described (164). EGFP cDNA was transfected as a control. Forty-

eight hours after transfection using Lipofectamine 2000, MIN6 cells were washed twice with PBS (minus $MgCl_2$ and $CaCl_2$) before adding trypsin, and resuspending the cell pellet in PBS with 2% fetal bovine serum. To ensure that only cells containing Raf-1 fusions were studied, GFP-positive cells were sorted by fluorescence-activated cell sorting (BD FACS Vantage SE/DIVA). The resulting cells (~200,000 cells) were washed twice with ice-cold PBS before adding lysis buffer with protease inhibitor (Cell Signalling Technology). Whole cell lysates were freeze-thawed before being subjected to Western blotting.

2.15 Insulin secretion analysis in islet perifusion

Mouse islets were cultured overnight after isolation after which groups of 100 size-matched islets were suspended with Cytodex microcarrier beads (Sigma, St Louis, MO) in the 300µl plastic chambers of an Acusyst-S perifusion apparatus (Endotronics, Minneapolis, MN). Islets were perifused at 37^{o}C and 5% CO_2 at 0.5 mL min^{-1} with a Krebs-Ringer buffer containing: 129mM NaCl, 5mM $NaHCO_3$, 4.8mM KCl, 2.5mM $CaCl_2$, 1.2mM $MgSO_4$, 1.2mM KH_2PO_4, 10mM HEPES, 3mM glucose, and 5g/L radioimmunoassay-grade BSA (Sigma). Prior to sample collection, islets were equilibrated under basal (3 mM glucose) conditions for 1 hour.

2.16 Insulin secretion analysis in glucose-stimulated insulin secretion and conditioned-media in MIN6 cells

Samples for glucose-stimulated insulin secretion were collected at time 0, 2, 15 and 30 minutes from tail and saphenous vein after intraperitoneal delivery of glucose (2 g/kg) to mice after 6 hour of fasting. Blood samples were spun down for 10 minutes in 10,000 RPM in 4^{o}C and sera were collected and stored in -20^{o}C before insulin levels were measured (Rat Insulin by an ELISA Kit, Crystal Chem Inc. Downers Grove, IL). To assess the relative contribution of endogenous insulin in our culture, we collected conditioned media from MIN6 cells treated with GW5074, Akti-1/2, UO126, and PD98059 for 3 hours. Insulin secretion was measured using a radioimmunoassay (Rat Insulin RIA Kit, Linco Research, St. Charles, MO).

2.17 Measurement of islet insulin content

Islet insulin content was measured by placing 15 size-matched islets into a 1.5 ml tube containing 100 µl Acid-Ethanol (5 µl of 0.4 mM HCl and 95 µl of 70% EtOH, pH=5) and stored at -20^{o}C over night prior to sonication. Samples were diluted 1:10, 1:500 and 1:1000 in Rat Insulin RIA kit diluent and insulin content was measured by radioimmunoassay (Rat Insulin RIA Kit, Linco Research, St. Charles, MO).

2.18 Glucose and insulin tolerance tests

Glucose and insulin tolerance tests were performed by intraperitoneal delivery of glucose (2 g/kg) or insulin (0.75 Unit/kg) to mice after 6 hour of fasting. Blood glucose was monitored for 2 h after glucose or insulin delivery. Where blood glucose levels exceeded the limit of detection for the blood glucose meter, samples were diluted 1:2 in saline.

2.19 Statistical analysis

Results are presented as mean ± SEM. Statistical significance was determined using un-paired, two-tailed Student's t-tests calculated in Excel (Microsoft, Redmond, WA). ANOVA with a Student-Newman-Keuls post-hoc test was used where appropriate. Results were considered to be statistically significant when $p < 0.05$.

Table 1: Antibodies utilized for Western Blot.

Antibody (Ab)	Size (kDa)	Manufacturer	Source/Type	1° Ab Dilution	2° Ab Dilution
Western Blot					
pASK-1 (Thr845)	155	Cell Signalling	Rabbit	1:1000	1:3000
ASK-1	155	Cell Signalling	Rabbit	1:1000	1:3000
β-actin	44	Novus	Mouse	1:10000	1:6000
Bridge-1	25	BD	Rabbit	1:1000	1:3000
Bad (Ser 112)	23	ell Signalling	Mouse	1:1000	1:3000
Bad	23	Cell Signalling	Rabbit	1:1000	1:3000
Bax	20	Cell Signalling	Rabbit	1:1000	1:3000
Bcl-2	26	Cell Signalling	Rabbit	1:1000	1:3000
Cleaved caspase 3	17/19	Cell Signalling	Rabbit	1:1000	1:3000
HOP/Gadd 153	28	anta Cruz	Rabbit	1:500	1:3000
CoxIV	17	Abcam	Rabbit	1:1000	1:3000
pERK1/2	42/44	Cell Signalling	Mouse	1:1000	1:3000

Table 1: Antibodies utilized for Western Blot.

ERK1/2	42/44	Cell Signalling	Rabbit	1:1000	1:3000
pFoxo-1, Foxo-1	78-82	Cell Signalling	Rabbit	1:1000	1:3000
Pdx-1	45	Chemicon	Rabbit	1:1000	1:3000
pRaf-1 (Ser 259 & Ser 338)	74	Cell Signalling	Rabbit	1:1000	1:3000
Raf-1	65-75	Cell Signalling	Rabbit	1:1000	1:3000
Raf-1	74	BD	Mouse	1:1000	1:3000
Raf-1	74	Epitomics	Rabbit	1:1000	1:3000

Table 2: Antibodies utilized for Immnunofluorescence Imaging.

Antibody (Ab)	Manufacturer	Source/Type	1° Ab Dilution	2° Ab Dilution
Insulin	Linco	Guinea Pig	1:750	
BrdU	Roche	Mouse	1:100	
DAPI	Vector Labs			
Draq5	Alexis Inc.		1:50	
TUNEL	Roche		1:100	
Texas Red	Jackson	Guinea Pig		1:200
FITC	Jackson	Goat α Mouse		1:200

Table 3: Summary of key phosphorylation sites on Raf-1, ERK, Akt, and Bad.

Phosphorylation Sites	Effect
Akt Ser 473	Stimulatory
Bad Ser 112	Inhibitory
ERK1/2 Thr202/Tyr204	Stimulatory
Raf-1 Ser 259	Inhibitory
Raf-1 Ser 338	Stimulatory

Table 4: Primers used for genotyping the RIP-Cre mouse line and to detect Raf transcripts.

Primers	Sequence
RIP Cre-DS	CTA ATC GCC ATC TTC CAG CAG G
RIP Cre-US	AGG TGT AGA GAA GGC ACT CAG C
Raf-1 DS Lox	AAC ATG AAG TGG TGT TCT CCG GGC GCC
Raf-1 US Lox	TGG CTG TGC CCT TGG AAC CTC AGC ACC
A-Raf Forward	AGCATCCAGGATCTGTCTGG
A-Raf Reverse	ACCTGCATGAGGCTGGAGTC
B-Raf Forward	GGCCAGGCTCTGTTCAATG
B-Raf Reverse	CTCTTTGCTGAAGGGCATCT
Raf-1 Forward	AGTTAGAGCCGAGCGGACTT
Raf-1 Reverse	AGTCCAAAGCCATTGCTGAT

3. Results I: Acute and chronic insulin signalling in pancreatic β-cells are mediated by multiple Raf-1 dependent pathways

3.1 Effects of insulin in primary β-cell survival in vitro

Insulin is a potent growth factor in many cell types. Exposing pancreatic β-cells to low concentrations of insulin can initiate many complex signals such as Ca^{2+} oscillations and activation of proteins required to protect islets from cell death (42, 102), but the functional significance of blocking insulin signalling in the β-cell remains controversial. Our own work suggested that insulin could prevent apoptosis, as indicated by DNA fragmentation, in serum-starved human and mouse islets *in vitro* (42). To test the functional effects of blocking endogenous insulin receptor signalling in primary mouse islets, we treated dispersed mouse islet cells with HNMPA-AM, a commercially available drug known to inhibit insulin receptor tyrosine autophosphorylation (165) and showed the real-time incorporation of propidium iodide, an intercalating agent taken up by cells which have lost membrane integrity (Figure 2A). Although HNMPA-AM was specifically designed to block the insulin receptor (165, 166), one cannot discount its effects on IGF-I signalling especially at high concentrations. Indeed, 100 uM of HNMPA-AM had been shown to block IGF-1 signalling in choriocarcinoma cell line JEG-3 (167), but remains to be tested in primary dispersed β-cells. We have examined basal concentrations of insulin in our cell culture on day 0 and day 3 to be 0 nM and 1.5 nM respectively (42). At these concentrations, we would anticipate little contribution of IGF-I-

mediated *vs.* insulin receptor-mediated signalling. It is also unlikely that dispersed β-cells are making and secreting large amounts of IGF-1, therefore, insulin is likely to be the more relevant local factor in our cell culture. In the present study, we conclude that blocking endogenous insulin signalling with HNMPA-AM caused robust cell death in a time and concentration-dependent manner supporting previous findings that insulin signalling plays an anti-apoptotic role in β-cells *in vitro* (42). Indeed, βIRKO mice show reduced pancreatic insulin content and islet number and β-cell mass, as well as an increase in apoptosis (84-86). Next, we tested whether various concentrations of insulin could prevent caspase-3 activation, a critical event in apoptosis that occurs before DNA fragmentation. We found that low concentrations of insulin, but not high concentrations, prevented caspase-3 cleavage in isolated human islets (Figure 2B). Together, these results indicate that low concentrations of insulin promote pancreatic β-cell survival.

3.2 Effects of insulin signalling on Akt in primary islets

Evidence from *in vivo* studies strongly suggested that insulin signalling is necessary to increase pancreatic β-cell mass both in the normal physiological condition and the insulin resistant state (42, 85, 86). However, the signal transduction mechanisms downstream of β-cell insulin receptors are not well understood and remain controversial. Akt has been suggested to be a critical component of both insulin and insulin-like growth factor signalling in β-cells (53). In the present study, we examined the effects of various concentrations of insulin on Akt phosphorylation and total Akt protein levels in primary islets. Interestingly, we did not observe sustained Akt phosphorylation

or robust increase in Akt protein level in mouse islets treated with insulin or serum for 48 hours (Figure 3A,B). As a positive control for the known acute effects of high concentrations of insulin and insulin-like growth factor (IGF-1), we also examined Akt phosphorylation after 15 minutes. In this experiment, both 200 nM insulin and 100 nM IGF-1 induced rapid Akt phosphorylation, whereas a concentration of 0.2 nM insulin had no effect on Akt phosphorylation at serine 473 residue (Figure 3C,D). This result suggests that 0.2 nM insulin, a concentration we found to have an anti-apoptotic role *in vitro* (42) and one that cannot cross-activate the IGF-1 receptor, may not stimulate Akt in primary β-cells. Together, these data suggest that signalling via physiological concentrations of insulin may work through alternative pathways in β-cells.

3.3 Effects of insulin on Pdx-1, Bridge-1, and Foxo-1

Potential antiapoptotic downstream targets of insulin signalling were evaluated next. We focused on the transcription factor Pdx-1 because it is required for pancreas development and differentiation (168). Pdx-1 also mediates the effects of pro-survival factors in adult β-cells *in vitro* (169)(42) and *in vivo* (89). Mice lacking one allele of Pdx-1 have increased islet apoptosis (170). Acting as a transcription factor, Pdx-1 translocation to the nucleus is a critical event for its activity (171). In pancreatic β-cells, nuclear translocation of active Pdx-1 increases its DNA-binding activity and insulin promoter activity (171, 172). For example, high glucose (20 mM) has been demonstrated to increase Pdx-1 (46kDa) in the nucleus of human and MIN6 β-cells by

Western blot (171). Through immunofluorescence imaging, we have shown that 0.2 nM insulin increased Pdx-1 localization in the nucleus compared with untreated controls (42). Thus, we tested whether insulin-induced Pdx-1 nuclear localization in mouse β-cells is associated with increased protein expression of Pdx-1. Indeed, 0.2 nM insulin caused a modest increase in active Pdx-1 protein in islets incubated under serum free conditions for 3 days (Figure 4A,B). A proteomics analysis uncovered Bridge-1 as a potential target of autocrine insulin signalling (42). Therefore, we examined the protein expression of Bridge-1, a positive binding partner of Pdx-1 (173). Both low and high concentrations of insulin increased total Bridge-1 protein expression (Figure 4C,D), suggesting that a high level of Bridge-1 is not always associated with β-cell survival. Indeed, overexpression of Bridge-1 caused a reduction in insulin expression and diabetes in a mouse model (174). Preliminary data suggest that insulin may not significantly alter the cytosolic localization of Bridge-1 (Figure 4E,F) or enhance its binding affinity to Pdx-1 as shown in our co-immunoprecipitation studies (Figure 4G). Together, our results indicate that insulin increases the expression of both Pdx-1 and Bridge-1, and thus may influence the pool and localization of these transcription factors to enhance β-cell survival.

One mechanism whereby insulin/Akt promotes survival is by the phosphorylation of the Forkhead transcription factor Foxo-1 (175), a repressor of the Pdx-1 promoter (176). Foxo-1 and Pdx1 exhibit mutually exclusive patterns of nuclear localization in β-cells (176). Thus, constitutive nuclear expression of a mutant Foxo-1 is associated with lack of Pdx-1 expression in the nucleus (176). In the present study, we examined the

protein expression and phosphorylation status of Foxo-1. In our experiments, insulin treatment did not result in a robust change in Foxo-1 phosphorylation or total protein levels (Figure 5A). In preliminary data we did observe a modest decrease in the percentage of β-cells exhibiting nuclear Foxo-1 staining in insulin-treated cultures, relative to serum-free conditions (Figure 5B). Together, these results suggest the possibility that sub-cellular location of Foxo-1 may participate in antiapoptotic insulin signalling in primary β-cells.

3.4 Identification and relative expression of Raf-1 kinase in β-cells

Despite its potential importance in pro-survival signalling, neither the presence nor the sub-cellular localization of Raf-1 have been directly assessed in pancreatic β-cells to our knowledge. First, we examined the gene expression of the three members of the Raf family kinase in MIN6 cells and mouse islets by RT-PCR and determined that all three isoforms were present (Figure 6A). Both B-Raf and Raf-1 were identified in human islets by immunohistochemistry and immunoblotting respectively (64). Due to the predominant anti-apoptotic role of Raf-1, we focused on this isoform and confirmed its protein expression in primary mouse and human islets and MIN6 cells using Western blotting (Figure 6B). Although these results demonstrate that Raf-1 is present in islets, they do not provide information regarding the β-cell specific expression or sub-cellular localization of this protein. To address this, we used immunofluorescence imaging. We observed that endogenous Raf-1 is expressed in dispersed β-cells from both mouse and human islets and in MIN6 cells (Figure 6C). In primary islet cells, Raf-1 was

localized in the cytoplasm and the nucleus, which was consistent with studies in other cell types (117). In MIN6 cells, we found Raf-1 immunoreactivity primarily in the cytoplasm, although some cells also exhibited nuclear staining. Together these findings indicate that Raf-1 is present in primary and transformed pancreatic β-cells.

3.5 Direct effects of Raf-1 on β-cell proliferation

Insulin has also been implicated in β-cell proliferation both *in vitro* and *in vivo* (44, 86). In the present study, we demonstrated that blocking Raf-1 signalling in β-cells using a small-molecule inhibitor blunted the effects of exogenous insulin, especially at the low concentration of insulin (44) (see Chapter 4). To further elucidate the role of Raf-1 in β-cell proliferation, we transiently overexpressed fusion proteins containing full-length Raf-1 and dominant-negative truncation mutants in MIN6 cells. Compared to EGFP-expressing control cells, MIN6 cells from cultures overexpressing Raf-1 had a significant increase in proliferation in serum-free conditions (Figure 7A). Notably, this was sufficient to increase proliferation to the same level as insulin. No increase in proliferation was observed in Raf-1-overexpressing cells cultured in insulin or serum, indicating that the effects of Raf-1 expression are not additive to those of insulin or serum. Conversely, expression of truncated dominant-negative Raf-1-GFP fusion protein had inhibitory effects on the total number of cells in each culture (see Chapter 4, Figure 19) and on the proliferation of GFP-positive cells in the presence of 0.2 nM insulin (Figure 7B). Therefore, the effects of the mutant Raf-1 protein lacking the kinase domain are similar to the effects of the chemical Raf-1 kinase inhibitor (164). The activation of Raf-1 at

serine 338 was also maximal at 0.2nM insulin in primary mouse islets (Figure 7C). Together, these results demonstrate that Raf-1 kinase-dependent pathways are important for the proliferative effects of insulin on primary mouse β-cells, and that Raf-1 appears to be specifically involved in mediating insulin signals at concentrations of the hormone expected to activate the insulin receptor, but not the IGF-1 receptor (177). Together, these results strongly implicate Raf-1 in the control of β-cell proliferation.

3.6 Insulin induces Raf-1 and ERK1/2 activation in primary islets and MIN6 cells

Insulin supports sufficient pancreatic β-cell mass by increasing the proliferation and enhancing the survival of β-cells (42, 85, 86). We have mentioned above that a low concentration of insulin (0.2 nM) was more effective at preventing β-cell apoptosis than higher concentrations (42, 178). Unfortunately, little is known about the signalling pathways activated by low concentrations of insulin, specifically those concentrations that would not activate IGF-1 receptors (179). Given our previous observations suggesting that low concentrations of insulin may not activate Akt (42), we hypothesized that insulin might activate Raf-1 in β-cells. In primary mouse islets, we found that insulin caused Raf-1 dephosphorylation at serine 259 (Figure 8A), an event which is known to initiate activation of this kinase (93). Subsequent to the dephosphorylation of Raf-1 at serine 259, we observed a significant increase in phosphorylation of the stimulatory site of Raf-1 at serine 338 (Figure 8B), suggesting activation of this kinase (180). To

determine the outcome of Raf-1 activation, we investigated the phosphorylation status of ERK1/2, the canonical downstream target of Raf-1 (58). Insulin increased ERK1/2 phosphorylation in mouse islets (Figure 8C). We observed a similar activation of Raf-1 and ERK1/2 by insulin in human islets (Figure 8D, E). In our preliminary experiments, 0.2 nM IGF-1 did not induce statistically significant ERK1/2 phosphorylation in primary mouse islets (Figure 8F,G), suggesting that insulin may be more potent in activating ERK1/2 at this concentration. These are the first data demonstrating that insulin rapidly activates both Raf-1 and ERK1/2 in primary islets. In each of these cases, the effects of 0.2 nM insulin were robust and reproducible. Higher concentrations of insulin showed more variable responses and were not consistently more effective than lower concentrations. These observations mirror previous findings that β-cell survival and proliferation are selectively enhanced by 0.2 nM insulin relative to higher concentrations (42).

Next, we examined the requirement of Raf-1 for the activation of ERK1/2 by insulin, as well as the concentration-dependence and time-course of this event. It should be noted that a Raf inhibitor (GW5074) and a dominant-negative Raf-1 mutant robustly inhibited basal ERK1/2 phosphorylation (see Chapter 4.3). Importantly, blocking Raf-1 kinase activity with GW5074 completely prevented insulin-induced ERK1/2 phosphorylation (Figure 9A). However, this inhibitor has been shown to also block B-Raf and other protein kinases at high concentrations (181). We also employed a dominant-negative Raf-1 mutant Raf-1^{51-131}-GFP and found that ERK1/2 phosphorylation induced by 0.2 nM insulin was similarly blocked (Figure 9B). Together,

these results are consistent with a critical role for Raf-1 in insulin-induced ERK1/2 activation.

We also examined the rapid kinetics of Raf-1 and ERK1/2 stimulation in MIN6 cells (Figure 9C,D). The temporal profile of Raf-1 activation was sustained. We observed significant phosphorylation on Raf-1 at serine 338 after 1 and 30 minutes with insulin and a trend towards significance after 5,10, and 15 minutes with insulin treatment. However, we only noted a trend towards significance on ERK1/2 phosphorylation. Thus, it appears that ERK1/2 activation may be transient in MIN6 cells. In many cell types including the β-cell, ERK1/2 phosphorylation in the cytoplasm is immediately followed by its translocation into the nucleus where it initiates the transcription of pro-survival proteins (182-184). MIN6 cells treated with insulin showed an increased number of cells with phosphorylated ERK1/2 in the nucleus (Figure 9E,F). These data suggest that insulin rapidly activates Raf-1, and its canonical downstream target, the ERK1/2 pathway in the β-cell.

3.7 Role of autocrine insulin signalling in glucose-induced ERK1/2 phosphorylation

Glucose has been shown to induce ERK1/2 phosphorylation in islets as well as in β-cell lines (182, 185). High glucose transiently induced ERK1/2 activation within 15

minutes (Figure 10A), after which ERK1/2 phosphorylation returned to baseline within 30 minutes in MIN6 cells (Figure 10B). However, since glucose is a potent insulin secretagogue, we tested whether these effects might be due in part to autocrine insulin signalling or whether they were mediated by an insulin-independent mechanism. We incubated MIN6 cells at 5 mM glucose or 25 mM glucose in the presence or absence of 1 μM somatostatin, which blocks glucose-induced insulin secretion. Glucose-induced ERK1/2 phosphorylation was completely blocked by somatostatin (Figure 10A), consistent with a requirement for insulin secretion for ERK1/2 activation. Similarly, $Ins1^{-/-}$:$Ins2^{+/-}$ mice (186, 187) with ~50% reduced insulin secretion *in vivo* (A. Mehran and J. Johnson, unpublished) and *in vitro* (Figure 10C, insulin secretion levels) displayed a ~50% decrease on ERK1/2 activation induced by high glucose, when compared with islets from phenotypically normal $Ins1^{-/-}$:$Ins2^{+/+}$ mice (Figure 10D). While additional experiments are required to elucidate the mechanism of glucose-stimulated ERK1/2 activation, these findings implicate insulin feedback signalling as a factor in the mitogenic effects of acute hyperglycemia and provides the basis for detailed future studies.

3.8 Raf-1 and Bcl-2 protein families in pancreatic-β-cells

An ERK1/2-independent mechanism involving Bcl-2-mediated targeting of Raf-1 to the mitochondria has been described (142). In this paradigm, Raf-1 is thought to act at the mitochondria to phosphorylate Bad at serine 112, which then inactivates the pro-

apoptotic Bad protein by tethering it to 14-3-3 proteins in the cytoplasm (188). A large fraction of Raf-1 localizes to the mitochondria in β-cells. In the present study, we observed a moderate but significant increase in mitochondrial Raf-1 localization in β-cells treated with 0.2 nM insulin compared to serum-free treated β-cells (Figure 11 A,B). Insulin also had clear effects on mitochondrial morphology, inducing networks of rod and thread-shaped mitochondria, which contrasted with the clustered and rounded mitochondria seen in serum-free conditions (Figure 11A). Furthermore, we detected increased phosphorylated Raf-1 at serine 338 in the mitochondrial fraction of MIN6 cells treated with insulin (Figure 11C). Using immunofluorescence imaging, endogenous Raf-1 and Bad exhibited overlapping staining patterns in human β-cells and mouse β-cells (Figure 12A). This association was specific for Raf-1, as the related kinase B-Raf showed little co-localization with Bad (Figure 12B). Using immunoprecipitation, we confirmed the close physical proximity of these two proteins with Bcl-2, Bcl-xL and Bad antibodies pulling down endogenous Raf-1 (Figure 13A). Moreover, insulin increased the protein-protein interaction between Raf-1 and Bad (Figure 13B). Since Raf-1 inhibitor causes a dramatic loss of Bad phosphorylation at serine 112 (Chapter 4.2), we would expect insulin to have the converse effect. Indeed, primary mouse islets treated with insulin showed an increase in Bad phosphorylation at serine 112 (Figure 13C), a step that prevents Bad from performing its pro-apoptotic actions. Our findings strongly suggest that insulin modulates Bad-dependent apoptosis via Raf-1 in β-cells.

3.9 Ca^{2+} signals are required for insulin-induced ERK1/2 phosphorylation

In human and mouse islet β-cells, acute treatment with insulin stimulates Ca^{2+} release from intracellular stores (102, 189, 190). We further confirmed the presence of these previously described Ca^{2+} signals (102) in MIN6 cells using both conventional Ca^{2+} dyes and fluorescent protein-based Ca^{2+} indicators (103). We observed both small cytosolic Ca^{2+} signals were evident in human β-cells or MIN6 cells exposed to increasing concentrations of insulin, as we have demonstrated previously with mouse β-cells (190). Next, we addressed whether these Ca^{2+} signals were required for insulin-induced ERK1/2 phosphorylation by treating β-cells with the Ca^{2+} chelator BAPTA-AM. BAPTA-AM blocked the activation of ERK1/2 by insulin (Figure 14A). We did not observe significant changes in total ERK1/2 in cells treated with BAPTA-AM for 10 minutes (Figure 14A). Chronic treatment with BAPTA-AM mimicked the effects of the Raf inhibitor, reducing both ERK1/2 and Bad phosphorylation and increasing cell death in a manner that was not additive to Raf-1 inhibition alone (Figure 14B,C,D,E,F). These data further support that insulin stimulates cytosolic Ca^{2+} signals and demonstrate that these anti-apoptotic signals are required for the activation of ERK1/2.

Insulin-stimulated Ca^{2+} signals are initiated by NAADP-sensitive Ca^{2+} stores (102) and reduced in mice lacking CD38, an enzyme responsible for generating some of the cellular NAADP (190). Here, we utilized a new NAADP receptor antagonist, Ned-19 (159) to assess the role of these Ca^{2+} stores in ERK1/2 phosphorylation. Ned-19 reduced the effects of insulin on ERK1/2 phosphorylation (Figure 14G). These data are the first to directly implicate NAADP-sensitive Ca^{2+} stores on ERK1/2 activation in any

cell type. NAADP-sensitive stores are thought to reside in acidic organelles, such as lysosomes (159) or VAMP-containing granules (191). Additional studies on the role of acidic organelles in autocrine insulin signaling are therefore warranted.

Our results with BAPTA-AM and Ned-19 pointed to Ca^{2+}-dependent regulation of Raf-1 and ERK1/2 phosphorylation status. However, BAPTA-AM alone can have effects on ERK1/2 phosphorylation and events associated with ER-stress (192, 193). Thus, we sought to further examine the role of Ca^{2+} on the regulation of ERK1/2 phosphorylation using inhibitors of a Ca^{2+} dependent kinase and a Ca^{2+} phosphatase. CaMKII is known to mediate the activation of Raf-1 and ERK1/2 by Ca^{2+} in other cell types (185, 194). The effects of insulin on ERK1/2 phosphorylation were abrogated by the inhibitor of CaMKII and KN93 (Figure 14H,I). KN93 did not change the amount of total ERK1/2 after 10 minutes of treatment. As noted above, the activation of ERK1/2 by insulin also required the dephosphorylation of serine 259 on Raf-1, pointing to a Ca^{2+}-dependent phosphatase such as calcineurin. Indeed, the clinically relevant calcineurin inhibitor FK506 blocked insulin-induced ERK1/2 phosphorylation (Figure 14H). These data further indicate the importance of Ca^{2+} signalling, and point to CaMKII and calcineurin as critical upstream regulators of Raf-1/ERK1/2 signalling in β-cells.

Figure 2

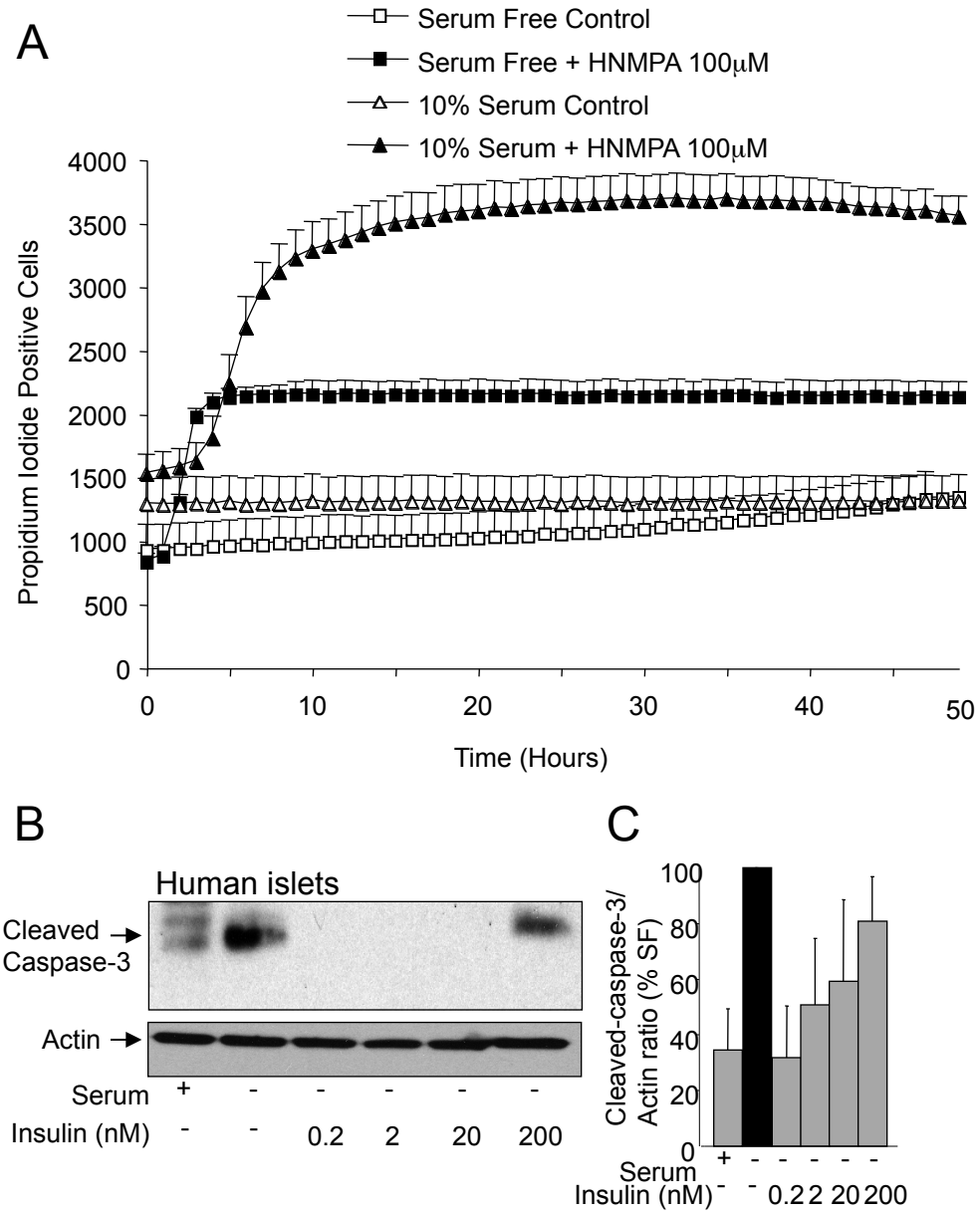

A
Serum Free Control
Serum Free + HNMPA 100μM
10% Serum Control
10% Serum + HNMPA 100μM
Propidium Iodide Positive Cells
4000
3500
3000
2500
2000
1500
1000
500
0
0
10
20
30
40
50
Time (Hours)
B
Human islets
Cleaved Caspase-3
Actin
Serum + - - - - -
Insulin (nM) - - 0.2 2 20 200
C
Cleaved-caspase-3/ Actin ratio (% SF)
100
80
60
40
20
0
Serum + - - - - -
Insulin (nM) - - 0.2 2 20 200

Figure 2. **Effects of endogenous insulin signalling in primary β-cell survival.** (A) Blocking endogenous insulin receptor autophosphorylation with HNMPA-AM caused a dose and time-dependent death in dispersed primary islet cells. Three independent cultures were analyzed per imaging run and each experimental run was performed on at least three different days. (B) Low doses of insulin selectively reduced cleaved caspase-3 in human islets. (C) Quantification of 3 Western blots from different cultures.

Figure 3

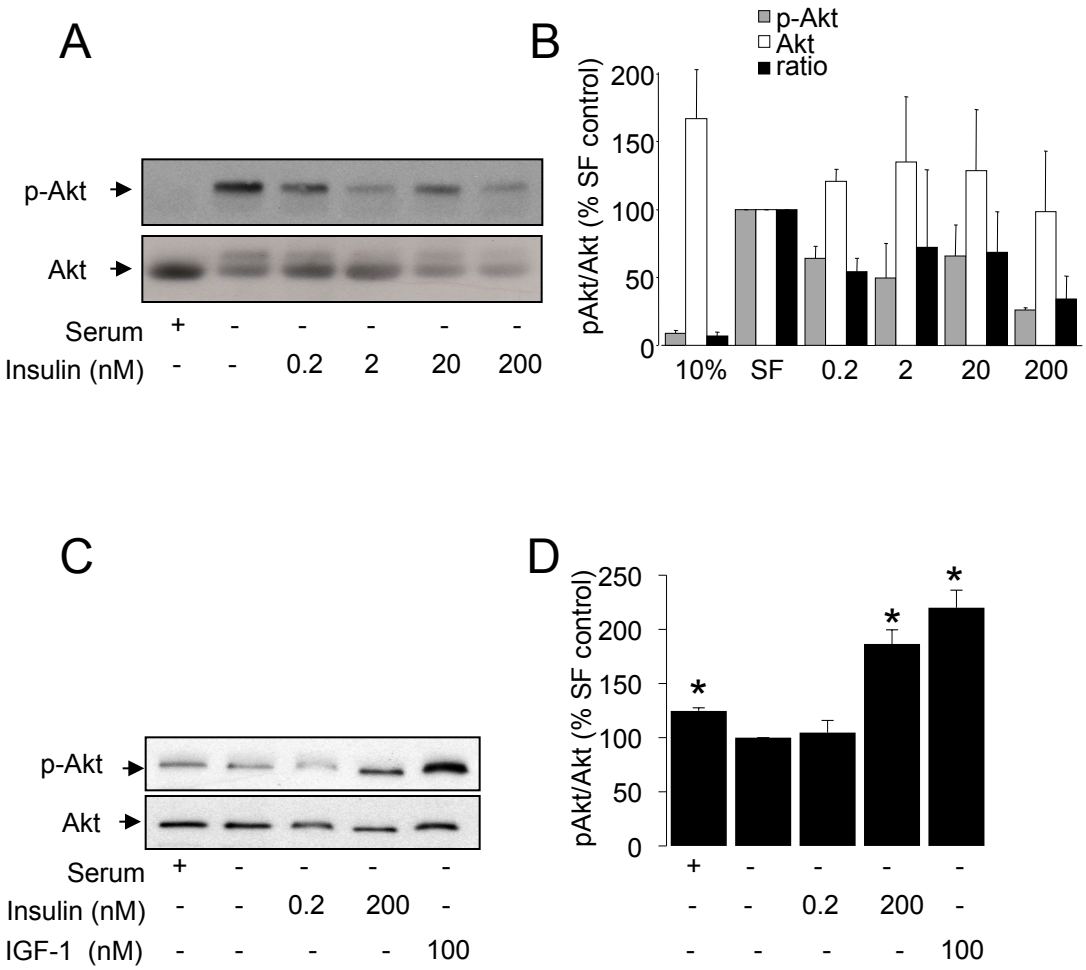

A
p-Akt
Akt
Serum + - - - - -
Insulin (nM) - - 0.2 2 20 200
B
p-Akt
Akt
ratio
pAkt/Akt (% SF control)
200
150
100
50
0
10% SF 0.2 2 20 200
C
p-Akt
Akt
Serum + - - - -
Insulin (nM) - - 0.2 200 -
IGF-1 (nM) - - - - 100
D
pAkt/Akt (% SF control)
250
200
150
100
50
0
*
*
*
+ - - - -
- - 0.2 200 -
- - - - 100

Figure 3. **Role of Akt in anti-apoptotic insulin signalling.** (A) Akt phosphorylation and total levels in mouse islets cultured for 2 days with various concentration of insulin. (B) Quantification of 3 Western blots from independent cultures. (C) Acute insulin and IGF-1 treatment (15 minutes) in primary mouse islets. (D) Quantification of 3 Western blots from independent mouse islet cultures. *Denotes significant difference ($p<0.05$, Student's *t* test) between the serum-free control and treatment.

Figure 4

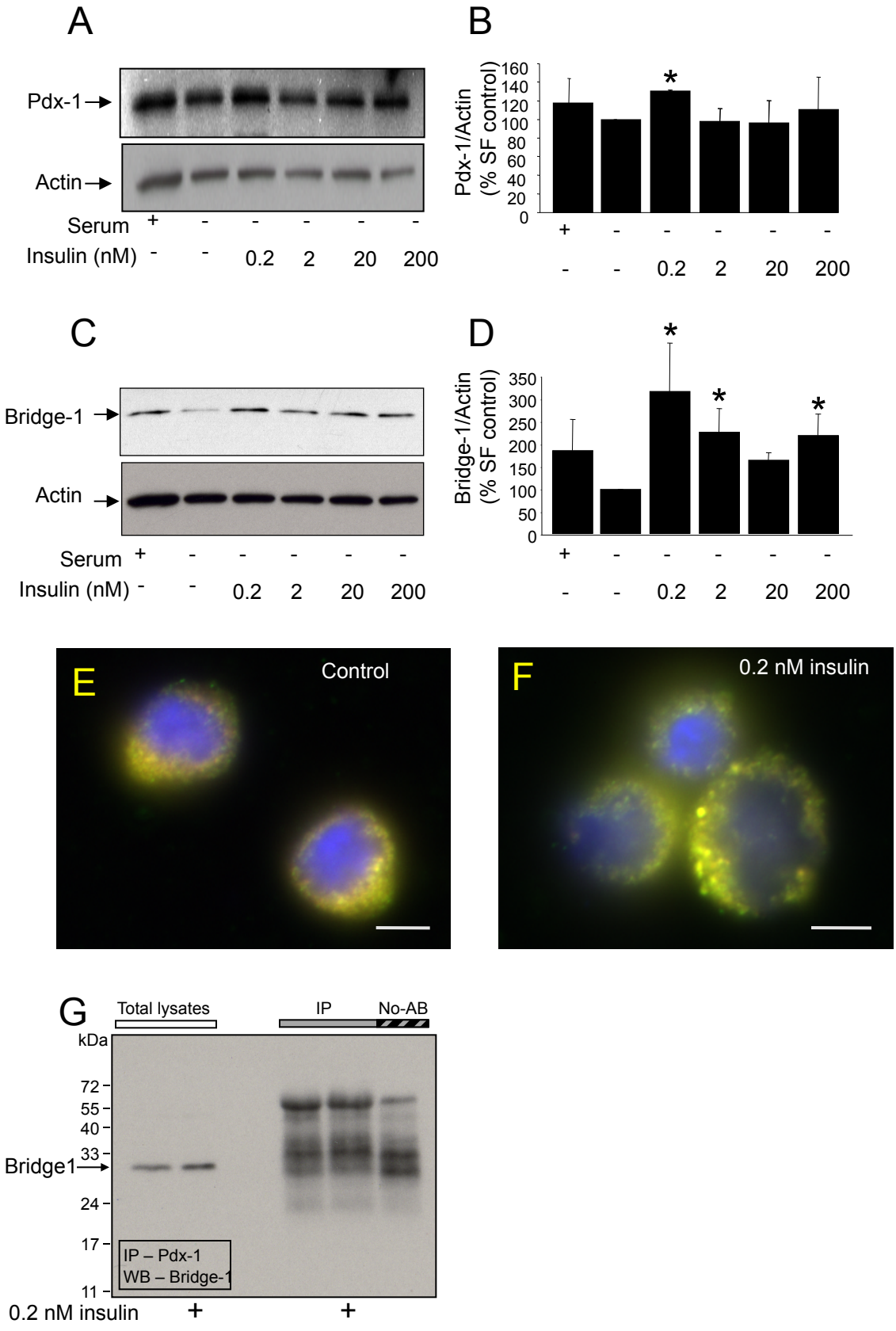

A
Pdx-1
Actin
Serum + - - - - -
Insulin (nM) - - 0.2 2 20 200
B
Pdx-1/Actin (% SF control)
160
140
120
100
80
60
40
20
0
*
+ - - - - -
- - 0.2 2 20 200
C
Bridge-1
Actin
Serum + - - - - -
Insulin (nM) - - 0.2 2 20 200
D
Bridge-1/Actin (% SF control)
350
300
250
200
150
100
50
0
*
*
*
+ - - - - -
- - 0.2 2 20 200
E
Control
F
0.2 nM insulin
G
Total lysates
IP
No-AB
kDa
72
55
40
33
24
17
11
Bridge1
IP – Pdx-1
WB – Bridge-1
0.2 nM insulin + +

Figure 4. **Effects of insulin on Pdx-1 and Bridge-1 protein expression and sub-cellular localization and interaction.** *(A*) Western blot of Pdx-1 and β-actin in mouse islets treated with insulin for 3 days. Pooled densitometry data from three independent experiments indicated that Pdx-1 protein expression in 0.2 nM insulin was increased compared to serum-free control. (*B*) Pooled densitometry results from three independent experiments are quantified. (*C*) Western blot analysis of Bridge-1 expression in mouse islets cultured for 3 days as indicated. (*D*) Pooled densitometry results from four independent experiments are quantified. (E and F) Merged immunofluorescence images of Bridge-1 (green), insulin (red), and DAPI (blue) staining in dispersed mouse β-cells treated with serum-free media (control) or 0.2 nM insulin for 3-4 days as indicated. Similar results were seen with 2-200 nM insulin and serum-containing media. Data shown is a representative of four independent experiments. (G) Co-immunoprecipitation of Bridge-1 with Pdx-1 in the presence of insulin was performed as described in Materials and Methods (n=5 different cultures). Reprobing the membrane with anti-Pdx-1 antibody demonstrated that equal amounts of Pdx-1 were immunoprecipitated. *p< 0.05 using Student's *t* test. Scale bars, 5 μm.

Figure 5

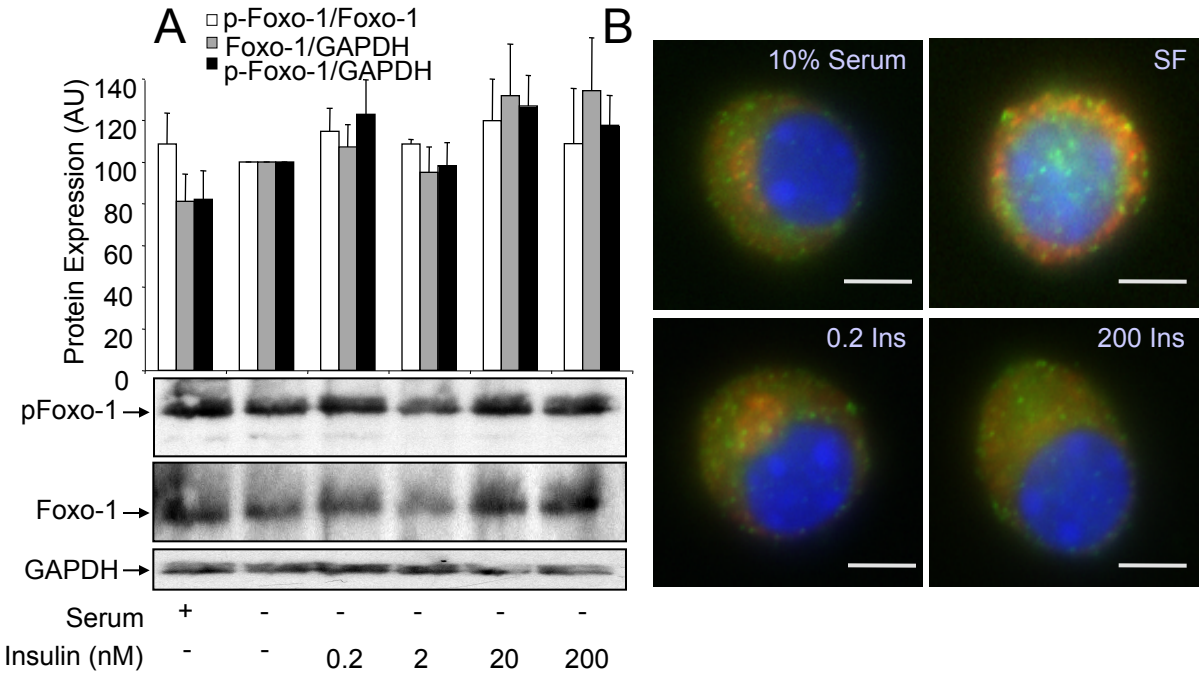

A
p-Foxo-1/Foxo-1
Foxo-1/GAPDH
p-Foxo-1/GAPDH
Protein Expression (AU)
140
120
100
80
60
40
20
0
pFoxo-1
Foxo-1
GAPDH
Serum + - - - - -
Insulin (nM) - - 0.2 2 20 200
B
10% Serum
SF
0.2 Ins
200 Ins

Figure 5. **Effects of insulin on Foxo-1 protein expression and sub-cellular localization.** (A) Western blot of phosphorylated Foxo-1, Foxo-1, and GAPDH in isolated primary mouse islets. Results are quantified by densitometry using Photoshop (n=5 Western blots from different cultures). (B) Triple immunofluorescence labeling of Foxo-1 (green), insulin (red), and nuclei (blue) in dispersed islet cells cultured for 3 days in 10% serum (normal media), serum-free media, 0.2 nM insulin, or 200 nM insulin. Image shown is a representative of cells from 3 different cultures. Scale bars, 5 μm.

Figure 6

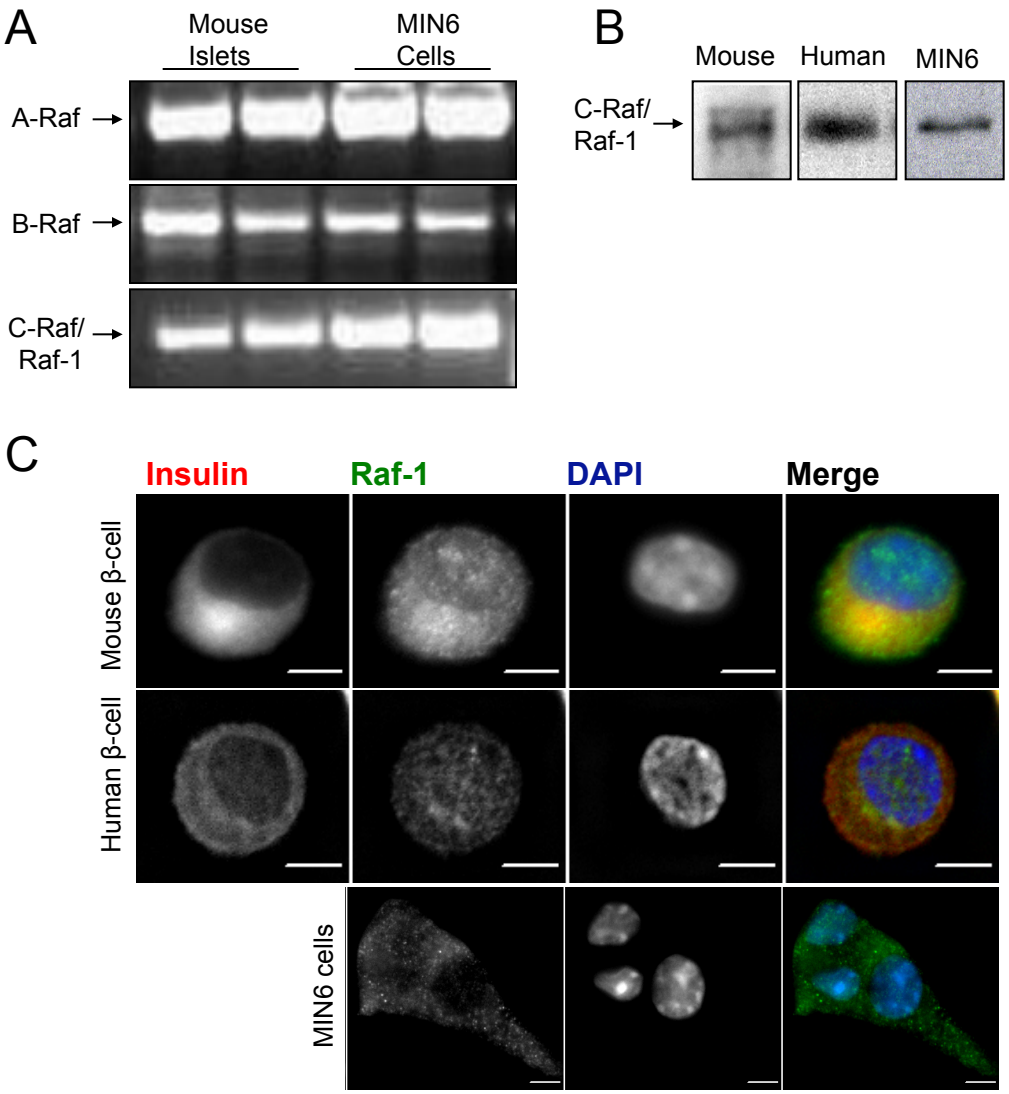

A
Mouse Islets
MIN6 Cells
A-Raf
B-Raf
C-Raf/ Raf-1
B
Mouse
Human
MIN6
C-Raf/ Raf-1
C
Insulin
Raf-1
DAPI
Merge
Mouse β-cell
Human β-cell
MIN6 cells

Figure 6. **Expression of Raf isoforms in pancreatic β-cells.** (A) Total RNA was extracted from mouse islets and MIN6 cells and gene expression levels were analyzed by semi-quantitative RT-PCR (n=3 different cultures). (B) Raf-1 protein expression was detected in human and mouse islets as well as in MIN6 cell line using Western blot (n=3 different cultures). (C) Immunofluorescence imaging of Raf-1 in mouse β-cells, human β-cells, and MIN6 cells dispersed islet cells showing Raf-1 localization in the cytoplasm and the nucleus (>1000 cells examined from 3 different cultures). Scale bars, 5 μm.

Figure 7

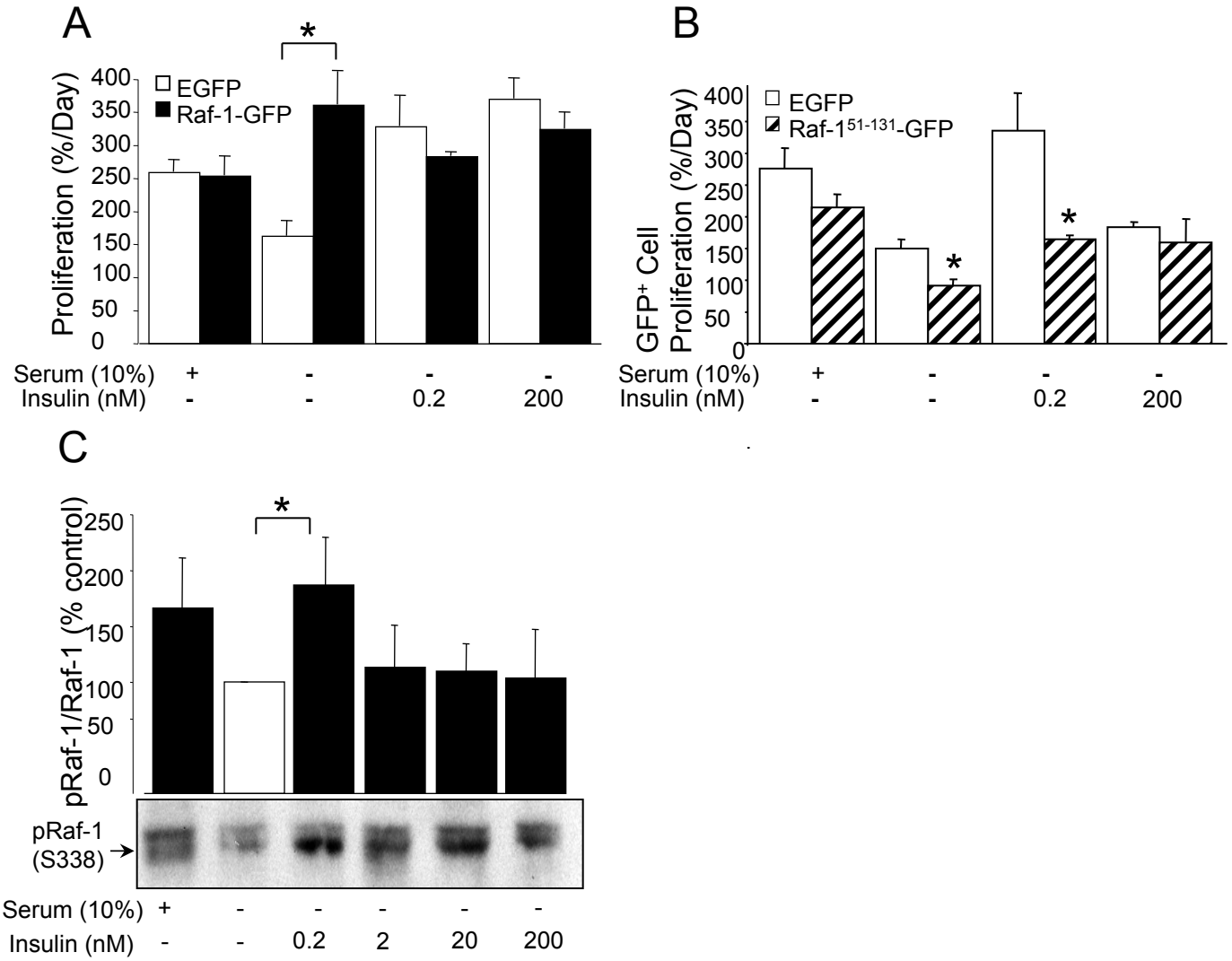

A
*
EGFP
Raf-1-GFP
Proliferation (%/Day)
400
350
300
250
200
150
100
50
0
Serum (10%) + - - -
Insulin (nM) - - 0.2 200
B
EGFP
Raf-1^51-131-GFP
GFP+ Cell Proliferation (%/Day)
400
350
300
250
200
150
100
50
0
*
*
Serum (10%) + - - -
Insulin (nM) - - 0.2 200
C
*
pRaf-1/Raf-1 (% control)
250
200
150
100
50
0
pRaf-1 (S338)
Serum (10%) + - - - - -
Insulin (nM) - - 0.2 2 20 200

Figure 7. **Effects of wild type and mutant Raf-1 on β-cell proliferation.** (A) MIN6 cells transfected with EGFP (control) or Raf-1-GFP were treated with insulin or 10% serum. Cells were cultured for six hours and percent proliferation was quantified from all cells using BrdU immunofluorescence in the red channel (n=3 different cultures). (B) Proliferation in MIN6 cells transfected with dominant-negative Raf-1 fusion protein (i.e. only GFP-positive cells), treated as indicated (n=3 different cultures). (C) Mouse islets were treated with insulin for 48 hour and phosphorylation of Raf-1 on serine 338 was examined and normalized to total Raf-1/glyceraldehyde-3-phosphate dehydrogenase (GAPDH) control (n=4 different Western blots from different cultures). *Denotes significant difference ($p<0.05$, Student's *t* test) between EGFP and Raf-1 fusion proteins or serum-free control and treatment.

Figure 8

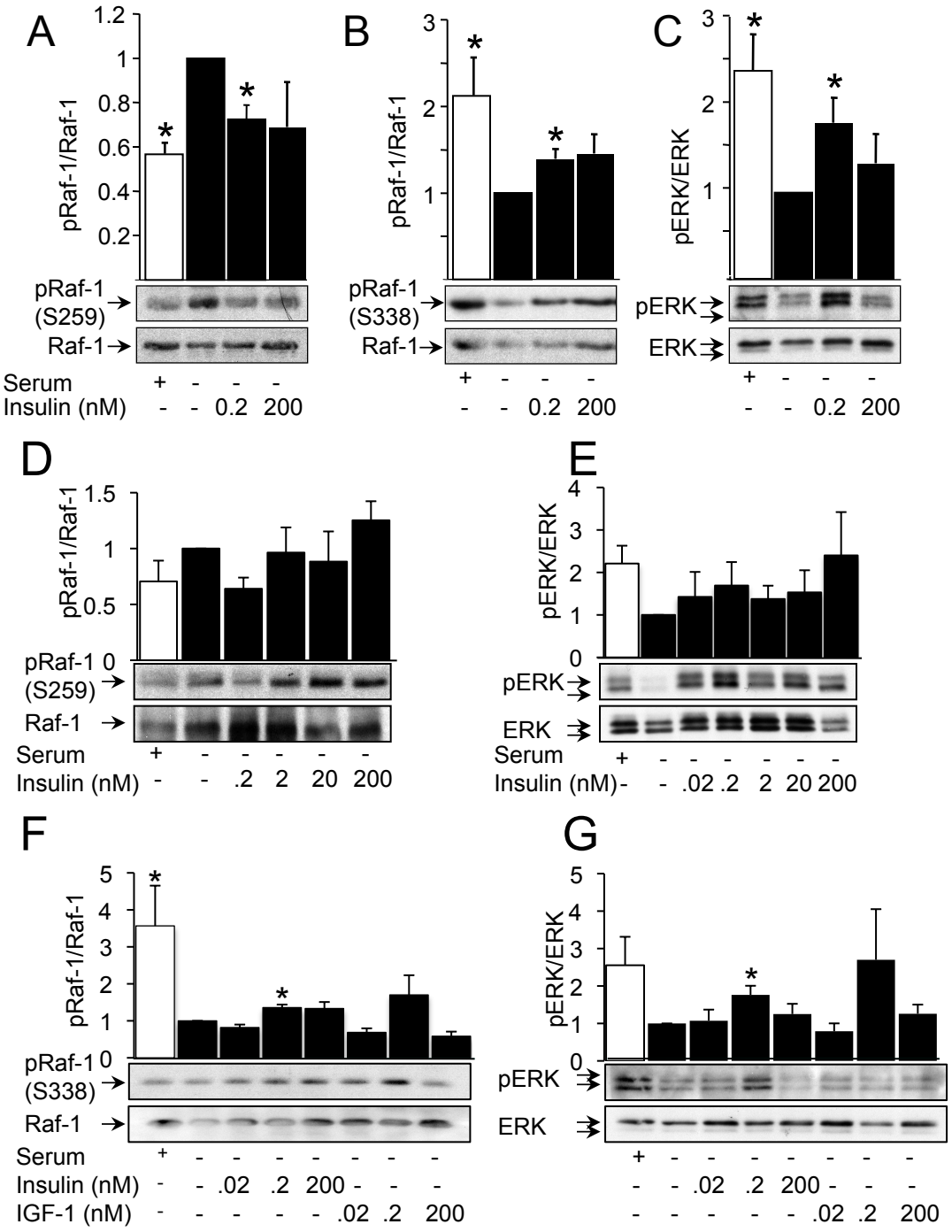

A
pRaf-1/Raf-1
1.2
1
0.8
0.6
0.4
0.2
pRaf-1 (S259)
Raf-1
Serum + - - -
Insulin (nM) - - 0.2 200
B
pRaf-1/Raf-1
3
2
1
pRaf-1 (S338)
Raf-1
+ - - -
- - 0.2 200
C
pERK/ERK
3
2
1
pERK
ERK
+ - - -
- - 0.2 200
D
pRaf-1/Raf-1
1.5
1
0.5
0
pRaf-1 (S259)
Raf-1
Serum + - - - - -
Insulin (nM) - - .2 2 20 200
E
pERK/ERK
4
3
2
1
0
pERK
ERK
Serum + - - - - - -
Insulin (nM) - - .02 .2 2 20 200
F
pRaf-1/Raf-1
5
4
3
2
1
0
pRaf-1 (S338)
Raf-1
Serum + - - - - - - -
Insulin (nM) - - .02 .2 200 - - -
IGF-1 (nM) - - - - - .02 .2 200
G
pERK/ERK
5
4
3
2
1
0
pERK
ERK
+ - - - - - - -
- - .02 .2 200 - - -
- - - - - .02 .2 200

Figure 8. **Insulin promotes Raf-1 and ERK1/2 activation in primary islets.** (A) Acute insulin signalling stimulation for 15 minutes in primary mouse islets with 0.2 nM and 200 nM insulin resulted in a loss of inhibitory phosphorylation of Raf-1 at serine 259. (B) Mouse islets treated with 0.2 nM and 200 nM insulin for 30 minutes also caused an increase of the stimulatory phosphorylation of Raf-1 at serine 338 and a pro-survival phosphorylation of ERK1/2 (C). (D) Acute 0.2 nM insulin treatment (30 minutes) in human islets showed a significant decrease in the inhibitory phosphorylation site of Raf-1 at serine 259 and an increasing trend of phosphorylated ERK1/2 (E). (F,G) Acute insulin and IGF-1 signalling stimulation for 15 minutes in primary mouse islets. Bar graphs are quantification of 4 Western blots using densitometry using Photoshop (n=4 different cultures). *Denotes significant difference ($p<0.05$, Student's *t* test) between serum-free control and treatment.

Figure 9

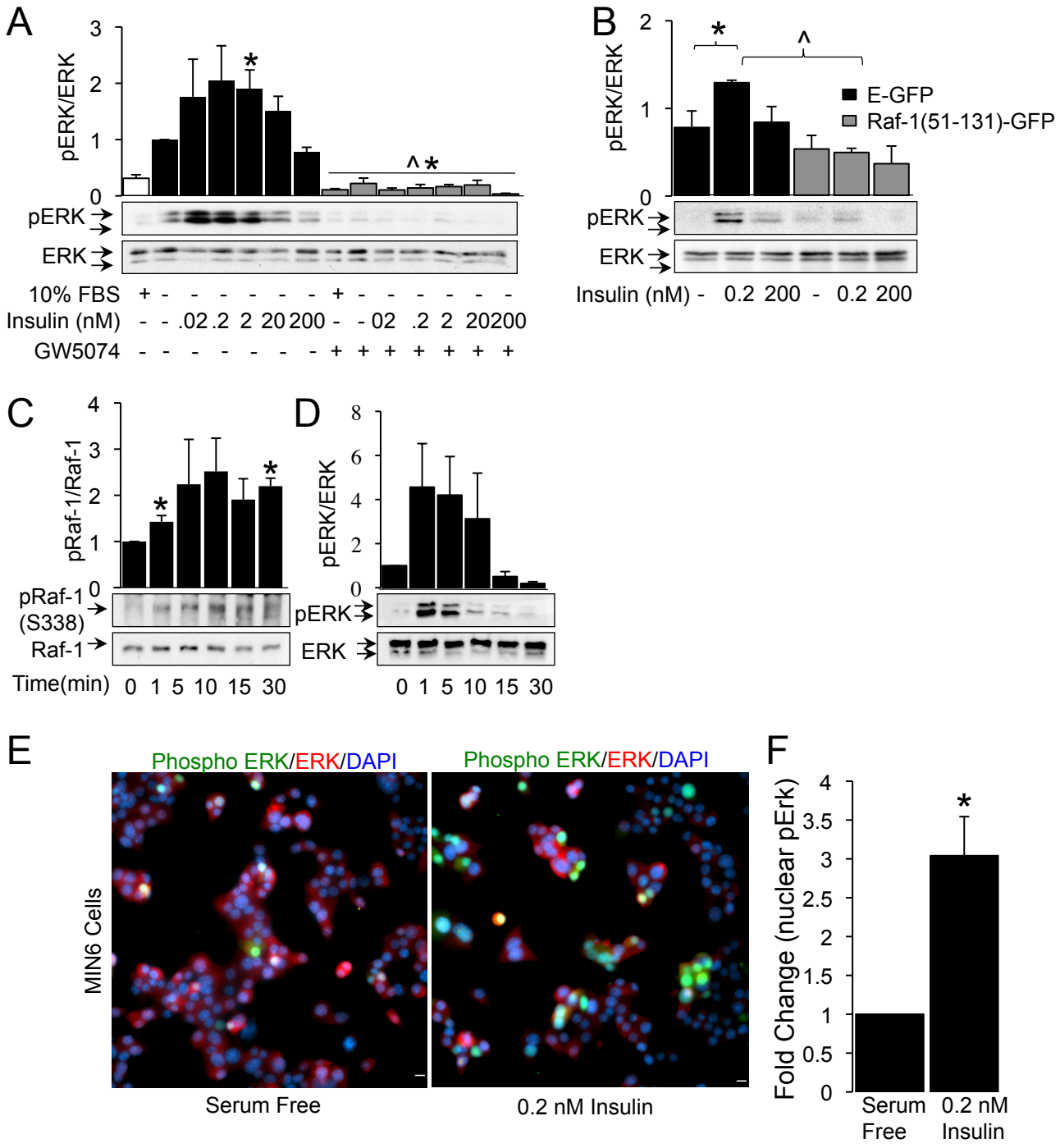

A
pERK/ERK
pERK
ERK
10% FBS
Insulin (nM)
GW5074
B
pERK/ERK
E-GFP
Raf-1(51-131)-GFP
pERK
ERK
Insulin (nM)
C
pRaf-1/Raf-1
pRaf-1 (S338)
Raf-1
Time(min)
D
pERK/ERK
pERK
ERK
E
Phospho ERK/ERK/DAPI
Phospho ERK/ERK/DAPI
MIN6 Cells
Serum Free
0.2 nM Insulin
F
Fold Change (nuclear pErk)
Serum Free
0.2 nM Insulin

Figure 9. **Insulin activates Raf-1 and ERK1/2 in transformed MIN6 cells.**

(A,B) Insulin-induced ERK1/2 phosphorylation was blocked by a Raf inhibitor (GW5074, 10 μM, n=4 Western blots from different cultures) or by a dominant-negative Raf-1^{51-131}-GFP protein (n=3 different cultures). (C, D) Insulin (0.2 nM) caused an increase in Raf-1 and ERK1/2 phosphorylation in a time-dependent manner (n=3 Western blots from different cultures). (E) Insulin induced phosphorylated ERK1/2 translocation in the nucleus of MIN6 cells. (F) Quantification of cells showing nuclear ERK1/2 phosphorylation analyzed by Slidebook, n=3 different cultures. *Denotes a significant difference ($p<0.05$, Student's *t* test) between treatment and control (serum-free). ^Denotes significant difference ($p<0.05$, Student's *t* test) between insulin and insulin with GW5074 or with dominant-negative Raf-1^{51-131}-GFP. Scale bars, 10 μm.

Figure 10

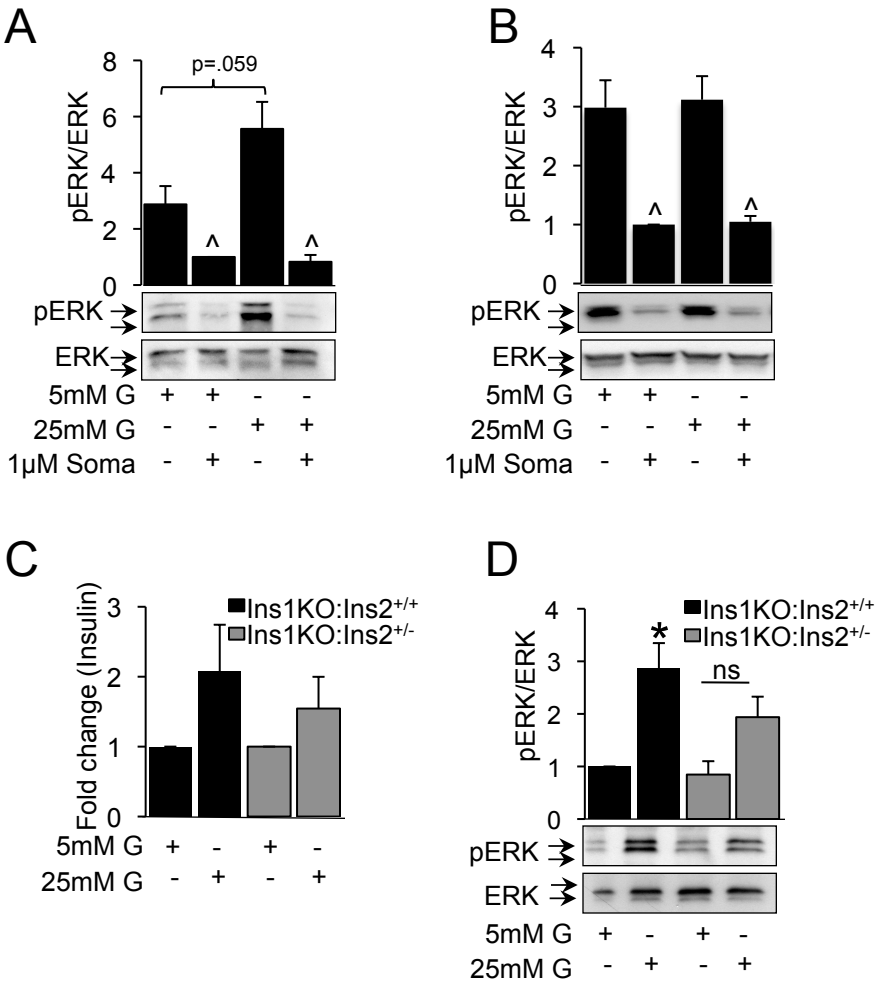

A
p=.059
pERK/ERK
pERK
ERK
5mM G
25mM G
1μM Soma
B
pERK/ERK
pERK
ERK
5mM G
25mM G
1μM Soma
C
Fold change (Insulin)
Ins1KO:Ins2+/+
Ins1KO:Ins2+/-
5mM G
25mM G
D
pERK/ERK
Ins1KO:Ins2+/+
Ins1KO:Ins2+/-
ns
pERK
ERK
5mM G
25mM G

Figure 10. **Role of autocrine insulin signalling in glucose-induced ERK1/2 phosphorylation.** (A,B) ERK1/2 phosphorylation in MIN6 cells treated with low glucose (5 mM) or high glucose (25 mM) for 15 (A) and 30 (B) minutes with or without somatostatin (Soma, 1 μM, n=3 Western blots from different cultures). (C) Insulin secretion levels were measured in conditioned media of islets from $Ins1^{-/-}$:$Ins2^{+/+}$ mice treated with low and high glucose (n=4 different conditioned media from different islet culture). Insulin levels were measured by radioimmunoassay. (D) ERK1/2 phoshorylation in islets from $Ins1^{-/-}$:$Ins2^{+/-}$ or $Ins1^{-/-}$:$Ins2^{+/+}$ mice or in response to 15 minutes treatment with 25 mM glucose or 5 mM glucose. *Denotes significant difference ($p<0.05$, Student's *t* test) between the serum-free control and glucose treatment. ^Denotes significant difference ($p<0.05$, Student's *t* test) between glucose only and glucose with Soma.

Figure 11

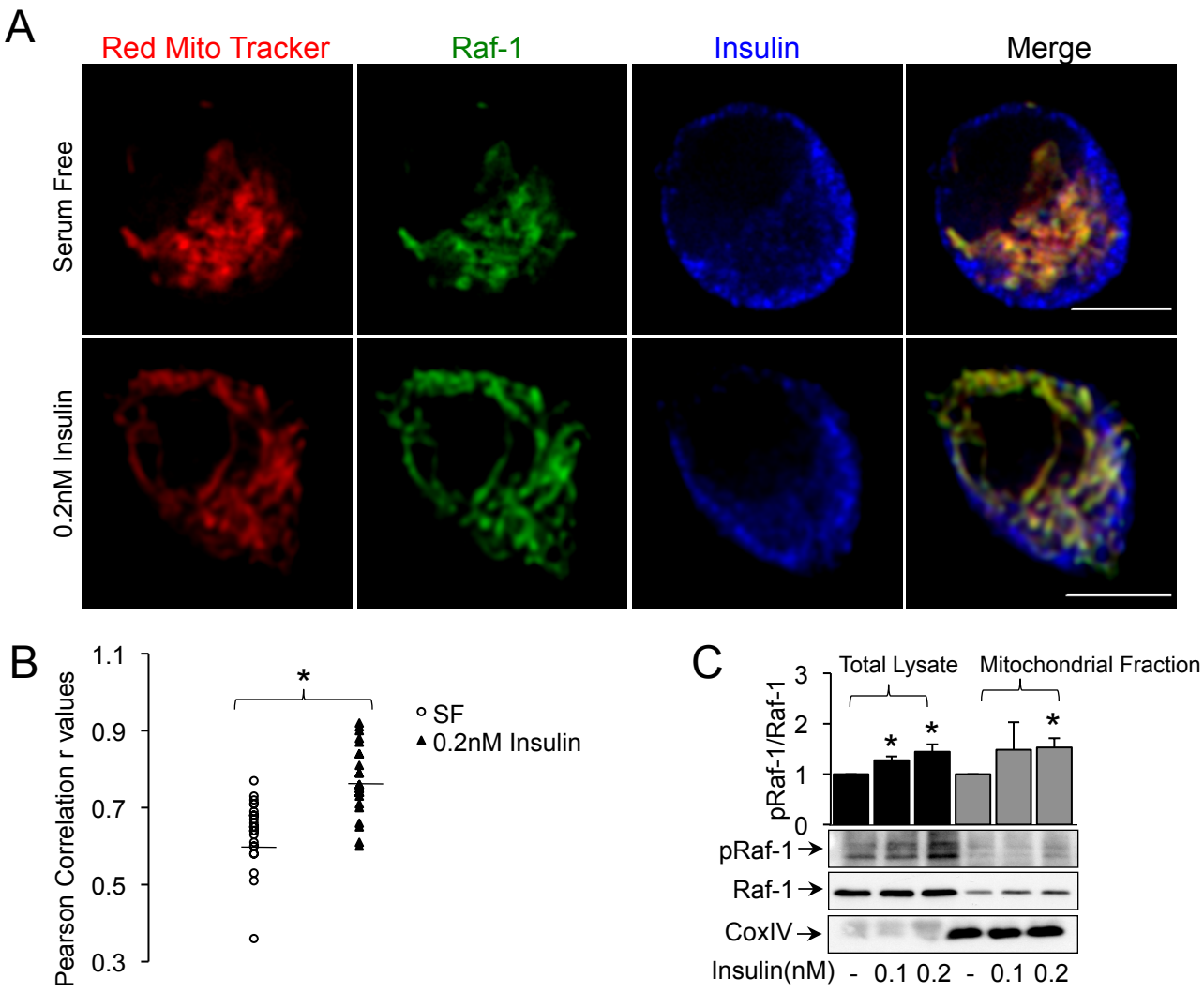

A
Red Mito Tracker
Raf-1
Insulin
Merge
Serum Free
0.2nM Insulin
B
Pearson Correlation r values
1.1
0.9
0.7
0.5
0.3
*
SF
0.2nM Insulin
C
Total Lysate
Mitochondrial Fraction
pRaf-1/Raf-1
3
2
1
0
*
*
*
pRaf-1
Raf-1
CoxIV
Insulin(nM) - 0.1 0.2 - 0.1 0.2

Figure 11. **Insulin promotes Raf-1 translocation to the mitochondria.** (A) Insulin (0.2 nM) promoted Raf-1 mitochondrial localization compared to serum-free in mouse pancreatic β-cell (n=3 different cultures). Scale bars, 10 μm. (B) A plot of Pearson correlation *r* values between Raf-1 and Red-mitochondria tracker in mouse pancreatic β-cells treated in serum-free or 0.2 nM insulin condition. (C) Phosphorylated Raf-1 (serine 338), Raf-1, and CoxIV levels in total cell lysates or mitochondrial fraction of MIN6 cells treated with insulin for 10 minutes. Bar graph is a densitometry quantification using Photoshop (n=3 Western blots from independent cultures). *Denotes a significant difference ($p<0.05$, Student's *t* test) between treatment and control (serum-free).

Figure 12

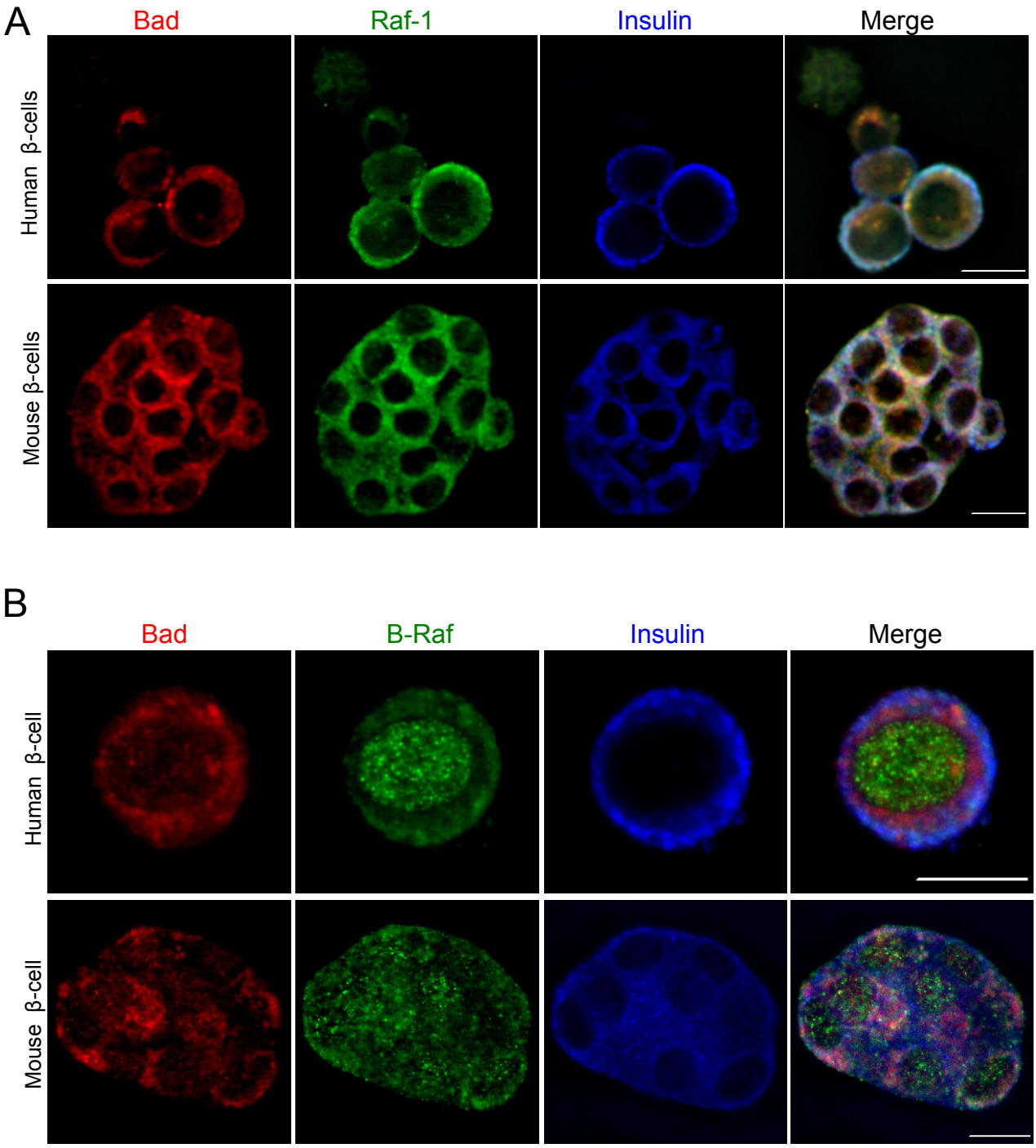

A
Bad
Raf-1
Insulin
Merge
Human β-cells
Mouse β-cells
B
Bad
B-Raf
Insulin
Merge
Human β-cell
Mouse β-cell

Figure 12. **Sub-cellular localization of endogenous Raf-1, B-Raf and Bad in mouse and human islets.** (A) Immunofluorescence imaging of endogenous Raf-1 and Bad in primary human and mouse β-cells. Pearson correlation *r* values between Raf-1 and Bad in human and mouse β-cells were 0.7 and 0.86 respectively. Scale bars, 10 μm. (B) Immunofluorescence imaging of B-Raf showing localization in the nucleus and cytoplasm, and a moderate co-localization with Bad in primary human and mouse β-cells. Pearson correlation *r* values between B-Raf and Bad in human and mouse β-cells were 0.5 and 0.3 respectively. Images shown are representative of 3 independent cultures. Scale bars, 10 μm.

Figure 13

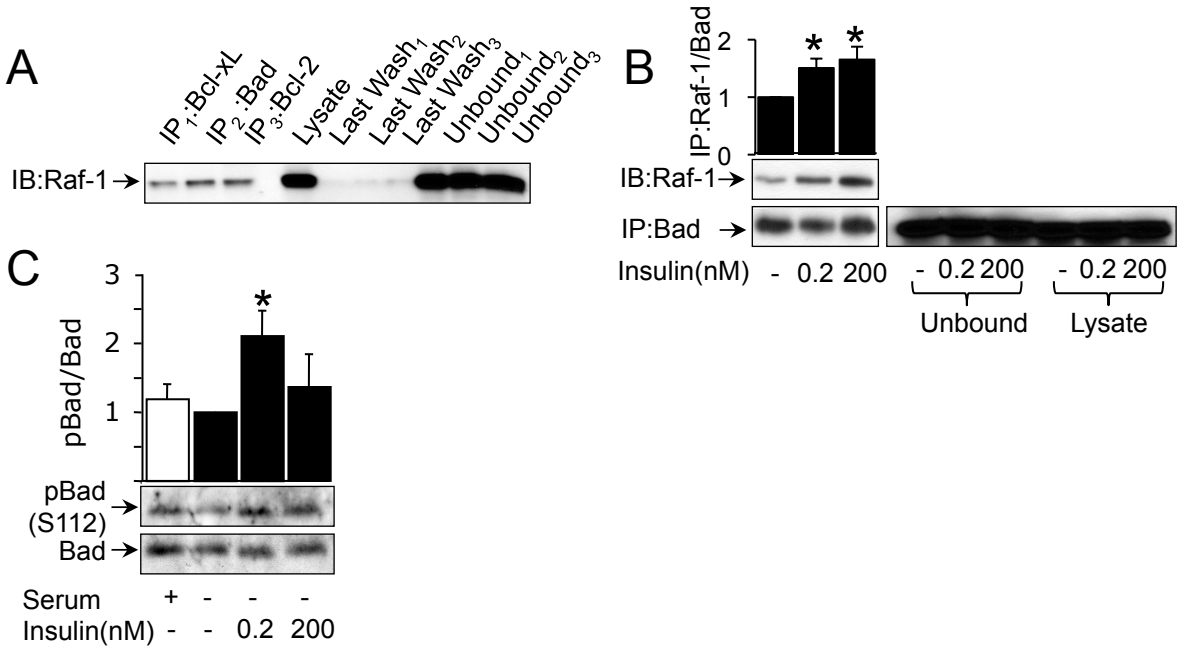

A
IP1:Bcl-xL
IP2:Bad
IP3:Bcl-2
Lysate
Last Wash1
Last Wash2
Last Wash3
Unbound1
Unbound2
Unbound3
IB:Raf-1
B
IP:Raf-1/Bad
IB:Raf-1
IP:Bad
Insulin(nM) - 0.2 200 - 0.2 200 - 0.2 200
Unbound
Lysate
C
pBad/Bad
pBad (S112)
Bad
Serum + - - -
Insulin(nM) - - 0.2 200

Figure 13. **Endogenous Bcl-2 family members and Raf-1 form protein-protein interactions in pancreatic β-cell.** (A) Immunoprecipitation demonstrating Raf-1 ability to form protein-protein interaction with Bcl-2 family proteins (Bcl-2, Bcl-xL, and Bad). (B) Insulin increased Raf-1 and Bad protein-protein interaction (n=3 different cultures). (C) Mouse islets treated with 0.2 nM and 200 nM insulin for 30 minutes caused an increase in the inhibitory phosphorylation Bad at serine 112 (n=3 Western blots from different cultures). Bar graphs are quantification of Western blots using Photoshop. *Denotes a significant difference ($p<0.05$, Student's *t* test) between treatment and control (serum-free).

Figure 14

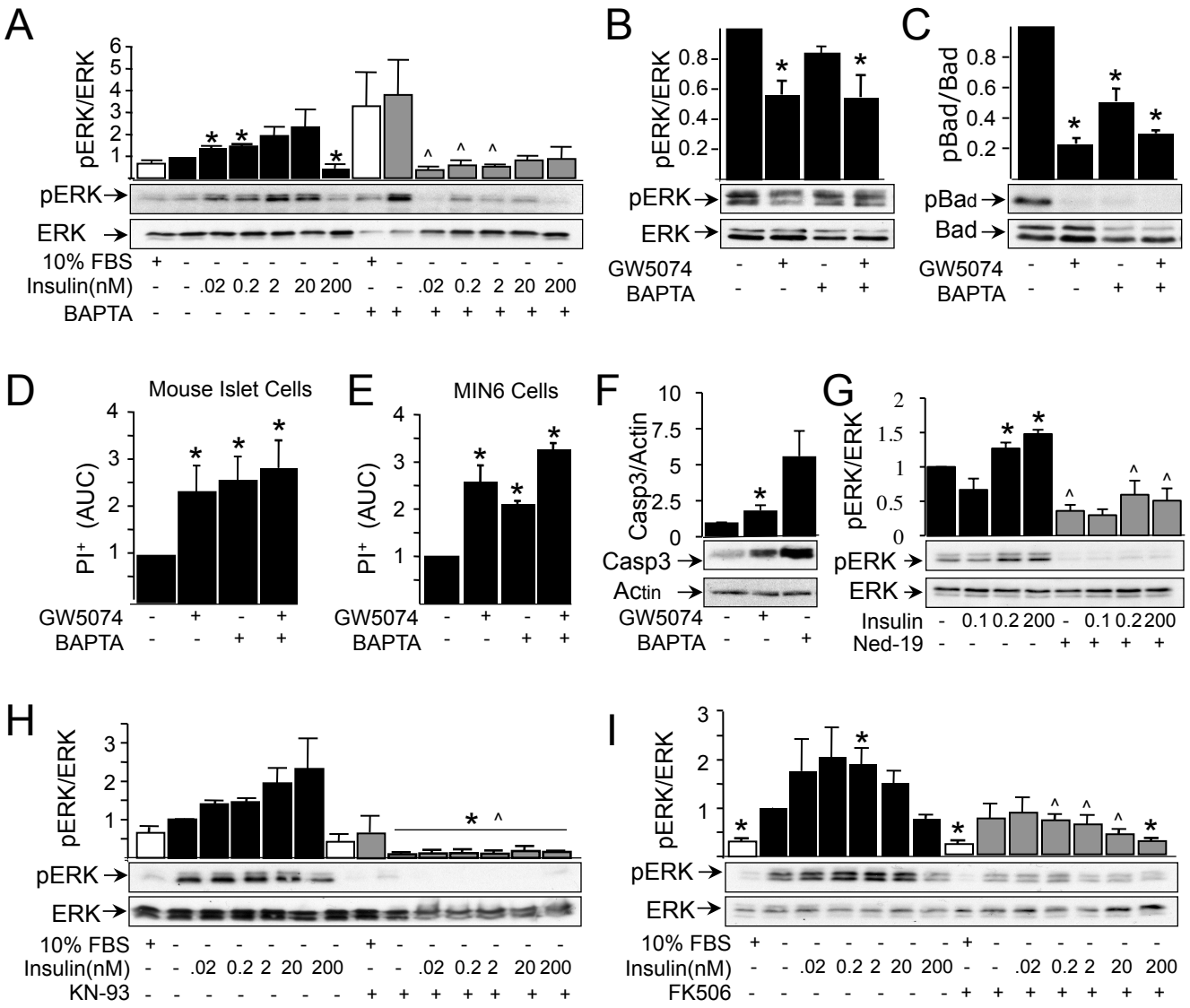

A
pERK/ERK
pERK
ERK
10% FBS
Insulin(nM)
BAPTA
B
pERK/ERK
pERK
ERK
GW5074
BAPTA
C
pBad/Bad
pBad
Bad
GW5074
BAPTA
D
Mouse Islet Cells
PI+ (AUC)
GW5074
BAPTA
E
MIN6 Cells
PI+ (AUC)
GW5074
BAPTA
F
Casp3/Actin
Casp3
Actin
GW5074
BAPTA
G
pERK/ERK
pERK
ERK
Insulin
Ned-19
H
pERK/ERK
pERK
ERK
10% FBS
Insulin(nM)
KN-93
I
pERK/ERK
pERK
ERK
10% FBS
Insulin(nM)
FK506

Figure 14. **Ca^{2+} signalling modulators abrogates insulin-induced ERK1/2 activation.** (A) MIN6 cells treated with insulin showed an increase on ERK1/2 phosphorylation in 10 minutes that was blocked in the presence of BAPTA-AM. (B) Phosphorylated ERK1/2 levels in MIN6 cells treated with GW5074 (10 μM) and BAPTA-AM (50 μM) individually or both for 6 hours (n=3 independent cultures). (C) Phosphorylated Bad (serine 112) levels in MIN6 cells treated with GW5074 and BAPTA-AM individually or both for 6 hours (n=3 independent cultures). Rapid increase in propidium iodide (PI) incorporation in dispersed mouse islet cells (D) and MIN6 cells (E) treated with GW5074, BAPTA-AM, or both (n=6 independent cultures). Area under the curve (AUC) of propidium iodide incorporation from 1 hour to 48 hours after treatment. (F) Cleaved-caspase-3 expression levels in MIN6 cells treated with GW5074 and BAPTA-AM for 6 hours (n=3 independent cultures). (G-I) MIN6 cells treated with insulin show an increase in ERK1/2 phosphorylation that was blocked in the presence of Ned-19 (G, NAADP-Receptor antagonist), KN-93 (H, CaMKII inhibitor), and FK506 (I, calcineurin inhibitor) for 10 minutes treatment (n= 3 different cultures). Bar graphs are quantification of Western blots using Photoshop. *Denotes significant difference ($p<0.05$, Student's *t* test and ANOVA with a Student-Newman-Keuls post-hoc test) between the serum-free control and treatment. ^Denotes significant difference between insulin and insulin with inhibitors: BAPTA-AM (50 μM), Ned-19 (100 μM), KN-93 (1 μM), and FK506 (50 nM).

4. Results II: Inhibition of Raf-1 alters multiple downstream pathways to induce pancreatic β-cell apoptosis *in vitro*

4.1 Inhibition of endogenous Raf-1 signalling induces β-cell apoptosis in vitro

We examined the consequences of blocking endogenous Raf-1 signalling in this cell type using a commercially available Raf-1 inhibitor (GW5074), reported to be highly selective for Raf-1 (100 times more selective compared to a panel of related kinases)(195). We hypothesized that disrupting Raf-1 signalling may lead to β-cell death. Indeed, inhibiting Raf-1 signalling in both primary mouse islet cells and transformed MIN6 β-cells increased cell death in a time- and concentration-dependent manner as determined by the real-time monitoring of propidium iodide incorporation (Figure 15A,B). A massive increase in apoptotic β-cell death in the presence of Raf-1 inhibitor (GW5074) was confirmed in MIN6 cells using the fluorescent TUNEL technique (Figure 15C,D). A similar increase in apoptosis was observed when TUNEL was combined with insulin staining in primary mouse islet cells (Figure 15C). The Raf-1 inhibitor also increased caspase-3 cleavage in a concentration- and time-dependent manner (Figure 15E,F), suggesting that a reduction in Raf-1 kinase activity leads to the activation of this executioner caspase. We further confirmed that GW5074 evokes apoptotic programmed cell death using DNA ladder analysis on mouse islets (Figure 15G). Together, these results clearly demonstrate that blocking Raf-1 kinase triggers a

rapid and robust apoptotic response in primary human and mouse β-cells and in the MIN6 β-cell line. Thus, endogenous Raf-1 activity appears to be essential for β-cell survival.

4.2 Raf-1 inhibitor reduces ERK1/2 and Bad phosphorylation

We next investigated the mechanisms by which Raf-1 inhibition caused apoptosis using MIN6 cells as a model. We first examined the phosphorylation and activation state of ERK1/2, the canonical downstream target of Raf-1. Western blot analysis revealed that GW5074 decreased ERK1/2 phosphorylation, but did not alter the total levels of ERK1/2 protein (Figure 16A,B,C). MEK is known to mediate the effects of Raf-1 on ERK1/2 phosphorylation. To test the hypothesis that endogenous MEK serves an anti-apoptotic role in β-cells, cell death in the presence of two MEK inhibitors, UO126 and PD98059, was examined. These inhibitors had more modest apoptosis-inducing effects in MIN6 cells and in primary mouse islet cells relative to the Raf-1 inhibitor (Figure 16D,E), suggesting that a component of the pro-survival actions of Raf-1 may be mediated via MEK and ERK1/2. As expected, both MEK inhibitors reduced ERK1/2 phosphorylation levels (Figure 16F,G,H). Together, these results implicate both MEK and ERK1/2 in β-cell survival.

The observation that the modest cell death subsequent to inhibiting MEK could not fully account for the effects of the Raf-1 inhibitor led us to examine alternative

pathways. Several recent studies have suggested that the pro-survival effects of Raf-1 can be mediated by phosphorylating Bad on serine 112, which contributes to the inactivation and sequesterization of this pro-apoptotic Bcl-2 family member by 14-3-3 proteins (143, 144). Thus, we hypothesized that inhibition of Raf-1 signalling might reduce Bad phosphorylation. Indeed, a marked and rapid reduction in Bad phosphorylation was observed in β-cells treated with the Raf-1 inhibitor (Figure 17A,B). Studies in other cell types have demonstrated that when Bad is dephosphorylated, it migrates to the mitochondria where it can dimerize with Bcl-xL (196), leading to the release of Bax, an initiator of apoptosis (197, 198). We found that Raf-1 inhibitor increased the protein expression of both Bad and Bax (Figure 17C,D,E). Since the ratio of Bcl-2 to Bax is thought to determine sensitivity to apoptosis, we also analyzed whether GW5074 affected Bcl-2 levels. Indeed, the ratio of Bax to Bcl-2 was increased (Figure 17F,G,H). Together, these data further implicate Raf-1 in the control of β-cell apoptosis and point to an emerging Bad-dependent pathway as a contributing mechanism in this action.

4.3 Effects of dominant-negative Raf-1 on β-cell death in vitro

Given the caveats inherent to pharmacological inhibitors, we also employed Raf-1-GFP fusion proteins (164) to inhibit or enhance Raf-1 signalling in β-cells. First, we confirmed the over-expression and examined the localization of three different Raf-1-GFP fusion proteins using live-cell imaging in MIN6 cells. The over-expressed full length

Raf-1-GFP fusion protein was found in both the cytoplasm and at the mitochondria, but not in the nucleus (Figure 18A). We also utilized two dominant-negative truncated mutants of Raf-1, Raf-1^{51-131}-GFP and Raf-1^{51-220}-GFP, both of which have been previously described to inhibit Raf-1 signalling (164). We observed that Raf-1^{51-220}-GFP was localized in the cytoplasm, mitochondria and partially in the nucleus. The short form of Raf-1^{51-131}-GFP was mainly localized in the cytoplasm and the nucleus (Figure 18A,B). Together, these studies demonstrate that Raf-1 localization in β-cells depends on the presence of specific domains.

To determine the effect of over-expressing Raf-1 mutants on β-cell death, we measured caspase-3 cleavage 24 hours after transient transfection. In initial experiments with about 10% transfection efficiency, we observed an increase of approximately 20% in caspase-3 cleavage compared to control by Western blotting. Thus, to enrich our Raf-1-GFP-expressing MIN6 cell population, fluorescence-activated cell sorting (FACS; Figure 19A) was employed prior to measuring ERK1/2 phosphorylation and caspase-3 cleavage. We observed that over-expression of Raf-1-GFP increased ERK1/2 phosphorylation and modestly reduced caspase-3 cleavage (Figure 19B,C,D), suggesting the possibility that over-expression of Raf-1 may protect β-cells from apoptosis. Conversely, over-expression of dominant negative Raf-1 mutants significantly reduced ERK1/2 phosphorylation and increased the expression of cleaved caspase 3 compared to control cells transfected with EGFP alone. Moreover, we observed an increase in propidium iodide incorporation in cells expressing dominant-negative Raf-1 mutants (Figure 19E). Overall, our data from experiments

using the pharmacological inhibitor and Raf-1 mutants point to Raf-1 as a critical pro-survival kinase in the β-cell.

4.4 Relative roles of Raf-1 and PI3-kinase/Akt signalling in β-cell apoptosis

Raf-1, ERK1/2, PI3-kinase and Akt are all vital components of numerous pro-survival signalling cascades in many cell types. Among these kinases, PI3-kinase and Akt have received the majority of the attention in the β-cell. To assess in parallel the relative importance of these kinases in the context of β-cell survival, we measured cell death in real-time in the presence of specific inhibitors in primary mouse islet cells and MIN6 cells. PI3-kinase was inhibited using LY294002, and two different Akt inhibitors were employed (TATAkt-i and Akti-1/2). In the presence of LY294002, an increase in the number of propidium iodide-positive cells was detected (Figure 20A,B), supporting the notion that PI3-kinase is important for β-cell survival (88, 199). Inhibiting Akt caused an increase in β-cell death, but to a lesser extent compared with the Raf-1 inhibitor (Figure 20A,B). The increase in apoptotic β-cell death in the presence of GW5074 and Akti-1/2 was associated with the cleavage of caspase-3 (Figure 21A). Multiple studies have implicated the ER-stress response in β-cell apoptosis under certain conditions (124, 125, 200-202) and pointed to Akt as a critical suppressor of this signal of programmed cell death (203). Treatment of MIN6 cells with Akti-1/2 caused a concentration-dependent increase in CHOP, a well-established marker of ER stress

(Figure 21A,B). In contrast, GW5074 induced only modest CHOP expression at low but not at high concentrations at this time point (Figure 21A,B). We also detected a trend towards increased CHOP levels in isolated human islets treated with GW5074 (Figure 21G,H). Together these experiments demonstrate that multiple kinase cascades play important roles in β-cell survival and ER-stress.

Next we examined the mechanisms involved in pro-survival signalling downstream of Akt and Raf-1 and assessed cross-talk between these signalling networks in the β-cell. The Raf-1 kinase has been previously identified as a point of cross-talk between Akt and ERK1/2 signalling cascades (204). Although it has been postulated that both the PI3-kinase/Akt and Raf-1/ERK1/2 signalling cascades are activated simultaneously to magnify cell survival signals, depending on the cell context Akt can negatively affect Raf-1 activity by phosphorylation at serine 259 (94). In the presence of Akti-1/2, this negative regulation appeared to be attenuated resulting in an increase in ERK1/2 phosphorylation (Figure 21A,F). This suggested the possibility that a compensatory mechanism can be upregulated to maintain β-cell survival under conditions when Akt is inhibited. Interestingly, unlike GW5074, Akti-1/2 did not alter Bad phosphorylation at serine 112 or total Bad protein levels (Figure 21A,D,E), pointing to a difference in the targets of these two kinases. The ability of Akt to negatively regulate Raf-1 may explain why we see a robust and enhanced β-cell death in the presence of GW5074 and only moderate death in the presence of Akt inhibitors.

It is not clear whether inhibition of Raf-1 kinase signalling directly affects Akt activity. Raf-1 gene ablation in the heart did not alter Akt phosphorylation or total protein level (141). Interestingly, MIN6 cells treated with GW5074 had reduced Akt phosphorylation (Figure 22A,B,C). Therefore, Raf-1 kinase may affect Akt directly or indirectly through an upstream kinase. We also examined the autoregulatory effects of blocking Raf-1 signalling. GW5074, as well as Akti-1/2 reduced the apparent total Raf-1 protein level (Figure 22D,E), supporting the notion that Raf-1 signalling can exert positive feedback on itself (205). These results strongly suggest important functional interactions between Raf-1- and Akt-dependent signalling networks in β-cells.

Given the differences in their downstream mechanisms, we hypothesized that simultaneously blocking both Raf-1 and Akt signalling would cause an additive effect on β-cell death. Indeed, a massive increase in β-cell death was observed in the presence of both Raf-1 and Akt inhibitors when compared to individual treatments (Figure 22F). These results further support the concept that the downstream anti-apoptotic targets of Raf-1 and Akt are distinct. Moreover, while inhibiting Akt alone results in an upregulation of ERK1/2 phosphorylation, blocking Raf-1 negates this compensatory response. Collectively, these results identify Raf-1 as a critical kinase mediating β-cell survival and point to complex interactions with the Akt signalling system.

The suppression of Raf-1 protein levels presented an opportunity to address whether any of the known kinase-independent downstream targets of Raf-1 may be

involved in β-cell survival. Aside from its ability to phosphorylate key downstream proteins such as ERK1/2 and Bad, Raf-1 has also been proposed to protect cells by serving as a scaffolding protein (206). Deletion of the Raf-1 gene has been associated with increased activity of Apoptosis Signalling Kinase-1 (ASK-1), an event that does not require Raf-1 catalytic activity (207). Therefore, we assessed ASK-1 phosphorylation and total ASK-1 protein levels in our model (208). Although we were unable to detect GW5074-induced changes in ASK-1 phosphorylation at Thr845, we did observe a trend towards increases in total ASK-1 expression at the highest concentration of the inhibitor (Figure 23A,B,C). Overall, our data point to a more important role for the kinase-dependent actions of Raf-1 in β-cell survival.

4.5 Effects of Raf-1 signalling on insulin secretion in vitro

In addition to mediating β-cell survival, many kinases have been implicated in β-cell function (52, 185). Thus, the effects of blocking Raf-1 signalling on basal and glucose-stimulated insulin secretion were examined. Using a perifusion approach to examine the dynamics of insulin secretion in primary mouse islets, we found that acute treatment with GW5074 had a moderate, negative effect on both phases of glucose-stimulated insulin secretion (Figure 24A), prior to the appearance of any cell death (see Figure 15). Similarly, inhibition of Raf-1 and ERK1/2, but not Akt, resulted in modestly decreased insulin secretion from MIN6 cells (Figure 24B,C). Thus, Raf-1 and ERK1/2 appear to regulate both the survival and, to a lesser degree, the function of pancreatic β-cells.

Figure 15

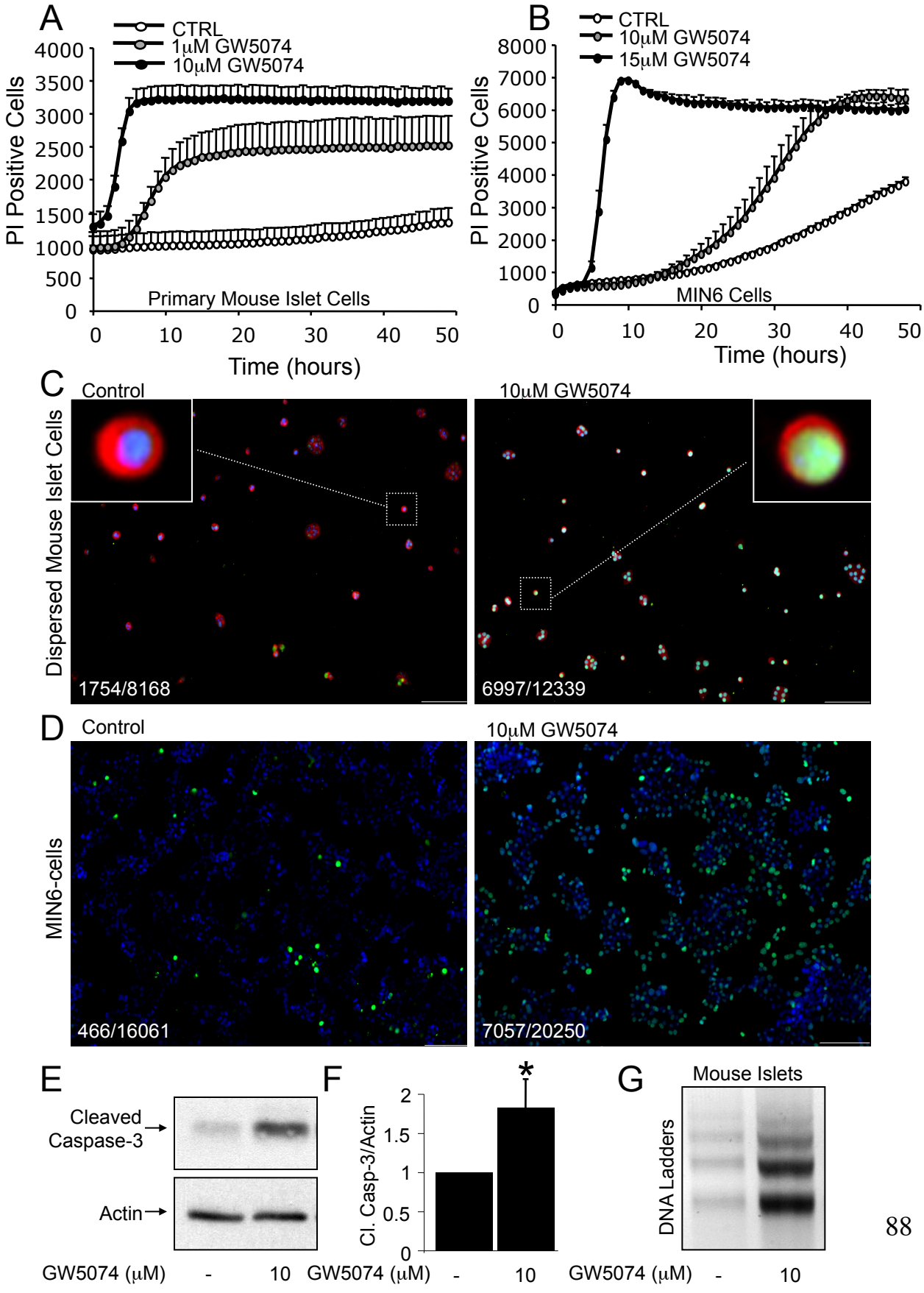

A
CTRL
1μM GW5074
10μM GW5074
PI Positive Cells
Primary Mouse Islet Cells
Time (hours)
B
CTRL
10μM GW5074
15μM GW5074
PI Positive Cells
MIN6 Cells
Time (hours)
C
Control
10μM GW5074
Dispersed Mouse Islet Cells
1754/8168
6997/12339
D
Control
10μM GW5074
MIN6-cells
466/16061
7057/20250
E
Cleaved Caspase-3
Actin
GW5074 (μM) - 10
F
Cl. Casp-3/Actin
GW5074 (μM) - 10
G
Mouse Islets
DNA Ladders
GW5074 (μM) - 10

Figure 15. **Inhibition of endogenous Raf-1 signalling in primary mouse islet cells and MIN6 cells causes β-cell death.** Rapid increase in propidium iodide incorporation in dispersed mouse islet cells (A) and MIN6 cells (B) treated with the specific inhibitor of Raf-1 kinase, GW5074 (n=9 different cultures). GW5074 increases β-cell death in a dose- and time-dependent manner. GW5074 also caused β-cell apoptosis assessed by TUNEL staining of mouse islet β-cells (C) and MIN6 cells within 24 hours (D). (C) Dispersed mouse β-cells stained for insulin are red, TUNEL positive cells are green, and cell nuclei stained with DAPI are blue. (D) TUNEL-positive MIN6 cells are green and nuclei are blue. TUNEL staining experiments were repeated independently with similar results using dispersed human and mouse islets (≥8168 cells examined for each condition per culture, 3 independent cultures) and MIN6 cells (≥16061 cells for each condition per culture, 3 in three different cultures). The ratio of TUNEL positive cells/total cells examined is shown as an inset. (E) Cleaved caspase-3 protein levels in MIN6 cells treated with GW5074 for 6 hours (n=3 Western blots from different cultures). (F) Quantification of cleaved caspase-3 protein levels corrected to control. (G) GW5074 induces DNA-laddering in intact mouse islets (n=3 different cultures). *Denotes significant difference ($p<0.05$, student's t test) between the control and treatment.

Figure 16

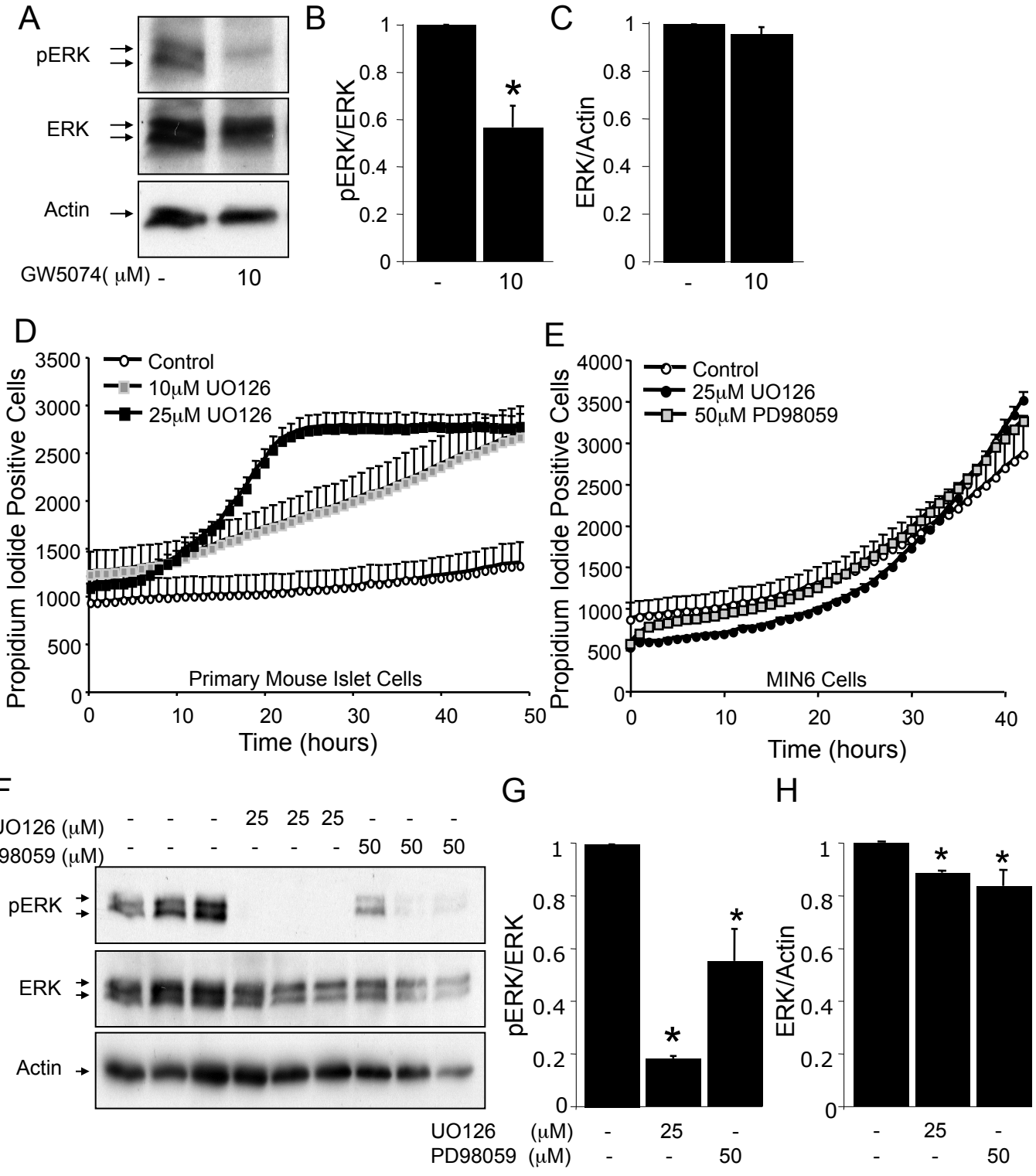

A
pERK
ERK
Actin
GW5074(μM) - 10
B
pERK/ERK
C
ERK/Actin
D
Control
10μM UO126
25μM UO126
Propidium Iodide Positive Cells
Primary Mouse Islet Cells
Time (hours)
E
Control
25μM UO126
50μM PD98059
Propidium Iodide Positive Cells
MIN6 Cells
Time (hours)
F
UO126 (μM) - - - 25 25 25 - - -
PD98059 (μM) - - - - - - 50 50 50
pERK
ERK
Actin
G
pERK/ERK
UO126 (μM) - 25 -
PD98059 (μM) - - 50
H
ERK/Actin
- 25 -
- - 50

Figure 16. **Roles of ERK1/2 and MEK in Raf-1 inhibitor-induced β-cell death.** (A) Phosphorylated and total ERK1/2 levels in MIN6 cells treated with GW5074 for 6 hours (n=3 different cultures). (B) Quantification of phosphorylated ERK1/2 to total ERK1/2 protein levels normalized to control. (C) Quantification of total ERK1/2/β-Actin protein levels normalized to control. Moderate increase in propidium iodide incorporation in dispersed mouse islet cells (D) and MIN6 cells (E) treated with MEK inhibitors, UO126 and PD98059. (F) Phosphorylated and total ERK1/2 levels in MIN6 cells treated with UO126 and PD98059 for 3 hours (n=3 different cultures). (G) Quantification of phosphorylated ERK1/2 to total ERK1/2 protein levels normalized to control. (H) Quantification total ERK1/2/β-Actin ratio normalized to control. *Denotes significant difference ($p<0.05$, student's t test) between the control and treatment.

Figure 17

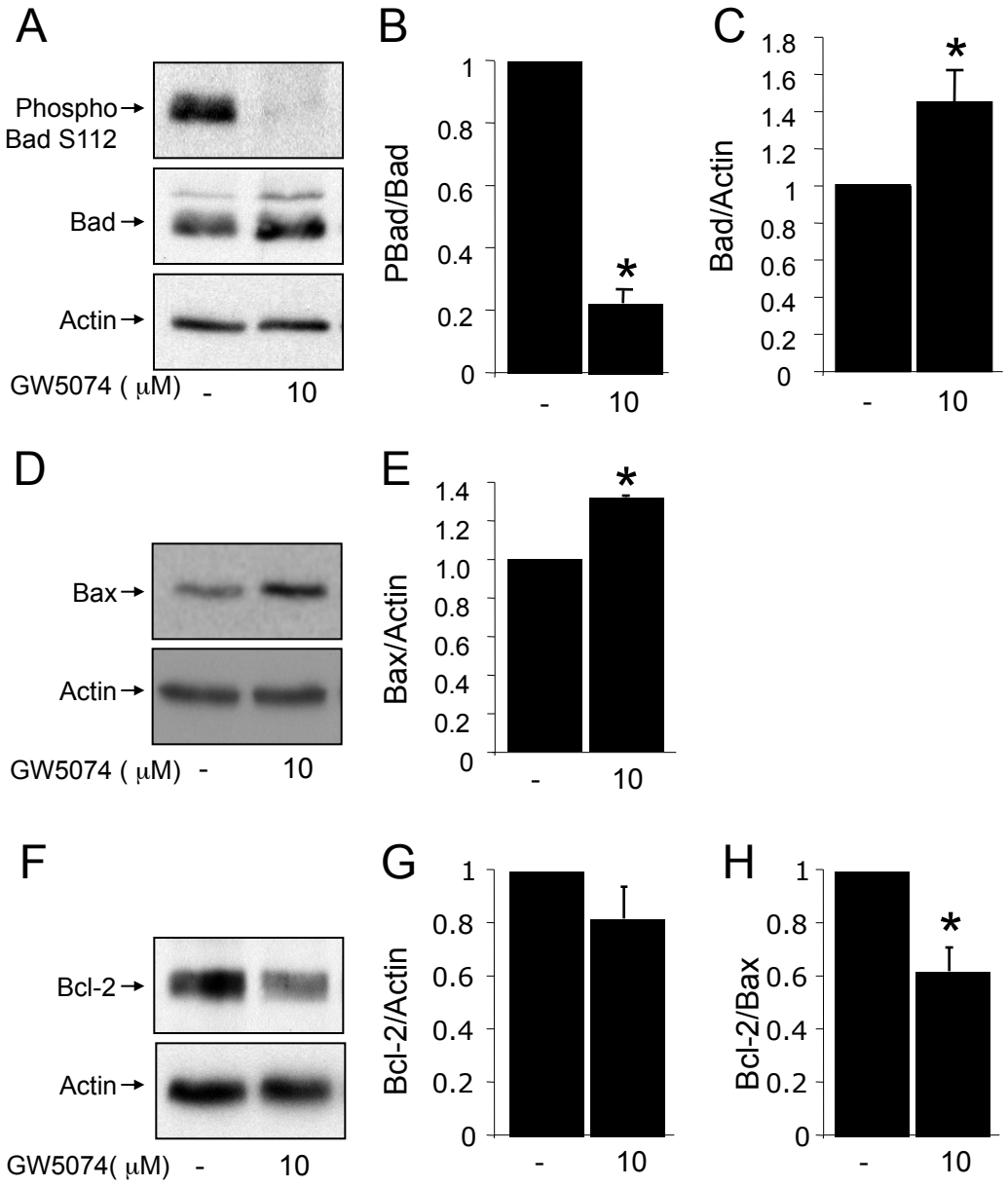
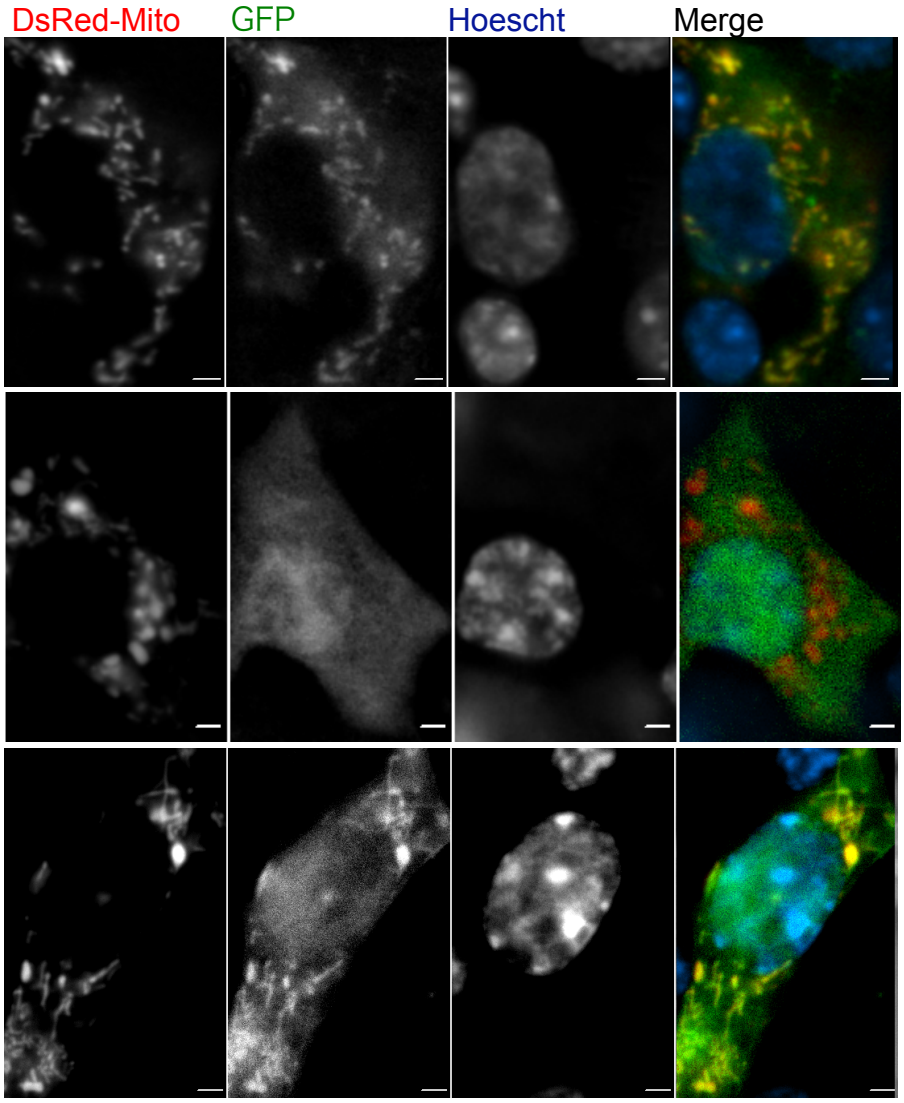

A
Phospho Bad S112
Bad
Actin
GW5074 (μM) - 10
B
PBad/Bad
1
0.8
0.6
0.4
0.2
0
*
- 10
C
Bad/Actin
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
*
- 10
D
Bax
Actin
GW5074 (μM) - 10
E
Bax/Actin
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
*
- 10
F
Bcl-2
Actin
GW5074(μM) - 10
G
Bcl-2/Actin
1
0.8
0.6
0.4
0.2
0
- 10
H
Bcl-2/Bax
1
0.8
0.6
0.4
0.2
0
*
- 10

Figure 17. **Effects of Raf-1 inhibitor on Bad, Bcl-2 and Bax**. (A) Phosphorylated Bad (serine 112) and total Bad in MIN6 cells treated with GW5074 for 6 hours (n=3 different cultures). (B) Quantification of the ratio of phosphorylated Bad serine 112 to total Bad protein (normalized to control). (C) Quantification of total Bad/β-Actin protein ratio normalized to control. (D) GW5074 increased Bax protein level in MIN6 cells treated for 6 hours (n=3 different cultures). (E) Quantification of total Bax/β-Actin protein ratio normalized to control. (F) Bcl-2 protein levels/β-Actin protein normalized to the control (n=3 different cultures). (G) Quantification of total Bcl-2/β-Actin protein levels normalized to control. (H) Bcl-2/Bax ratio. *Denotes a significant difference ($p<0.05$, Student's *t* test) between treatment and control (serum-free).

Figure 18

A



B

GFP-Raf-1 Constructs	PC between GFP and DsRed-Mito	PC between GFP and Hoescht
Raf-1-GFP	**r = 0.88**	r = -0.23
Raf-1$^{51-131}$-GFP (DN1)	r = -0.06	r = 0.25
Raf-1$^{51-220}$-GFP (DN2)	r = 0.58	r = 0.08

Figure 18. **Localization of Raf-1-GFP fusion proteins in MIN6 cells.**

Wide-field, deconvolution fluorescence imaging of Raf-1-GFP fusion proteins and mitochondrial-targeted red fluorescent protein (DsRed-Mito) co-transfected into MIN6 cells. (A) DsRed-Mito colocalizes strongly with Raf-1-GFP, modestly with Raf-1^{51-220}-GFP, but not with Raf-1^{51-131}-GFP. (B) Pearson's Correlation (PC) between Raf-1-GFP fusion proteins and DsRed-Mito or Hoechst DNA dye (nucleus) were calculated as described in the Materials and Methods. Scale bar, 2 μm.

Figure 19

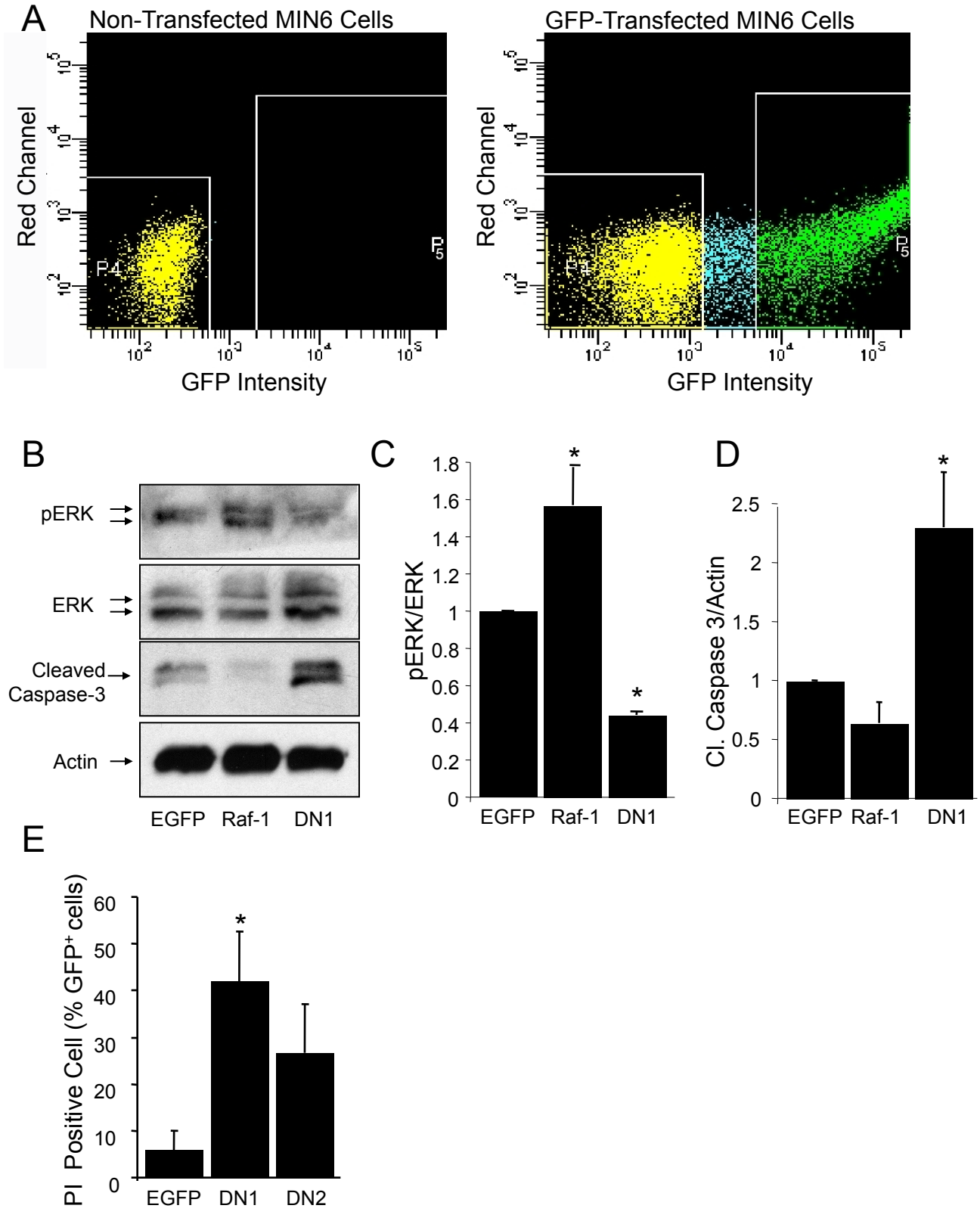

A
Non-Transfected MIN6 Cells
GFP-Transfected MIN6 Cells
Red Channel
GFP Intensity
P4
P5
B
pERK
ERK
Cleaved Caspase-3
Actin
EGFP
Raf-1
DN1
C
pERK/ERK
D
Cl. Caspase 3/Actin
E
PI Positive Cell (% GFP+ cells)
DN2

Figure19. **Expressed Raf-1-GFP fusion proteins inhibit Raf-1 mediated ERK1/2 activation and induces MIN6 cells β-cell death.** (A) Sample FACS scatter plots showing non-GFP expressing MIN6 cells and GFP-positive cells. (B) Phosphorylated ERK1/2 and cleaved caspase-3, total ERK1/2 and β-Actin in FACS-enriched GFP-positive MIN6-cells (n=3 different cultures). (B) Quantification of phosphorylated ERK1/2/total ERK1/2 protein ratio normalized to control. (C) Quantification of cleaved caspase-3 protein/β-Actin ratio normalized to control. (D) Expression of dominant-negative Raf-1 fusion proteins caused an increased in propidium iodide incorporation in MIN6 cells. *Denotes a significant difference ($p<0.05$, Student's *t* test) between treatment and control (EGFP).

Figure 20

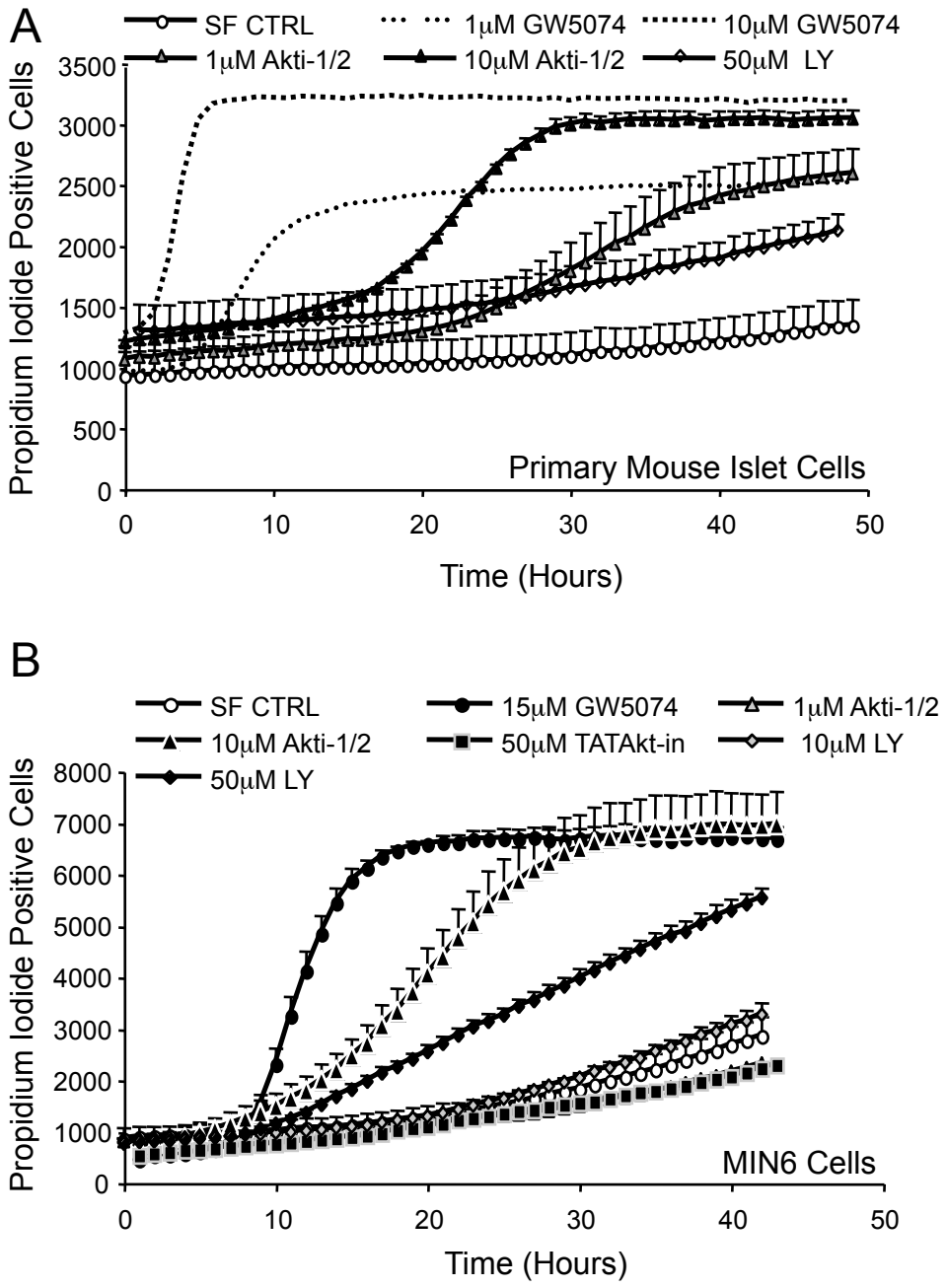

A
SF CTRL
1μM GW5074
10μM GW5074
1μM Akti-1/2
10μM Akti-1/2
50μM LY
Propidium Iodide Positive Cells
Primary Mouse Islet Cells
Time (Hours)
B
SF CTRL
15μM GW5074
1μM Akti-1/2
10μM Akti-1/2
50μM TATAkt-in
10μM LY
50μM LY
Propidium Iodide Positive Cells
MIN6 Cells
Time (Hours)

Figure 20. **The relative roles of Raf-1 and PI3-kinase/Akt signalling in β-cell apoptosis.** Rapid increase in propidium iodide incorporation in dispersed mouse islet cells (A) and MIN6 cells (B) treated with specific inhibitors of Raf-1 kinase (GW5074), PI3-kinase (LY294002) or Akt kinase (Akti-1/2 and TATAkt-in). Like GW5074, LY294002 and Akti-1/2 increased β-cell death in a dose and time-dependent manner, suggesting that all three kinases play a role in β-cell survival (n= 9 different cultures conducted in three different days).

Figure 21

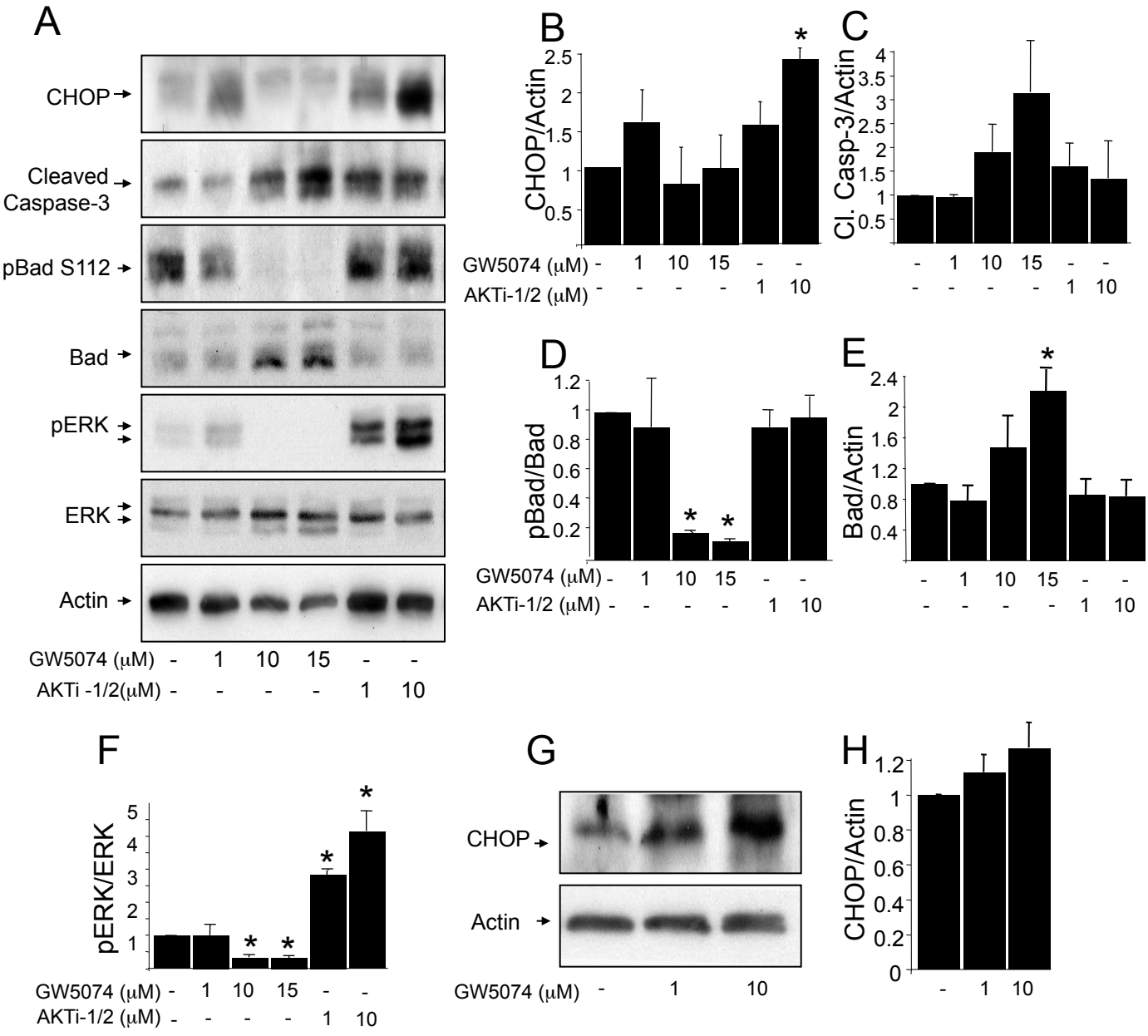

A
CHOP
Cleaved Caspase-3
pBad S112
Bad
pERK
ERK
Actin
GW5074 (μM) - 1 10 15 - -
AKTi -1/2(μM) - - - - 1 10
B
CHOP/Actin
2.5
2
1.5
1
0.5
*
GW5074 (μM) - 1 10 15 - -
AKTi-1/2 (μM) - - - - 1 10
C
Cl. Casp-3/Actin
4
3.5
3
2.5
2
1.5
1
0.5
- 1 10 15 - -
- - - - 1 10
D
pBad/Bad
1.2
1
0.8
0.6
0.4
0.2
* *
GW5074 (μM) - 1 10 15 - -
AKTi-1/2 (μM) - - - - 1 10
E
Bad/Actin
2.4
2
1.6
1.2
0.8
0.4
*
- 1 10 15 - -
- - - - 1 10
F
pERK/ERK
5
4
3
2
1
* * * *
GW5074 (μM) - 1 10 15 - -
AKTi-1/2 (μM) - - - - 1 10
G
CHOP
Actin
GW5074 (μM) - 1 10
H
CHOP/Actin
1.2
1
0.8
0.6
0.4
0.2
0
- 1 10

Figure 21. **The relative roles of Raf-1 and PI3-kinase/Akt signalling on ER Stress and in β-cell apoptosis.** (A) Effects of GW5074 and Akti-1/2 on the expression of CHOP, an ER stress marker, and cleavage of caspase-3 in MIN6 cells treated for 3 hours (n=3 Western blots from different cultures). (A) Phosphorylated and total ERK1/2 and phosphorylated Bad (serine 112) and total Bad levels in MIN6 cells treated with GW5074 and Akti-1/2 for 3 hours (n=3 Western blots from different cultures). Quantification of CHOP/β-Actin ratio (B) and cleaved caspase-3/β-Actin ratio (C) normalized to control. (D) Quantification of Bad serine 112/total Bad protein ratio normalized to the control. (E) Quantification of total Bad/β-Actin ratio corrected to control. (F) Quantification of phosphorylated and total ERK1/2 levels ratio normalized to control. (G,H) GW5074 increased CHOP expression in human islets (n=3 Western blots from different human islets) (D). Quantification of CHOP/β-Actin levels. *Denotes a significant difference ($p<0.05$, Student's *t* test) between treatment and control.

Figure 22

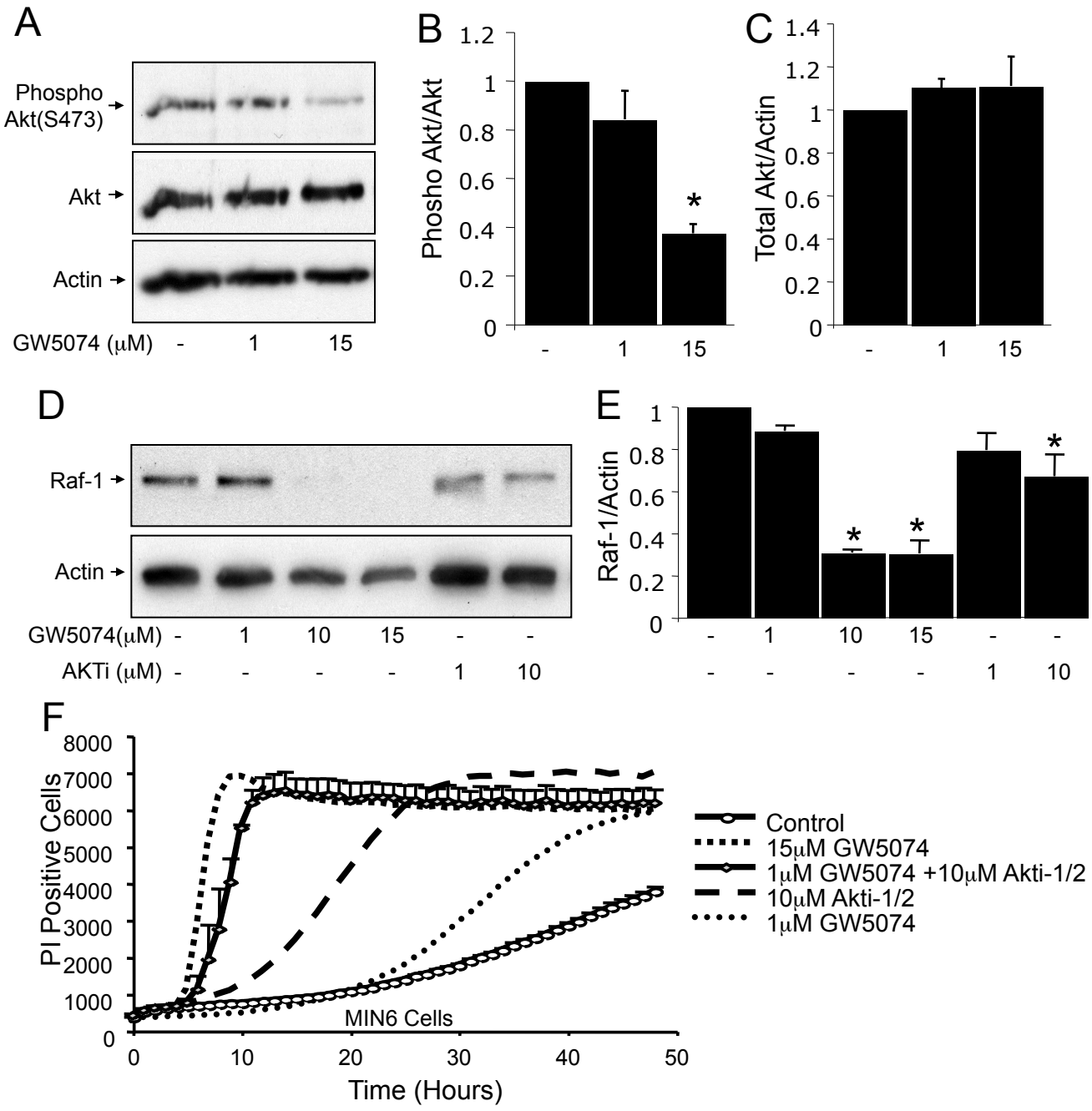

A
Phospho Akt(S473)
Akt
Actin
GW5074 (μM) - 1 15
B
Phosho Akt/Akt
1.2
1
0.8
0.6
0.4
0.2
0
*
- 1 15
C
Total Akt/Actin
1.4
1.2
1
0.8
0.6
0.4
0.2
0
- 1 15
D
Raf-1
Actin
GW5074(μM) - 1 10 15 - -
AKTi (μM) - - - - 1 10
E
Raf-1/Actin
1
0.8
0.6
0.4
0.2
0
*
*
*
- 1 10 15 - -
- - - - 1 10
F
PI Positive Cells
8000
7000
6000
5000
4000
3000
2000
1000
0
MIN6 Cells
0 10 20 30 40 50
Time (Hours)
Control
15μM GW5074
1μM GW5074 +10μM Akti-1/2
10μM Akti-1/2
1μM GW5074

Figure 22. **Raf-1 inhibitor reduces Akt activity and Raf-1 protein levels.** (A) Phosphorylated Akt (serine 473) and total Akt in MIN6 cells treated with GW5074 for 3 hours (n=3 different cultures). (B) Quantification of phosphorylated Akt (serine 473) and total Akt protein levels normalized to the control. (C) Quantification of total Akt normalized to the control. (D) GW5074 and Akti-1/2 both reduced total Raf-1 protein levels in MIN6 cells and quantification as shown (n=3 different cultures)(E). Note that this is the same β-Actin loading control shown in Figure 21A. (F) Additive effects of blocking both Raf-1 and Akt on β-cell death. Rapid increase in propidium iodide incorporation in the presence of GW5074 alone, or the combination of GW5074 and Akti-1/2 MIN6 cells (n=9 different cultures). *Denotes a significant difference ($p<0.05$, Student's *t* test) between treatment and control.

Figure 23

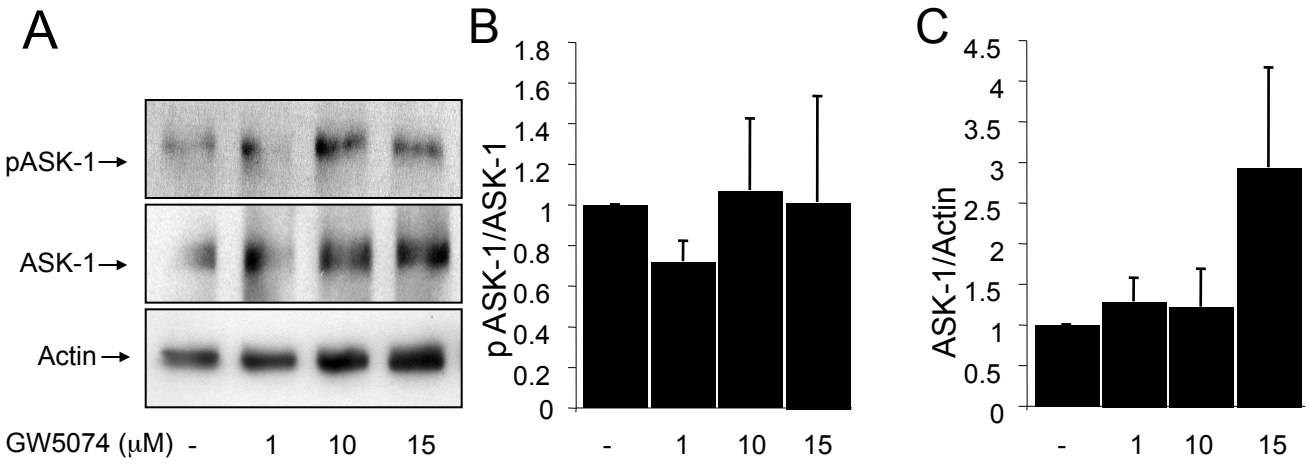

A
pASK-1
ASK-1
Actin
GW5074 (μM) - 1 10 15
B
pASK-1/ASK-1
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
- 1 10 15
C
ASK-1/Actin
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
- 1 10 15

Figure 23. **Effects of GW5074, a Raf-1 inhibitor on Apoptosis-Signaling Kinase-1.**

(A) GW5074, a Raf-1 inhibitor, did not significantly change the level of phosphoryalated ASK-1(Thr845) or total ASK-1 protein levels. (B) Quantification of phosphorylated ASK-1 (Thr845) and total ASK-1 protein levels corrected to the control. (C) Quantification of total ASK-1/actin levels corrected to the control. N=3 Western blots from three independent cultures.

Figure 24

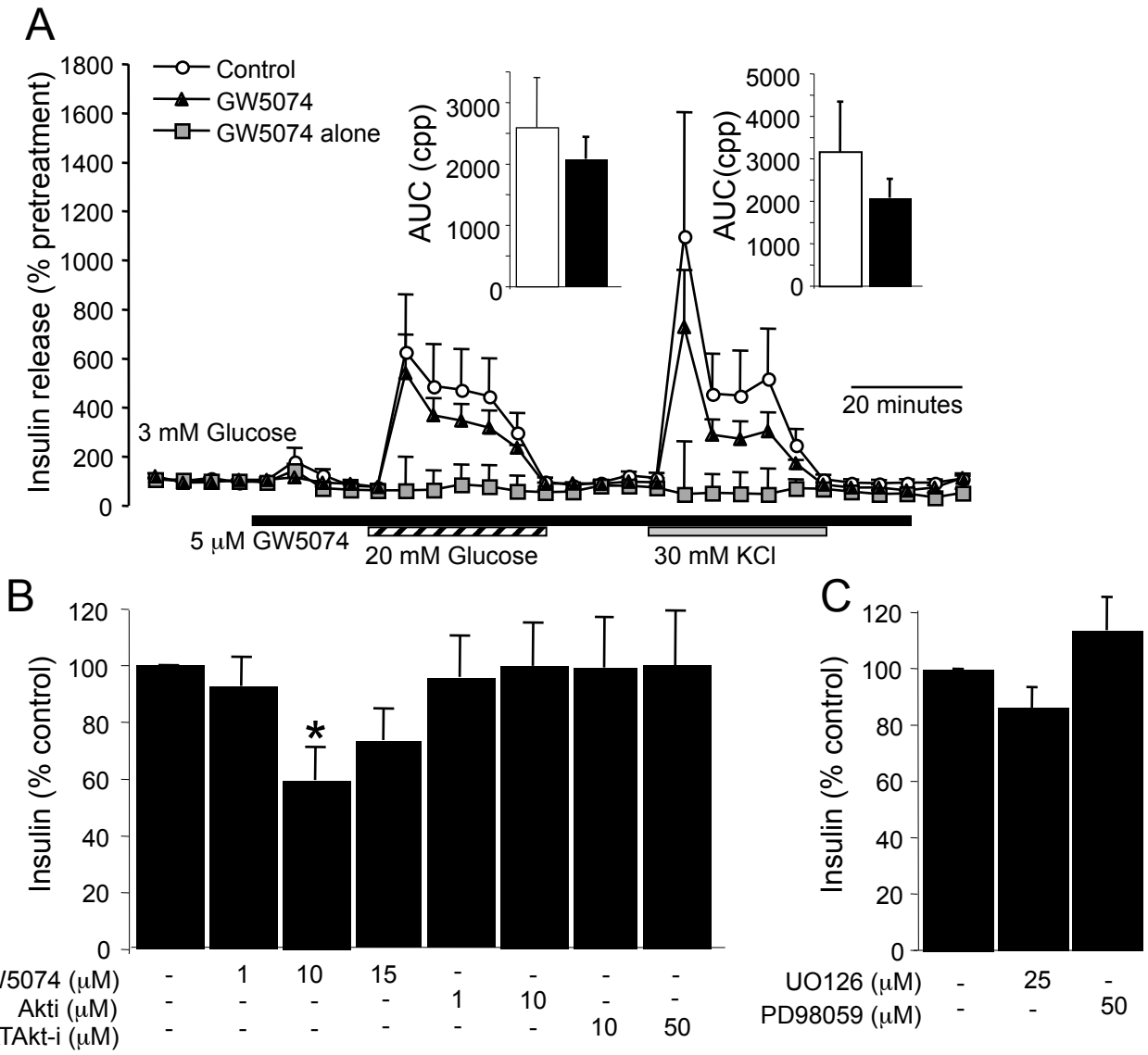

A
Control
GW5074
GW5074 alone
Insulin release (% pretreatment)
AUC (cpp)
AUC(cpp)
3 mM Glucose
5 μM GW5074
20 mM Glucose
30 mM KCl
20 minutes
B
Insulin (% control)
GW5074 (μM)
Akti (μM)
TATAkt-i (μM)
C
Insulin (% control)
UO126 (μM)
PD98059 (μM)

Figure 24. **Effects of Raf-1 inhibitor on insulin secretion in primary islets and MIN6 cells**. (A) Groups of 100 mouse islets were perifused with Krebs-Ringer buffer containing 3 mM glucose. Islets were exposed to 20mM glucose (striped bar), 30mM KCl (grey bar), in the presence or absence of 5μM GW5074 (black bar). Control islets (open circles) were exposed to glucose and KCl without GW5074. Islets treated with GW5074 (closed triangle) were also exposed to glucose and KCl. Some islets were exposed to GW5074 alone (grey squares) without glucose or KCl. Values are normalized to the pretreatment levels of insulin secretion to compensate for uneven numbers of islets in each column (n=4 different cultures). Area under the curve (AUC) was measured as the cumulative percent pretreatment (cpp) and shown as insets. Insulin levels were measured in conditioned media of MIN6 cells treated with GW5074, Akti-1/2, UO126 and PD98059 for 3 hours (n=3 independent cultures)(B,C). *Denotes a significant difference ($p<0.05$, Student's *t* test) between treatment and control (serum-free).

5. Results III: Conditional deletion of Raf-1 kinase in β-cells *in vivo*

5.1 Generation of pancreatic β-cell–specific Raf-1 knockout mice

We have previously reported that insulin protects β-cells from apoptosis and promotes β-cells proliferation via Raf-1 signalling *in vitro* (42). Thus, we hypothesized that Raf-1 may play an important role in β-cell growth and survival *in vivo*. To test this hypothesis, we conditionally targeted the β-cell Raf-1 gene using a transgenic mouse line expressing Cre recombinase driven by a 668 bp promoter fragment of the rat insulin II gene (RIP-Cre) (209, 210). While insulin promoter fragments of this size are thought to direct expression only to the pancreatic β-cell, RIP-Cre expression has been reported in the brain, consistent with reports describing insulin in the hypothalamus, olfactory bulb, and cerebral cortex (211). Although endogenous insulin gene transcription begins at 9.0 days post coitum (dpc) in mice (212), RIP-Cre-mediated recombination can be detected at 11.5 dpc in insulin-producing cells within the pancreas (213). There are caveats of using the RIP-Cre system to drive β-cell inactivation of the Raf-1 gene. For example, populations of cells within the pancreas may activate the insulin promoter at early stages of development but fail to become β-cells. A major caveat of this system is that the RIP-Cre mice alone display glucose intolerance (210). Thus, we employed the following breeding scheme to achieve conditional Raf-1 deletion in the β-cell and to obtain the proper controls: $RIPCre^{+/-}$:$Raf\text{-}1^{flox/wt}$ male mice were crossed with female $RIPCre^{-/-}$:$Raf\text{-}1^{flox/wt}$ mice (Figure 25A). To demonstrate tissue-specific deficiency of Raf-

1, we used Western blot analysis to examine the protein levels of Raf-1 in islets isolated from RIPCre$^{+/-}$:Raf-1$^{flox/flox}$ and RIPCre$^{+/-}$:Raf-1$^{wt/wt}$ mice. Immunoblotting indicated a marked reduction of approximately 80% of Raf-1 protein in islets isolated from RIPCre$^{+/-}$:Raf-1$^{flox/flox}$ mice compared to control RIPCre$^{+/-}$:Raf-1$^{wt/wt}$ mice (Figure 25B,C). Indeed, we expected less than 100% knockdown of Raf-1 protein because the islets are made up of several types of cells not targeted by RIP-Cre. We did not observe significant changes in B-Raf and ERK1/2 protein levels (Figure 25C). Collectively, these data suggest that we were successful in generating a pancreatic β-cell–specific Raf-1 knockout mouse line.

5.2 Body weight and fasting blood glucose levels of Raf-1 knockout mice

The RIPCre$^{+/-}$:Raf-1$^{flox/flox}$ mice were viable and appeared to be grossly phenotypically indistinguishable from other genotypes and wild-type animals. The mice developed to adulthood, were fertile, and exhibited a normal lifespan. Thus, we conclude that the Raf-1 gene is not essential for mouse islet development. Next, we investigated whether the β-cell-specific Raf-1 knockout would affect body weight and fasting blood glucose levels. We observed no significant change in body weight between RIPCre$^{+/-}$:Raf-1$^{flox/flox}$ and RIPCre$^{+/-}$:Raf-1$^{wt/wt}$ (Figure 26A,B). However, we noted an increased fasting basal blood glucose level in female RIPCre$^{+/-}$:Raf-1$^{flox/flox}$ compared to RIPCre$^{+/-}$:Raf-1$^{wt/wt}$, especially at a younger age (Figure 26C). These observations suggest that Raf-1 may regulate glucose homeostasis *in vivo*.

5.3 Raf-1 knockout mice have impaired glucose tolerance

To investigate whether conditional depletion of Raf-1 gene causes β-cell dysfunction, we performed glucose tolerance tests. We observed that RIPCre$^{+/-}$:Raf-1$^{flox/flox}$ female mice have a delayed glucose response compared to littermate control RIPCre$^{+/-}$:Raf-1$^{wt/wt}$ mice at both 8 and 20 weeks of age (Figure 27A,C). We observed a similar trend in our male cohort, although there was a less robust difference between the RIPCre$^{+/-}$:Raf-1$^{flox/flox}$ mice and RIPCre$^{+/-}$:Raf-1$^{wt/wt}$ mice (Figure 27B,D). Next, we performed insulin tolerance tests. Female RIPCre$^{+/-}$:Raf-1$^{flox/flox}$ mice displayed normal insulin tolerance response compared to littermate control RIPCre$^{+/-}$:Raf-1$^{wt/wt}$ mice (Figure 28A,B). The male cohort displayed a similar trend, but the heterozygous RIPCre$^{+/-}$:Raf-1$^{flox/wt}$ mice appeared to be more insulin resistant than RIPCre$^{+/-}$:Raf-1$^{flox/flox}$ and RIPCre$^{+/-}$:Raf-1$^{wt/wt}$ mice (Figure 28C,D). These observations further suggest that Raf-1 may regulate glucose homeostasis *in vivo* and warrant further thorough investigation.

5.4 Insulin secretion defect in Raf-1 knockout mice

Because we observed a normal response in the insulin tolerance test, we investigated whether the impairment in glucose tolerance was due to defect in insulin secretion. We obtained islets from RIPCre$^{+/-}$:Raf-1$^{flox/flox}$ and RIPCre$^{+/-}$:Raf-1$^{wt/wt}$ mice and subjected them to perifusion with high glucose and KCl. We observed a blunted response to both

glucose and KCl (Figure 29A), suggesting that the defect in glucose-stimulated insulin release was distal to the activation of voltage-gated calcium channels. We also observed a similar effect in RIPCre$^{+/-}$:Raf-1$^{flox/wt}$. Thus, Raf-1 appears to play a role in the distal stages of exocytosis, either directly or indirectly. In preliminary data, we did not observe a significant reduction in insulin content (Figure 29B) in Raf-1 knockout mice compared to controls, which suggest a defect in insulin secretion. Together with our *in vitro* findings, these data are consistent with the concept that Raf-1 plays an important role in the maintenance of functional β-cell mass.

Figure 25

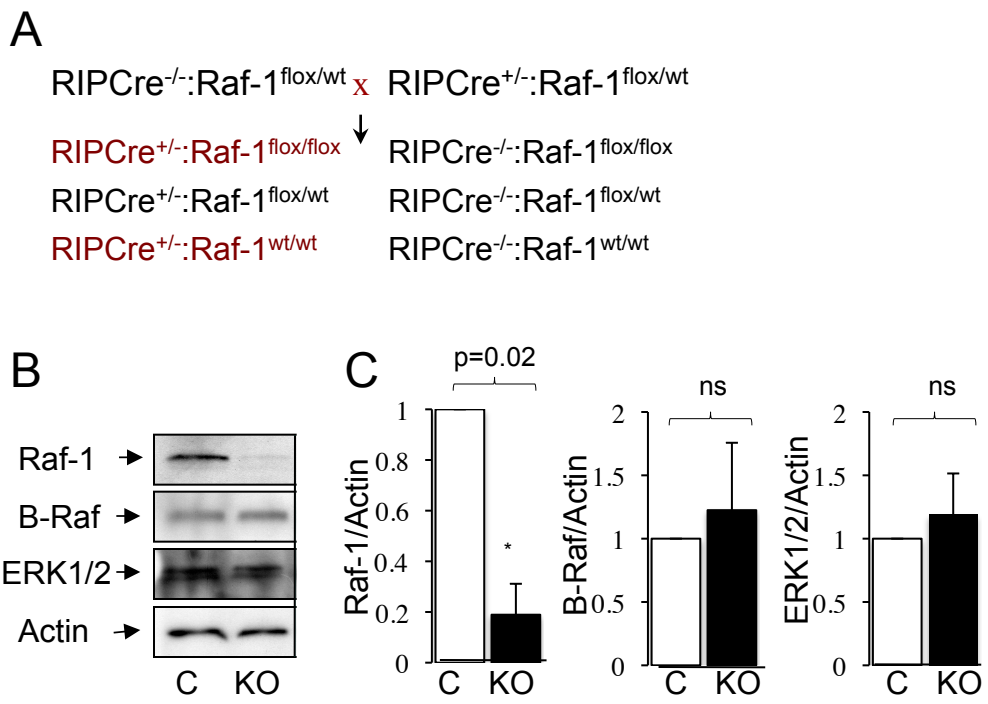

A
RIPCre-/-:Raf-1flox/wt x RIPCre+/-:Raf-1flox/wt
RIPCre+/-:Raf-1flox/flox
RIPCre-/-:Raf-1flox/flox
RIPCre+/-:Raf-1flox/wt
RIPCre-/-:Raf-1flox/wt
RIPCre+/-:Raf-1wt/wt
RIPCre-/-:Raf-1wt/wt
B
Raf-1
B-Raf
ERK1/2
Actin
C
KO
C
p=0.02
Raf-1/Actin
1
0.8
0.6
0.4
0.2
0
*
C
KO
ns
B-Raf/Actin
2
1.5
1
0.5
0
C
KO
ns
ERK1/2/Actin
2
1.5
1
0.5
0
C
KO

Figure 25. **Generation of pancreatic β-cell–specific Raf-1 knockout mice (RIPCre$^{+/-}$:Raf-1$^{flox/flox}$).** (A) Breeding scheme diagram. (B) Isolated islets from female RIPCre$^{+/-}$:Raf-1$^{flox/flox}$ (KO) and RIPCre$^{+/-}$:Raf-1$^{wt/wt}$ (control, C) mice at 12 wks were subjected to Western blotting for protein expression levels of Raf-1, B-Raf, and ERK1/2. (C) Quantification of protein levels in B corrected to control. *Denotes P<0.05 between RIPCre$^{+/-}$:Raf-1$^{wt/wt}$ (control, C) and RIPCre$^{+/-}$:Raf-1$^{flox/flox}$ (KO) or RIPCre$^{+/-}$:Raf-1$^{flox/wt}$ (Het) by Student's t-test, n=3 animal with littermate control.

Figure 26

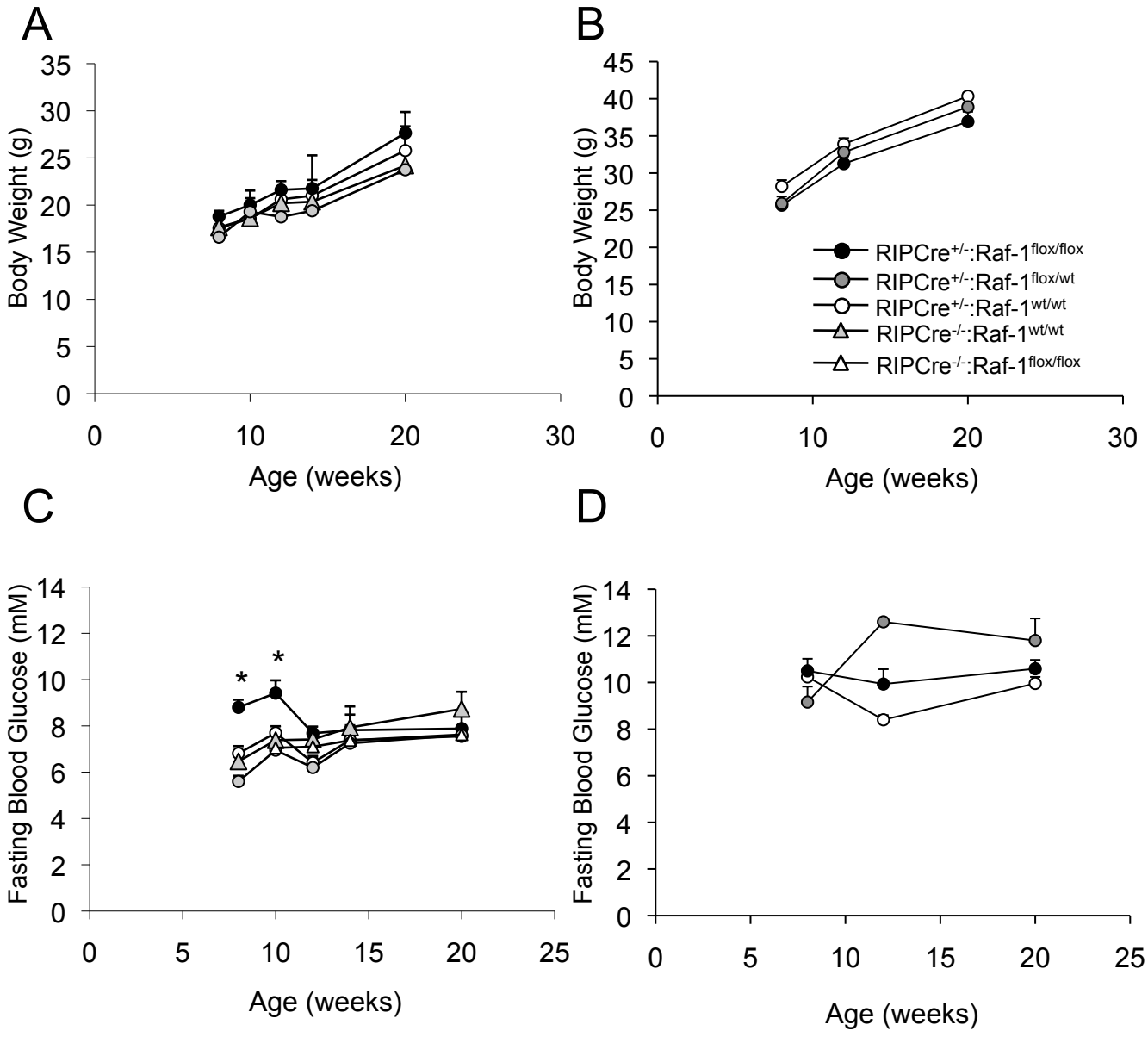

A
Body Weight (g)
Age (weeks)
B
Body Weight (g)
Age (weeks)
RIPCre+/-:Raf-1flox/flox
RIPCre+/-:Raf-1flox/wt
RIPCre+/-:Raf-1wt/wt
RIPCre-/-:Raf-1wt/wt
RIPCre-/-:Raf-1flox/flox
C
Fasting Blood Glucose (mM)
Age (weeks)
*
*
D
Fasting Blood Glucose (mM)
Age (weeks)

Figure 26. **Body weight and fasting blood glucose levels of RIPCre$^{+/-}$:Raf-1$^{flox/flox}$ mice.** RIPCre$^{+/-}$:Raf-1$^{flox/flox}$ (closed circle) and RIPCre$^{+/-}$:Raf-1$^{wt/wt}$ littermates (open circle) were fasted for 6 hr, and body weight (A,B) and fasting blood glucose levels (C,D) were measured. *Denotes $p<0.05$ between the 2 groups of mice as determined by Student's t-test, A,C (female, n= 6 knockout mice with littermate control) and B,D (male, n=5 knockout mice with littermate control except on week 12, where there is only one knockout mouse with littermate control).

Figure 27

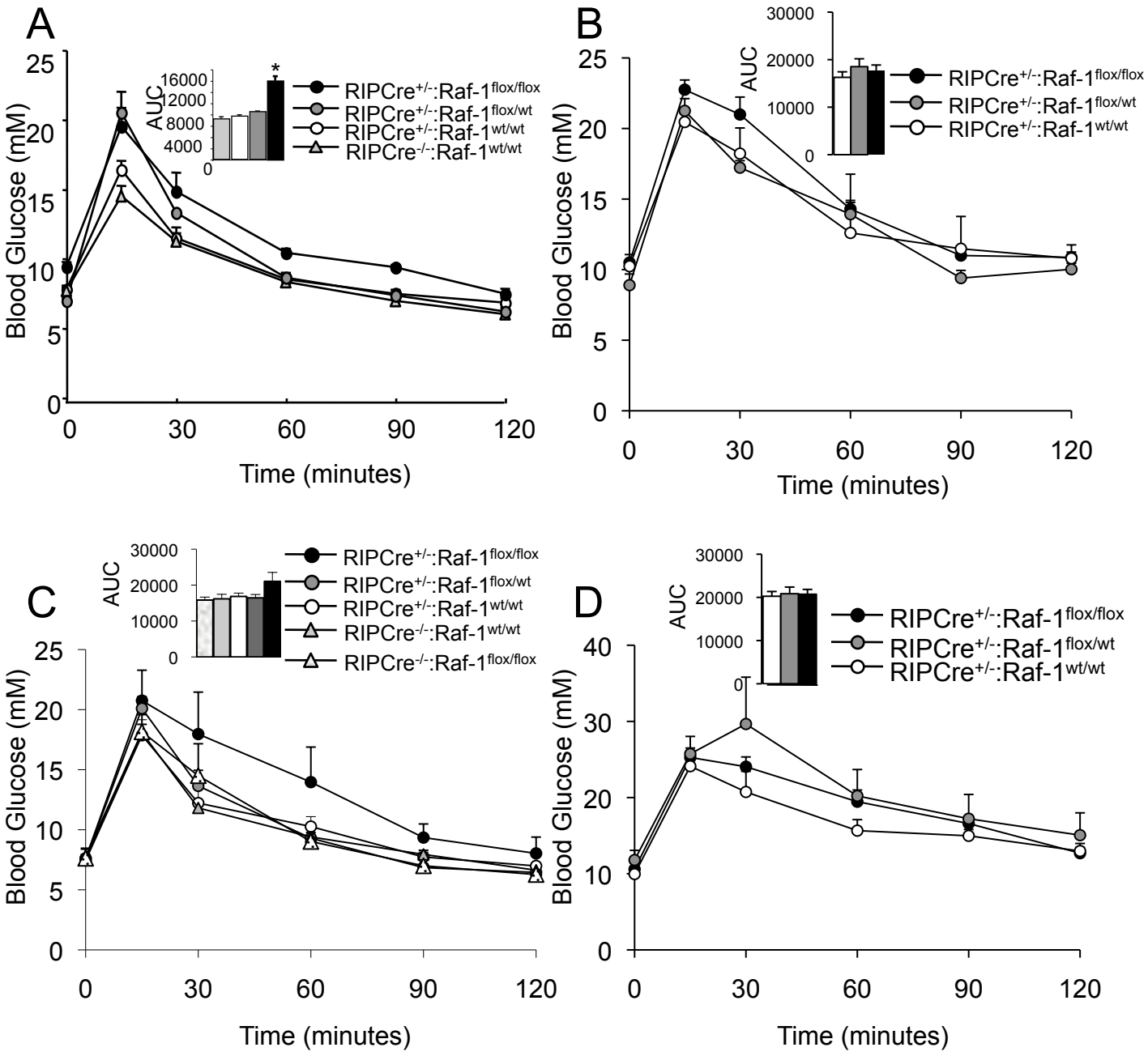

A
B
C
D
Blood Glucose (mM)
Time (minutes)
AUC
RIPCre+/-:Raf-1flox/flox
RIPCre+/-:Raf-1flox/wt
RIPCre+/-:Raf-1wt/wt
RIPCre-/-:Raf-1wt/wt
RIPCre-/-:Raf-1flox/flox
*

Figure 27. **Glucose tolerance test on RIPCre$^{+/-}$:Raf-1$^{flox/flox}$ mice.** Four groups of littermate mice (RIPCre$^{-/-}$:Raf-1$^{wt/wt}$ (light-gray triangle), RIPCre$^{-/-}$:Raf-1$^{wt/wt}$ (open circle), RIPCre$^{-/-}$:Raf-1$^{flox/wt}$ (dark-gray triangle), and RIPCre$^{+/-}$:Raf-1$^{flox/flox}$ (closed circle)) were fasted for 6 hr before glucose tolerance tests were performed by intraperitoneal delivery of glucose (2 g/kg of BW) at 8 wks (A,C) and 20 wks (B,D). Blood glucose level was monitored for 2 hour after glucose delivery. *Denotes $p<0.05$ between RIPCre$^{+/-}$:Raf-1$^{flox/flox}$ RIPCre$^{+/-}$:Raf-1$^{wt/wt}$ mice as determined by Student's t-test. A,C (female, n=6 knockout mice with littermate control) and B,D (male, n=5 knockout mice with littermate control). Area under the curve (AUC) was measured as the cumulative percent to fasting blood glucose level (time 0) and shown as insets.

Figure 28

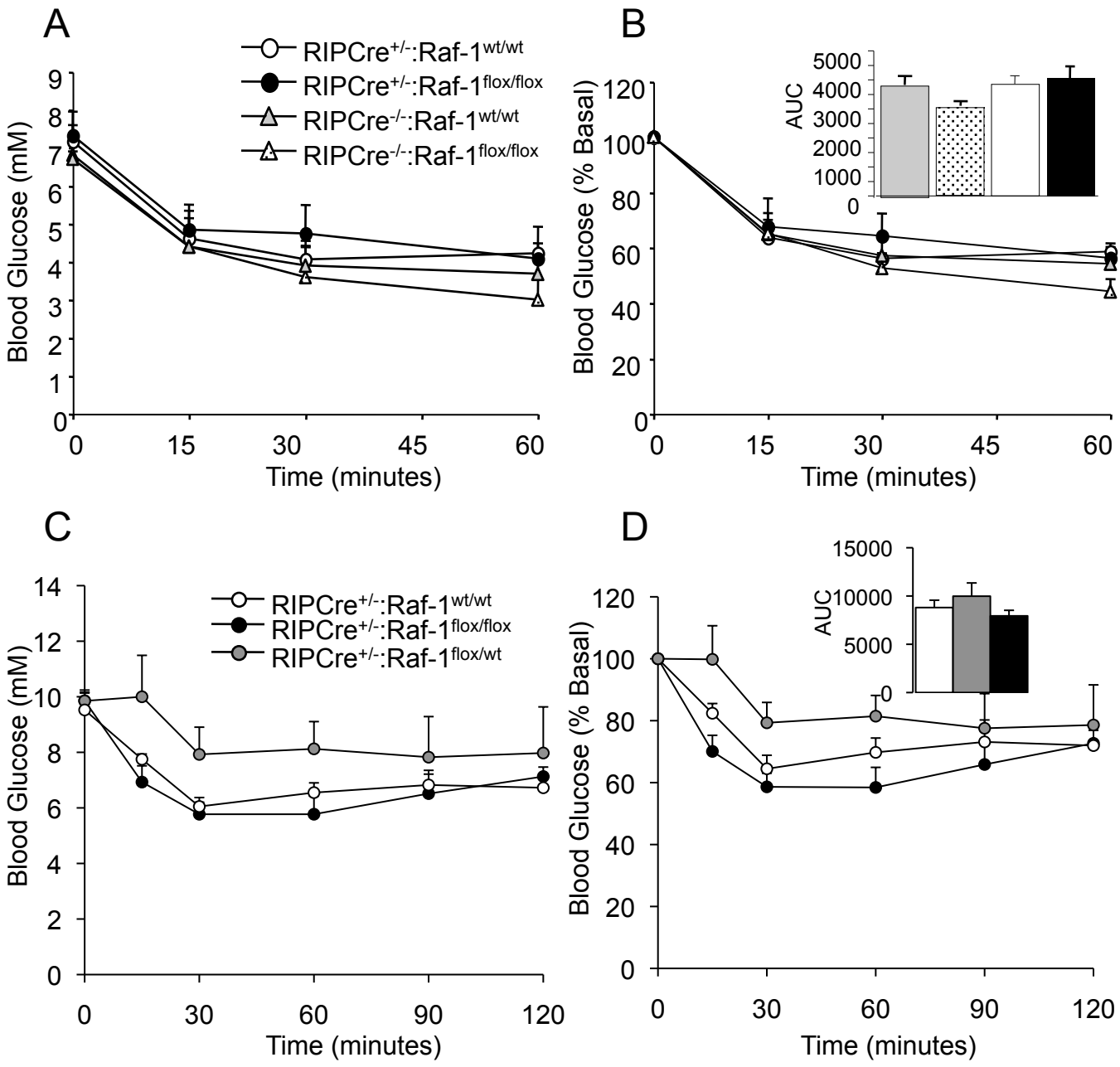
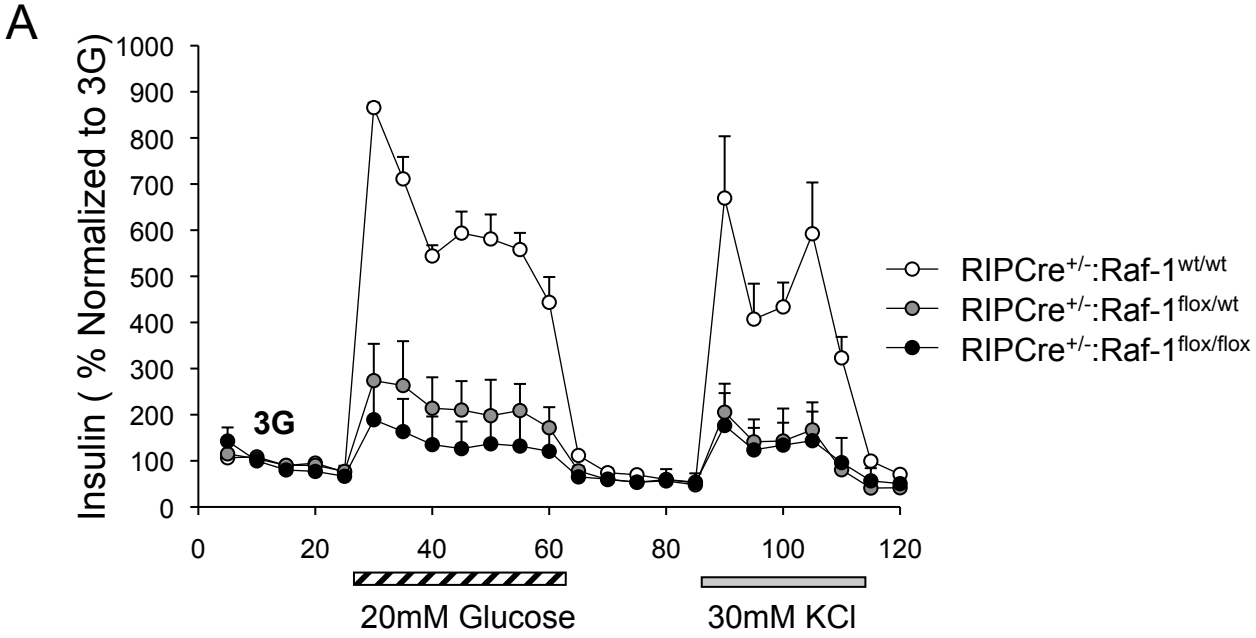

A
RIPCre+/-:Raf-1wt/wt
RIPCre+/-:Raf-1flox/flox
RIPCre-/-:Raf-1wt/wt
RIPCre-/-:Raf-1flox/flox
Blood Glucose (mM)
Time (minutes)
B
Blood Glucose (% Basal)
AUC
Time (minutes)
C
RIPCre+/-:Raf-1wt/wt
RIPCre+/-:Raf-1flox/flox
RIPCre+/-:Raf-1flox/wt
Blood Glucose (mM)
Time (minutes)
D
Blood Glucose (% Basal)
AUC
Time (minutes)

Figure 28. **Insulin tolerance test on RIPCre$^{+/-}$:Raf-1$^{flox/flox}$ mice.** Four groups of littermate mice (RIPCre$^{-/-}$:Raf-1$^{wt/wt}$ (light-gray triangle), RIPCre$^{-/-}$:Raf-1$^{flox/flox}$(dotted triangle), RIPCre$^{+/-}$:Raf-1$^{wt/wt}$ (open circle), and RIPCre$^{+/-}$:Raf-1$^{flox/flox}$(closed circle)) were fasted for 6 hr before insulin tolerance tests were performed by intraperitoneal delivery of insulin (.75units/kg of BW) at 8 wks. Blood glucose was monitored for 1 hour after insulin delivery. A,C Shows blood glucose (mM) values and B,D shows blood glucose corrected from basal glucose levels. A,B (female, n=6 animal with littermate control) and C,D (male, n=4 animal with littermate control).

Figure 29


A
Insulin (% Normalized to 3G)
1000
900
800
700
600
500
400
300
200
100
0
0
20
40
60
80
100
120
3G
20mM Glucose
30mM KCl
RIPCre+/-:Raf-1wt/wt
RIPCre+/-:Raf-1flox/wt
RIPCre+/-:Raf-1flox/flox

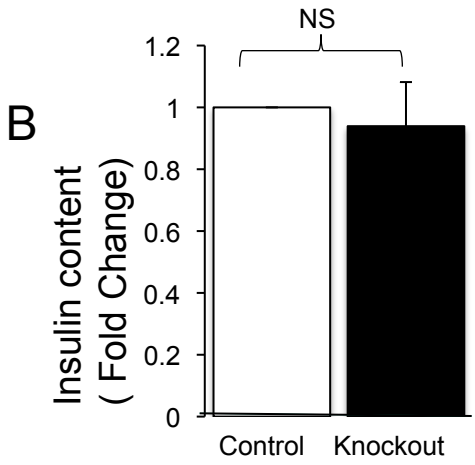

B
NS
Insulin content
(Fold Change)
1.2
1
0.8
0.6
0.4
0.2
0
Control
Knockout

Figure 29. **Insulin secretion defect in RIPCre$^{+/-}$:Raf-1$^{flox/flox}$ mice**. Islets isolated from RIPCre$^{+/-}$:Raf-1$^{flox/flox}$ (closed circle), RIPCre$^{+/-}$:Raf-1$^{flox/wt}$ (grey circle), and RIPCre $^{+/-}$:Raf-1$^{wt/wt}$ (open circle) littermates were perifused with Krebs-Ringer buffer containing 3mM glucose. Islets were exposed to 20mM glucose (striped bar) and 30 mM KCl (gray bar). Values are normalized to the pretreatment levels of insulin secretion to compensate for uneven numbers of islets in each column (n=3 different cultures, female, 6-8 wks of age). (B) insulin content were measured in size-matched islets isolated from RIPCre$^{+/-}$:Raf-1$^{flox/flox}$ (KO), and RIPCre$^{+/-}$:Raf-1$^{wt/wt}$ (Control), n=3 animal with littermate control.

6. Discussion

6.1 Summary of major findings

The ultimate goal of this research is to understand the mechanisms controlling pancreatic β-cell apoptosis so that we can apply this information therapeutically to type 1 diabetes, type 2 diabetes and clinical islet transplantation. In the present thesis, we demonstrated that insulin promotes β-cell survival and proliferation via the activation of Raf-1 kinase. We showed that Raf-1 kinase is expressed and functional in the β-cell. We further demonstrated that inhibiting basal Raf-1 kinase activity results in a rapid and robust programmed cell death in both primary islets and the MIN6 β-cell line. We also showed that Raf-1 protects β-cells via both ERK1/2-dependent and -independent modes (see Figure 30A). In the latter cascade, Raf-1 inhibition dephosphorylates Bad at serine 112 and increases the levels of pro-apoptotic Bax. We also found that Raf-1 and Akt work together to promote β-cell survival and that blocking both Raf-1 and Akt signalling caused additive β-cell death. We observed that Raf-1 plays a small but significant role in insulin secretion *in vitro*. Furthermore, our preliminary data suggest that Raf-1 may play a role in glucose homeostasis and insulin secretion *in vivo*. Collectively, these findings establish Raf-1 as a key node in the network of proteins controlling β-cell survival and function.

6.2 Local concentrations of insulin in the islets

The present findings suggest that insulin stimulates Raf-1/ERK1/2 activation and Bad deactivation at concentrations expected in the systemic and portal circulation, around 40-1000 pM (214). The *in vivo* significance of these findings remains to be determined. Specifically, it is not yet clear whether our results mean that insulin signalling is constitutively activated in islets *in vivo* or whether the local concentrations of insulin that β-cells are exposed to are lower than expected. Although it may seem intuitive that β-cells are exposed to higher concentrations of insulin than those observed in the systemic or portal circulation, no direct *in vivo* measurements of insulin levels in the intact local micro-environment have been reported to date. Indeed, there are a few clues that point to the possibility that local concentrations of bioactive, monomeric insulin may be within the picomolar or low nanomolar range. Theoretically, the concentration of free monomeric insulin available for binding to insulin receptors is dictated by a number of factors, including the rate of local blood flow, the rate of insulin exocytosis, and the rate at which insoluble zinc-containing insulin crystals and hexamers dissolve into monomeric insulin. Our group has proposed that a mathematical model might be useful in estimating the contribution of each factor (215). It is also important to consider the complex architecture of the islet microvasculature, which allows for some β-cells to be perfused first (216-218). Regardless of the rates of insulin secretion, decrystalization and blood flow, this would mean that a population of β-cells would be exposed to insulin concentrations in the picomolar range. Thus, our studies with 0.2 nM insulin are likely to be physiologically relevant to at least a sub-set

population of β-cells.

6.3 Effects of insulin in pancreatic β-cell fate

Numerous studies point to insulin as a key regulator of β-cell apoptosis and proliferation *in vitro* (38, 42) and *in vivo* (84-86). *In vivo* experiments have established the requirement for insulin receptor signalling for β-cell mass homeostasis, but they do not distinguish between acute and chronic effects of insulin or provide information on the optimum concentration of circulating insulin. While the exact concentration of dissolved insulin available for β-cell insulin receptors *in vivo* remains a subject of speculation and debate, our *in vitro* studies and others indicated that low nanomolar (0.2-20 nM) concentrations of insulin rescued human and rodent islets from serum-withdrawal-induced apoptosis (38, 42). Higher concentrations of insulin (200 nM) were relatively ineffective at reducing human β-cell apoptosis (42). Similarly, work from another group found that low insulin (0.1 nM) reduced caspase 3-activity in glucose-deprived β-cells, while 100 nM insulin increased caspase-3-mediated death (219), further suggesting that insulin signalling can be biphasic (102, 220). These and other observations led us to postulate that the pro-survival and mitogenic autocrine effects of insulin might be lost outside a relatively narrow concentration range (42, 178). It is also notable that concentrations of insulin in the picomolar to low nanomolar range have higher affinity for the insulin receptor than superphysiological concentrations (177). Both low (0.2 nM) and high (200 nM) concentrations of insulin stimulate β-cell proliferation,

but the high concentrations of insulin readily cross-activate the IGF-1 receptor (177). Indeed, the effects of 100 nM insulin and 100 nM IGF-1 on MIN6 cell apoptosis were not additive (221), suggesting common mediators. Collectively, our new data point to ERK1/2 and Bad as downstream targets that can be significantly regulated by 0.2 nM insulin. Consistently, the effects of insulin at the high 200 nM concentration were either non-significant or did not show a stronger effect than 0.2nM insulin. Negative feedback in insulin signalling may be an adaptation to prevent the oncogenic effects of excess mitogenic and anti-apoptotic signalling (222).

6.4 Novel mechanisms of the pro-survival effects of insulin

Insulin is known to activate multiple downstream pathways to suppress apoptosis in other cell types (223), but the specific pathways targeted by insulin in β-cells had not been elucidated. Previous evidence suggested that Akt was not likely to play a dominant role in β-cell insulin signalling (42) or the regulation of β-cell mass (52). Most of the protective effects of insulin were maintained in mice severely deficient in islet Akt activity (42). Moreover, insulin did not stimulate Akt phosphorylation at serine 473 in primary islets (42). Because we did not investigate other key phosphorylation sites of Akt, we cannot fully rule out the effects of insulin on this kinase. Nevertheless, instead of Akt, we focused the present study on Raf-1, a kinase that has not previously been studied in the pancreatic β-cells but is an established insulin target in other cell lines (73). Our studies indicate that treatment of islets and MIN6 cells with 0.2 nM insulin,

which is likely to interact primarily with the insulin receptor rather than the IGF-1 receptor, caused an increase in Raf-1 phosphorylation at serine 338. This is in accordance with earlier studies demonstrating that 0.1 nM or 7 nM insulin significantly increased Raf-1 activity and serine phosphorylation in cell lines expressing insulin receptors (73, 74). We have previously demonstrated that Raf inhibitor and dominant-negative Raf-1 specifically blocked the effects of 0.2 nM insulin on β-cell proliferation, but did not decrease the proliferation stimulated by 200 nM insulin. Together, our findings support the notion that the Raf/MEK/ERK1/2 arm of the insulin signalling pathway plays a critical role in both β-cell survival and proliferation.

Our data presented here and work from other groups are in agreement that insulin and glucose stimulate the rapid translocation of ERK1/2 to the nucleus (182), an event likely to promote β-cell survival and growth via ERK1/2-dependent transcription (182). Additional support for the hypothesis that insulin and glucose may have overlapping mechanisms comes from the observations that the activation of ERK1/2 by insulin and glucose share many common signalling requirements, including Ca^{2+} mobilization, and the activation of FK506-sensitive calcineurin, and KN93-sensitive CaMKII (182, 185, 224). The present study provides additional evidence that Ca^{2+} mobilization is important for insulin-induced ERK1/2 phosphorylation. Blocking Raf-1, ERK1/2, or Ca^{2+} signals induced β-cell apoptosis. Furthermore, we have now identified the NAADP-sensitive Ca^{2+} store as a mediator of ERK1/2 activation in β-cells. Like insulin, NAADP-stimulated Ca^{2+} mobilization follows a bell-shaped concentration-response profile in human β-cells (102). Together with our previous data on CD38-deficient islets (190), these

observations point to the complex role of Ca^{2+} signalling in the β-cell that warrant further investigation.

Although ERK1/2 is known as a pro-survival kinase in many cell types (184), the role of ERK1/2 in β-cell survival is enigmatic. Some investigations point towards an anti-apoptotic role for ERK1/2, while other studies suggest a requirement for sustained ERK1/2 phosphorylation in various forms of β-cell apoptosis (58, 80). Multiple studies have reported that cAMP/PKA mediated-ERK1/2 signalling regulates β-cell growth and survival via Bcl-2 and IRS-2 (58, 61). Our own work suggests that basal Raf-1/ERK1/2 signalling is essential for survival in primary β-cells. For instance, a dominant-negative Raf-1 mutant or a Raf inhibitor caused rapid death that was associated with down-regulated ERK1/2. In the present study, we observed increased death in β-cells treated with Raf and Ca^{2+} signalling inhibitors that was partly attributable to blunted ERK1/2 activation. On the other hand, sustained ERK1/2 activation of up to four days has been shown to be required for β-cell apoptosis induced by chronic high glucose (33.3 mM) or interleukin-1β treatment (80). The mechanisms of a putative dual role for ERK1/2 remain to be elucidated, but clearly the duration, strength and sub-cellular localization of ERK1/2 signalling may be important in determining its net effect on cell fate (83).

Another key finding of the present study involves insulin's effect on Raf-1 at the mitochondria. In the present study, we showed that over-expressed Raf-1:GFP fusion proteins were targeted to the mitochondria in the MIN6 β-cell line. Furthermore, we

confirmed that endogenous Raf-1 localized to the mitochondria of islet β-cells and MIN6 cells. In addition to inducing ERK1/2 activation, insulin tightened the localization of Raf-1 to mitochondria and promoted mitochondrial Raf-1 activity. These events allow Raf-1 to contribute to cellular survival by phosphorylating Bad. The possibility that Raf-1 might also function by interacting with other proteins in the vicinity of mitochondria remains to be addressed. The effects of insulin on Raf-1 at the mitochondria of islets and MIN6 cells are consistent with studies done on other cell types (143, 144). In other models, Bcl-2 appears to be critical for Raf-1 translocation to the mitochondria (143). We detected relatively stable protein-protein interactions between Raf-1 and Bcl-2 in β-cells. Because Raf-1 physically associates with Bad, it is possible that Raf-1 may regulate the function of Bad in glucose metabolism and insulin secretion (147). Thus, it will be of interest in the future to unravel exactly how insulin regulates Raf-1 binding to Bcl-2 proteins, the mitochondrial translocation of this complex, and the phosphorylation of Bad.

6.5 Autocrine insulin signalling in pancreatic β-cells treated with glucose

Like insulin, glucose has been reported to regulate proliferation and survival of β-cells (182). Glucose has been shown to act on the same set of genes as insulin (225, 226). However, it is unclear if the pro-survival effects of glucose occur via a mechanism independent of autocrine insulin signalling. In the present study, glucose-induced ERK1/2 phosphorylation was completely blocked in the presence of somatostatin in MIN6 cells. These data are in agreement with previous observation that somatostatin or

neutralizing anti-insulin antibodies reduced β-cell proliferation in the presence of high glucose in dispersed primary mouse β-cells and MIN6 cells (44, 221). In contrast to the effects of somatostatin on glucose-treated MIN6 cells, Wicksteed *et al* reported that somatostatin had no effect on high glucose-induced activation of ERK1/2 in rat islets (227). Differences in experimental procedures, the species, or microenvironment (dispersed vs. intact islets) may explain this discrepancy. These experiments also come with the caveat that somatostatin might directly inhibit glucose-dependent ERK1/2 phosphorylation that has been proposed to occur via cAMP and PKA (66). Therefore, we also directly assessed the role of secreted insulin and demonstrated that islets from mice with a ~50% reduction in glucose-stimulated insulin secretion have a proportional ~50% loss of ERK1/2 activation in response to glucose. Ideally, these studies would be complemented with experiments using primary islets made acutely deficient in insulin receptors using conditional gene ablation. It was recently shown that β-cell lines lacking insulin receptors (but with IGF-1 receptor up-regulation) had impaired activation of PI3-kinase and Akt in response to glucose, but intact ERK1/2 phosphorylation remained (228). Together, these data suggest that both glucose and insulin can activate ERK1/2, although a large proportion of the effects of glucose can be accounted for by autocrine insulin signalling. While additional work is clearly required, studies from multiple groups point to an important role for autocrine insulin signalling in β-cells.

6.6 Raf-1 function in vitro and its downstream mechanisms

Although ours is the first study to directly examine the consequences of blocking Raf-1 in primary and transformed β-cells, a possible role for Raf-1 in β-cell fate could be inferred from several previous studies. For example, over-expression of RKIP, a protein known to inhibit Raf-1 kinase, prevented proliferation of transformed β-cells, suggesting the possibility that Raf-1 may be involved in β-cell growth (63). ERK1/2, a well-known downstream target of Raf-1, has also been shown to participate in pathways controlling β-cell survival. It has been reported in several studies that acute glucose and PKA signalling may regulate β-cell growth and survival through ERK1/2 (11, 58, 66). On the other hand, others have shown that activation of ERK1/2 is required for human islet apoptosis in response to chronic exposure to high glucose concentrations or Interleukin-1β (78). Thus, ERK1/2 signalling is activated in both pro-survival and pro-apoptotic conditions, but the outcome may depend on the timing and duration of ERK1/2 activation (83). As reported by Longuet *et al*, ERK1/2 may also regulate insulin secretion by phosphorylating synapsin I, a protein that is involved in insulin exocytosis (154, 155). In the present study, we confirm that in both MIN6 cells and isolated mouse islets, blocking Raf-1 moderately reduces insulin secretion. Overall, our data further support the concept that ERK1/2 can transmit pro-survival signals and may promote insulin secretion in β-cells. However, our results with the Raf-1 inhibitor also point to the presence of important mechanisms downstream of Raf-1 that may not involve ERK1/2.

Attenuation of Raf-1 kinase activity or ablation of Raf-1 protein levels is known to cause apoptosis in many cell types (153, 207) including the pancreatic β-cells (229) as demonstrated for the first time in the present study. The most well known signalling action of Raf-1 kinase involves the activation of the MEK/ERK1/2 transduction cascade. However, the generation of mice with whole body Raf-1 gene deletion yet intact MEK/ERK1/2 activity enabled the identification of multiple downstream effectors other than MEK/ERK1/2. Specifically, Raf-1 directly interacts with and phosphorylates Bad (143). Our data in pancreatic β-cells support the observation that Raf-1 can reside at the mitochondria and deactivate Bad, as previously reported in HEK 293T cells (143, 144). However, the mechanisms of how Raf-1 translocates to the mitochondria remains elusive. Wang *et al* have demonstrated that Bcl-2 proteins shuttle Raf-1 to the mitochondria (143). Indeed, we have demonstrated that Raf-1 co-immunoprecipitated with Bcl-2 and Bcl-xL, suggesting a direct or indirect interaction. But it remains unclear whether such an interaction is required for Raf-1 to translocate to the mitochondria. Studies using cells devoid of individual or multiple Bcl-2 proteins will be helpful to elucidate whether Bcl-2 proteins are required for Raf-1 translocation to the mitochondria in the β-cells.

Additional non-Bcl-2 dependent action of Raf-1 has also been described. Deletion of the Raf-1 gene was also associated with increased activity of Apoptosis Signalling Kinase-1 (ASK-1)(141) and mice lacking the ASK-1 gene are resistant to apoptosis induced by blocking Raf-1 signalling. Raf-1 and ASK-1 have been reported to interact directly and it has been suggested that Raf-1 catalytic activity is not required for

inhibition of ASK1-induced cell death (207). Our data were inconclusive on the role of ASK-1 in β-cells and future studies will be required. On the other hand, our data provide strong evidence for the notion that Raf-1 acts via both ERK1/2 and Bad in β-cells. Because Raf-1 is a central hub of numerous pro-survival signalling pathways, it is conceivable that simultaneous activation of MEK/ERK1/2 pathways and inhibition of Bad may be required to maximize β-cell survival (141).

6.7 Raf-1 function in vivo

The direct role of Raf-1 in β-cell function *in vivo* is not known. However, blocking Raf-1 signalling reduced basal insulin secretion levels in MIN6 cells (229) and glucose-induced insulin secretion in primary islets (229) and INS-1 cells (230). Inhibiting ERK1/2 signalling also reduced basal insulin secretion in MIN6 cells, suggesting that the effects of Raf-1 on insulin secretion is mediated partly by ERK1/2 (229). In preliminary data, we have demonstrated that lack of Raf-1 protein in pancreatic β-cells resulted in increased fasting blood glucose in Raf-1 knockout RIPCre$^{+/-}$:Raf-1$^{flox/flox}$ mice compared to control RIPCre$^{+/-}$:Raf-1$^{wt/wt}$ mice. RIPCre$^{+/-}$:Raf-1$^{flox/flox}$ have impaired glucose tolerance, consistent with the idea that there is a defect in insulin secretion. Although these are only preliminary data, we did not observe a significant reduction in insulin content in Raf-1 knockout mice compared to controls, further pointing to a defect in insulin secretion. To rule out the effect of β-cell mass, it will be imperative to investigate the architecture and the size of the islets as well as the rate of apoptosis and proliferation of

β-cells *ex vivo*. Elucidating the mechanisms of how this occurs is not within the scope of the present thesis, however, we can speculate that cytoskeleton remodeling may play a role. Recently, Kowluru *et al* demonstrated that protein farnesylation via Raf-1/ERK1/2 signalling pathway leads to the activation of Rac1, which is known to be important for cytoskeleton reorganization, and the consequent exocytotic secretion of insulin (230). Raf-1 inhibition caused impairment of glucose stimulated insulin secretion in the β-cells (230). Thus, it will be of interest to examine the protein expression of the SNARE proteins (syntaxin-1, SNAP-25 and VAMP) in islets from Raf-1 knockout mice compared to controls. Taken together, the current data and these future studies will help define the mechanisms of how Raf-1 affects pancreatic β-cell function *in vivo*.

6.8 Limitations of the study

Although the present study illuminated novel mechanisms of insulin signalling in the β-cells, there are caveats that come with the use of chemical inhibitors, dominant-negative proteins and the Cre-lox system. The chemical inhibitors may have non-specific targets. For example, the GW5074 may not only inhibit Raf-1 but also related proteins like B-Raf and A-Raf at higher concentrations. Because these proteins have the same downstream substrates it is difficult to isolate the inhibitors' effects and to interpret the results accurately. The dominant-negative proteins may also have similar disadvantages. Raf-1^{51-131}-GFP may bind to activated Ras, thereby potentially affecting multiple downstream pathways. Thus, it will be important in the future to examine the effects of specific deletion of the Raf-1 gene in β-cells on ERK1/2 activation and

apoptosis *in vitro* via siRNA. One other drawback of the study is that the Cre recombinase itself may have effects on the host cells. Although, it has long been assumed that the expression of Cre recombinase does not adversely affect the physiology of the host cells, a growing body of evidence suggests that Cre recombinase has deleterious effects in some systems (210, 231, 232). For example, experiments in mouse embryonic fibroblasts have demonstrated effects on cell growth and DNA damage (231). Experimental evidence from various diabetes laboratories also suggest that RIP-Cre mice alone display glucose intolerance (210). In the present study, we addressed the effects of Cre recombinase by limiting each mouse to one Cre recombinase allele and we employed a specific breeding scheme (see Figure 25) to obtain the proper 'Cre-only' controls. Unfortunately, the expression of Cre recombinase could be mosaic, thus each animal may still have varying Cre-recombinase expression. A reporter strain for monitoring Cre recombinase-mediated DNA excisions in mice will be necessary to address this issue.

There are also limitations of performing mechanistic studies using the transformed MIN6 cell line. Primary human and mouse islets are ideal experimental systems, but there is an inherent technical difficulty in obtaining sufficient tissue for some of the experiments that address the temporal profile of Raf-1 activation or the Raf-1/Bcl-2 protein complex. We tried to be efficient in our use of primary islet protein by cutting the membranes to assess more than one protein. However, in cases where an antibody's specificity is not well known or when a protein of interest has various isoforms, we were only able to assess one protein per membrane. Certain antibodies

such as cleaved-caspase 3 also require high protein level for detection. Another technical difficulty is the true levels of insulin in our experiments where islets are cultured in various concentrations of exogenous insulin because islets themselves release ‘basal’ insulin constitutively. Indeed, we minimized this by using relatively few islets in a large culture volume, thereby diluting released insulin. We have also previously demonstrated our ability to ‘clamp’ insulin levels in our culture systems with a relatively high degree of accuracy (42, 44). Despite these shortcomings, the present study does provide a greater understanding of the action of insulin and Raf-1 in β-cell survival.

7. Future Directions

Emerging evidence suggests that Raf-1 kinase is a critical regulator of survival and proliferation, both key factors in maintaining a functional pancreatic β-cell mass. Therefore, an understanding of upstream growth factors, such as insulin, that activate Raf-1 warrant further study to increase our knowledge of signalling transduction controlling β-cell mass. A more thorough characterization of mice with pancreatic β-cell-specific deletion of Raf-1 will also be important to understand the physiological role of Raf-1 in β-cell survival, proliferation, and insulin secretion *in vivo*. It will be of interest to investigate whether Raf-1 plays a role in the ability of the β-cell to increase mass in response to pregnancy and in high-fat feeding. Future studies using tamoxifen-inducible Raf-1 deletion with RIP-Cre-ER mice or Pdx-1-Cre-ER mice will be important to confirm the preliminary data from the β-cell-specific Raf-1 deletion using RIP-Cre-Lox recombination model. Future studies may also be directed at determining whether Raf-1 directly or indirectly affects the interactions of Bad, glucokinase, and PKA complex, and thus, regulates their role in glucose metabolism and insulin secretion. The interaction of Raf-1 and Bcl-2 protein family members at the mitochondria, where apoptosis and cellular metabolism converge, highlights the critical role of impaired metabolism, β-cell dysfunction, and a relative decrease in β-cell mass as major factors causing type 2 diabetes. In conclusion, a better understanding of the multi-functionality of Raf-1 may provide exciting new information necessary for improving our understanding of β-cell

biology and possible means to combat the loss of functional β-cell mass that occurs in both type 1 and type 2 diabetes.

Figure 30

A

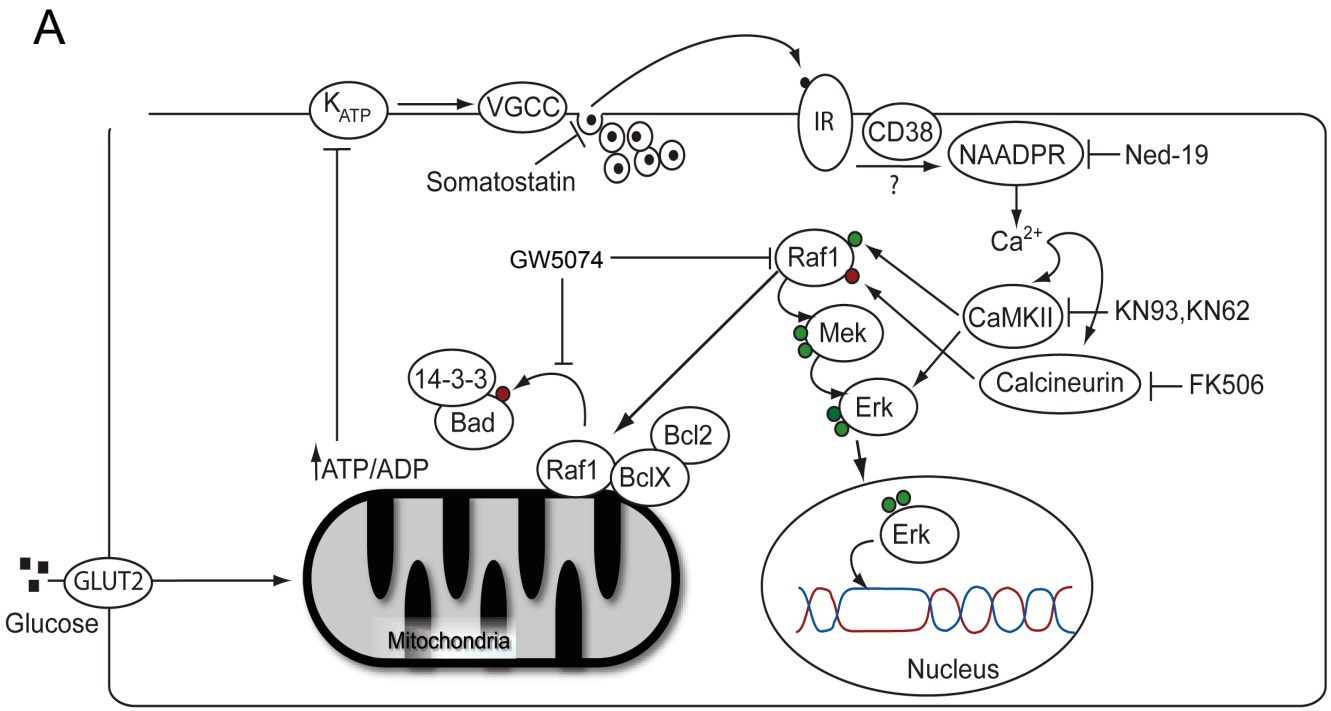

K_ATP
VGCC
Somatostatin
IR
CD38
?
NAADPR
Ned-19
Ca2+
GW5074
Raf1
CaMKII
KN93,KN62
Mek
14-3-3
Bad
Calcineurin
FK506
Erk
Bcl2
ATP/ADP
Raf1
BclX
Erk
GLUT2
Glucose
Mitochondria
Nucleus

Figure 30. **Simplified model of anti-apoptotic signalling of insulin via Raf-1/ERK1/2 and Bad pathways.** Activation of the insulin receptor may lead to the following pathways to promote β-cell survival: 1) signalling through the Raf/MEK and ERK1/2 cascade, 2) the Bcl-2-mediated translocation of Raf-1 to the mitochondria, where Bad is phosphorylated and inactivated, 3) signalling via Ca^{2+} mobilization via NAADP-sensitive intracellular stores. Green and red circles denote stimulatory and inhibitory phosphorylation respectively. Many signalling molecules have been omitted for clarity.

References

1. Millar WJ, Young TK 2003 Tracking diabetes: prevalence, incidence and risk factors. Health Rep 14:35-47
2. Hegele RA, Zinman B, Hanley AJ, Harris SB, Barrett PH, Cao H 2003 Genes, environment and Oji-Cree type 2 diabetes. Clin Biochem 36:163-170
3. Donath MY, Halban PA 2004 Decreased beta-cell mass in diabetes: significance, mechanisms and therapeutic implications. Diabetologia 47:581-589
4. O'Brien BA, Harmon BV, Cameron DP, Allan DJ 1997 Apoptosis is the mode of beta-cell death responsible for the development of IDDM in the nonobese diabetic (NOD) mouse. Diabetes 46:750-757
5. Kurrer MO, Pakala SV, Hanson HL, Katz JD 1997 Beta cell apoptosis in T cell-mediated autoimmune diabetes. Proc Natl Acad Sci U S A 94:213-218
6. Augstein P, Stephens LA, Allison J, Elefanty AG, Ekberg M, Kay TW, Harrison LC 1998 Beta-cell apoptosis in an accelerated model of autoimmune diabetes. Mol Med 4:495-501
7. Augstein P, Elefanty AG, Allison J, Harrison LC 1998 Apoptosis and beta-cell destruction in pancreatic islets of NOD mice with spontaneous and cyclophosphamide-accelerated diabetes. Diabetologia 41:1381-1388
8. Mathis D, Vence L, Benoist C 2001 beta-Cell death during progression to diabetes. Nature 414:792-798

9. Davalli AM, Scaglia L, Zangen DH, Hollister J, Bonner-Weir S, Weir GC 1996 Vulnerability of islets in the immediate posttransplantation period. Dynamic changes in structure and function. Diabetes 45:1161-1167

10. Rhodes CJ 2005 Type 2 diabetes-a matter of beta-cell life and death? Science 307:380-384

11. Mandrup-Poulsen T 2001 beta-cell apoptosis: stimuli and signaling. Diabetes 50 Suppl 1:S58-63

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

13. Marchetti P, Del Guerra S, Marselli L, Lupi R, Masini M, Pollera M, Bugliani M, Boggi U, Vistoli F, Mosca F, Del Prato S 2004 Pancreatic islets from type 2 diabetic patients have functional defects and increased apoptosis that are ameliorated by metformin. J Clin Endocrinol Metab 89:5535-5541

14. Vaxillaire M, Froguel P 2008 Monogenic diabetes in the young, pharmacogenetics and relevance to multifactorial forms of type 2 diabetes. Endocr Rev 29:254-264

15. Boden G 1997 Role of fatty acids in the pathogenesis of insulin resistance and NIDDM. Diabetes 46:3-10

16. Kahn SE, Hull RL, Utzschneider KM 2006 Mechanisms linking obesity to insulin resistance and type 2 diabetes. Nature 444:840-846

17. Polonsky KS, Sturis J, Bell GI 1996 Seminars in Medicine of the Beth Israel Hospital, Boston. Non-insulin-dependent diabetes mellitus - a genetically programmed failure of the beta cell to compensate for insulin resistance. N Engl J Med 334:777-783

18. Lingohr MK, Buettner R, Rhodes CJ 2002 Pancreatic beta-cell growth and survival--a role in obesity-linked type 2 diabetes? Trends Mol Med 8:375-384

19. Leahy JL, Bonner-Weir S, Weir GC 1992 Beta-cell dysfunction induced by chronic hyperglycemia. Current ideas on mechanism of impaired glucose-induced insulin secretion. Diabetes Care 15:442-455

20. Poitout V, Robertson RP 2002 Minireview: Secondary beta-cell failure in type 2 diabetes--a convergence of glucotoxicity and lipotoxicity. Endocrinology 143:339-342

21. Robertson RP, Harmon J, Tran PO, Tanaka Y, Takahashi H 2003 Glucose toxicity in beta-cells: type 2 diabetes, good radicals gone bad, and the glutathione connection. Diabetes 52:581-587

22. Scaglia L, Cahill CJ, Finegood DT, Bonner-Weir S 1997 Apoptosis participates in the remodeling of the endocrine pancreas in the neonatal rat. Endocrinology 138:1736-1741

23. Johnson JD, Han Z, Otani K, Ye H, Zhang Y, Wu H, Horikawa Y, Misler S, Bell GI, Polonsky KS 2004 RyR2 and calpain-10 delineate a novel apoptosis pathway in pancreatic islets. J Biol Chem 279:24794-24802

24. Mandrup-Poulsen T 2003 Apoptotic signal transduction pathways in diabetes. Biochem Pharmacol 66:1433-1440

25. Gallaher BW, Hille R, Raile K, Kiess W 2001 Apoptosis: live or die--hard work either way! Horm Metab Res 33:511-519

26. Hui H, Dotta F, Di Mario U, Perfetti R 2004 Role of caspases in the regulation of apoptotic pancreatic islet beta-cells death. J Cell Physiol 200:177-200

27. Augstein P, Bahr J, Wachlin G, Heinke P, Berg S, Salzsieder E, Harrison LC 2004 Cytokines activate caspase-3 in insulinoma cells of diabetes-prone NOD mice directly and via upregulation of Fas. J Autoimmun 23:301-309

28. Nakano M, Matsumoto I, Sawada T, Ansite J, Oberbroeckling J, Zhang HJ, Kirchhof N, Shearer J, Sutherland DE, Hering BJ 2004 Caspase-3 inhibitor prevents apoptosis of human islets immediately after isolation and improves islet graft function. Pancreas 29:104-109

29. Thomas D, Yang H, Boffa DJ, Ding R, Sharma VK, Lagman M, Li B, Hering B, Mohanakumar T, Lakey J, Kapur S, Hancock WW, Suthanthiran M 2002 Proapoptotic Bax is hyperexpressed in isolated human islets compared with antiapoptotic Bcl-2. Transplantation 74:1489-1496

30. Hanke J 2001 Apoptosis in cultured rat islets of langerhans and occurrence of Bcl-2, Bak, Bax, Fas and Fas ligand. Cells Tissues Organs 169:113-124

31. Rabinovitch A, Suarez-Pinzon W, Strynadka K, Ju Q, Edelstein D, Brownlee M, Korbutt GS, Rajotte RV 1999 Transfection of human pancreatic islets with an anti-apoptotic gene (bcl-2) protects beta-cells from cytokine-induced destruction. Diabetes 48:1223-1229

32. Liu Y, Rabinovitch A, Suarez-Pinzon W, Muhkerjee B, Brownlee M, Edelstein D, Federoff HJ 1996 Expression of the bcl-2 gene from a defective HSV-1 amplicon

vector protects pancreatic beta-cells from apoptosis. Hum Gene Ther 7:1719-1726

33. Klein D, Ribeiro MM, Mendoza V, Jayaraman S, Kenyon NS, Pileggi A, Molano RD, Inverardi L, Ricordi C, Pastori RL 2004 Delivery of Bcl-XL or its BH4 domain by protein transduction inhibits apoptosis in human islets. Biochem Biophys Res Commun 323:473-478

34. Allison J, Thomas H, Beck D, Brady JL, Lew AM, Elefanty A, Kosaka H, Kay TW, Huang DC, Strasser A 2000 Transgenic overexpression of human Bcl-2 in islet beta cells inhibits apoptosis but does not prevent autoimmune destruction. Int Immunol 12:9-17

35. Contreras JL, Bilbao G, Smyth CA, Jiang XL, Eckhoff DE, Jenkins SM, Thomas FT, Curiel DT, Thomas JM 2001 Cytoprotection of pancreatic islets before and soon after transplantation by gene transfer of the anti-apoptotic Bcl-2 gene. Transplantation 71:1015-1023

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

37. Zhou YP, Pena JC, Roe MW, Mittal A, Levisetti M, Baldwin AC, Pugh W, Ostrega D, Ahmed N, Bindokas VP, Philipson LH, Hanahan D, Thompson CB, Polonsky KS 2000 Overexpression of Bcl-x(L) in beta-cells prevents cell death but impairs mitochondrial signal for insulin secretion. Am J Physiol Endocrinol Metab 278:E340-351

38. Navarro-Tableros V, Sanchez-Soto MC, Garcia S, Hiriart M 2004 Autocrine regulation of single pancreatic beta-cell survival. Diabetes 53:2018-2023

39. Lingohr MK, Dickson LM, McCuaig JF, Hugl SR, Twardzik DR, Rhodes CJ 2002 Activation of IRS-2-mediated signal transduction by IGF-1, but not TGF-alpha or EGF, augments pancreatic beta-cell proliferation. Diabetes 51:966-976

40. Hugl SR, White MF, Rhodes CJ 1998 Insulin-like growth factor I (IGF-I)-stimulated pancreatic beta-cell growth is glucose-dependent. Synergistic activation of insulin receptor substrate-mediated signal transduction pathways by glucose and IGF-I in INS-1 cells. J Biol Chem 273:17771-17779

41. Raile K, Berthold A, Banning U, Horn F, Pfeiffer G, Kiess W 2003 IGFs, basic FGF, and glucose modulate proliferation and apoptosis induced by IFNgamma but not by IL-1beta in rat INS-1E beta-cells. Horm Metab Res 35:407-414

42. Johnson JD, Bernal-Mizrachi E, Alejandro EU, Han Z, Kalynyak TB, Li H, Beith JL, Gross J, Warnock GL, Townsend RR, Permutt MA, Polonsky KS 2006 Insulin protects islets from apoptosis via Pdx1 and specific changes in the human islet proteome. Proc Natl Acad Sci U S A 103:19575-19580

43. Pierucci D, Cicconi S, Bonini P, Ferrelli F, Pastore D, Matteucci C, Marselli L, Marchetti P, Ris F, Halban P, Oberholzer J, Federici M, Cozzolino F, Lauro R, Borboni P, Marlier LN 2001 NGF-withdrawal induces apoptosis in pancreatic beta cells in vitro. Diabetologia 44:1281-1295

44. Beith JL, Alejandro EU, Johnson JD 2008 Insulin stimulates primary beta-cell proliferation via Raf-1 kinase. Endocrinology 149:2251-2260

45. Saltiel AR, Kahn CR 2001 Insulin signalling and the regulation of glucose and lipid metabolism. Nature 414:799-806

46. Rhodes CJ, White MF 2002 Molecular insights into insulin action and secretion. Eur J Clin Invest 32 Suppl 3:3-13

47. Blakesley VA, Scrimgeour A, Esposito D, Le Roith D 1996 Signaling via the insulin-like growth factor-I receptor: does it differ from insulin receptor signaling? Cytokine Growth Factor Rev 7:153-159

48. Sun XJ, Wang LM, Zhang Y, Yenush L, Myers MG, Jr., Glasheen E, Lane WS, Pierce JH, White MF 1995 Role of IRS-2 in insulin and cytokine signalling. Nature 377:173-177

49. Rhodes CJ 2000 IGF-I and GH post-receptor signaling mechanisms for pancreatic beta-cell replication. J Mol Endocrinol 24:303-311

50. White MF 1998 The IRS-signaling system: a network of docking proteins that mediate insulin and cytokine action. Recent Prog Horm Res 53:119-138

51. Bernal-Mizrachi E, Wen W, Stahlhut S, Welling CM, Permutt MA 2001 Islet beta cell expression of constitutively active Akt1/PKB alpha induces striking hypertrophy, hyperplasia, and hyperinsulinemia. J Clin Invest 108:1631-1638

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

53. Dickson LM, Rhodes CJ 2004 Pancreatic beta-cell growth and survival in the onset of type 2 diabetes: a role for protein kinase B in the Akt? Am J Physiol Endocrinol Metab 287:E192-198

54. Wrede CE, Dickson LM, Lingohr MK, Briaud I, Rhodes CJ 2002 Protein kinase B/Akt prevents fatty acid-induced apoptosis in pancreatic beta-cells (INS-1). J Biol Chem 277:49676-49684

55. Tuttle RL, Gill NS, Pugh W, Lee JP, Koeberlein B, Furth EE, Polonsky KS, Naji A, Birnbaum MJ 2001 Regulation of pancreatic beta-cell growth and survival by the serine/threonine protein kinase Akt1/PKBalpha. Nat Med 7:1133-1137

56. Aikin R, Hanley S, Maysinger D, Lipsett M, Castellarin M, Paraskevas S, Rosenberg L 2006 Autocrine insulin action activates Akt and increases survival of isolated human islets. Diabetologia 49:2900-2909

57. Dalle S, Longuet C, Costes S, Broca C, Faruque O, Fontes G, Hani EH, Bataille D 2004 Glucagon promotes cAMP-response element-binding protein phosphorylation via activation of ERK1/2 in MIN6 cell line and isolated islets of Langerhans. J Biol Chem 279:20345-20355

58. Costes S, Broca C, Bertrand G, Lajoix AD, Bataille D, Bockaert J, Dalle S 2006 ERK1/2 control phosphorylation and protein level of cAMP-responsive element-binding protein: a key role in glucose-mediated pancreatic beta-cell survival. Diabetes 55:2220-2230

59. Wilson BE, Mochon E, Boxer LM 1996 Induction of bcl-2 expression by phosphorylated CREB proteins during B-cell activation and rescue from apoptosis. Mol Cell Biol 16:5546-5556

60. Xiang H, Wang J, Boxer LM 2006 Role of the cyclic AMP response element in the bcl-2 promoter in the regulation of endogenous Bcl-2 expression and apoptosis in murine B cells. Mol Cell Biol 26:8599-8606

61. Jhala US, Canettieri G, Screaton RA, Kulkarni RN, Krajewski S, Reed J, Walker J, Lin X, White M, Montminy M 2003 cAMP promotes pancreatic beta-cell survival via CREB-mediated induction of IRS2. Genes Dev 17:1575-1580

62. Baccarini M 2005 Second nature: biological functions of the Raf-1 "kinase". FEBS Lett 579:3271-3277

63. Zhang L, Fu Z, Binkley C, Giordano T, Burant CF, Logsdon CD, Simeone DM 2004 Raf kinase inhibitory protein inhibits beta-cell proliferation. Surgery 136:708-715

64. Trumper J, Ross D, Jahr H, Brendel MD, Goke R, Horsch D 2005 The Rap-B-Raf signalling pathway is activated by glucose and glucagon-like peptide-1 in human islet cells. Diabetologia 48:1534-1540

65. Gomez E, Pritchard C, Herbert TP 2002 cAMP-dependent protein kinase and Ca2+ influx through L-type voltage-gated calcium channels mediate Raf-independent activation of extracellular regulated kinase in response to glucagon-like peptide-1 in pancreatic beta-cells. J Biol Chem 277:48146-48151

66. Briaud I, Lingohr MK, Dickson LM, Wrede CE, Rhodes CJ 2003 Differential activation mechanisms of Erk-1/2 and p70(S6K) by glucose in pancreatic beta-cells. Diabetes 52:974-983

67. Koide H, Satoh T, Nakafuku M, Kaziro Y 1993 GTP-dependent association of Raf-1 with Ha-Ras: identification of Raf as a target downstream of Ras in mammalian cells. Proc Natl Acad Sci U S A 90:8683-8686

68. Marais R, Light Y, Paterson HF, Marshall CJ 1995 Ras recruits Raf-1 to the plasma membrane for activation by tyrosine phosphorylation. Embo J 14:3136-3145

69. Warne PH, Viciana PR, Downward J 1993 Direct interaction of Ras and the amino-terminal region of Raf-1 in vitro. Nature 364:352-355

70. Kyriakis JM 2007 The integration of signaling by multiprotein complexes containing Raf kinases. Biochim Biophys Acta 1773:1238-1247

71. Dent P, Haser W, Haystead TA, Vincent LA, Roberts TM, Sturgill TW 1992 Activation of mitogen-activated protein kinase kinase by v-Raf in NIH 3T3 cells and in vitro. Science 257:1404-1407

72. Howe LR, Leevers SJ, Gomez N, Nakielny S, Cohen P, Marshall CJ 1992 Activation of the MAP kinase pathway by the protein kinase raf. Cell 71:335-342

73. Kovacina KS, Yonezawa K, Brautigan DL, Tonks NK, Rapp UR, Roth RA 1990 Insulin activates the kinase activity of the Raf-1 proto-oncogene by increasing its serine phosphorylation. J Biol Chem 265:12115-12118

74. Blackshear PJ, Haupt DM, App H, Rapp UR 1990 Insulin activates the Raf-1 protein kinase. J Biol Chem 265:12131-12134

75. App H, Hazan R, Zilberstein A, Ullrich A, Schlessinger J, Rapp U 1991 Epidermal growth factor (EGF) stimulates association and kinase activity of Raf-1 with the EGF receptor. Mol Cell Biol 11:913-919

76. Galmiche A, Fueller J, Santel A, Krohne G, Wittig I, Doye A, Rolando M, Flatau G, Lemichez E, Rapp UR 2008 Isoform-specific interaction of C-RAF with mitochondria. J Biol Chem 283:14857-14866

77. Pavlovic D, Andersen NA, Mandrup-Poulsen T, Eizirik DL 2000 Activation of extracellular signal-regulated kinase (ERK)1/2 contributes to cytokine-induced apoptosis in purified rat pancreatic beta-cells. Eur Cytokine Netw 11:267-274

78. Maedler K, Storling J, Sturis J, Zuellig RA, Spinas GA, Arkhammar PO, Mandrup-Poulsen T, Donath MY 2004 Glucose- and interleukin-1beta-induced beta-cell apoptosis requires Ca2+ influx and extracellular signal-regulated kinase (ERK) 1/2 activation and is prevented by a sulfonylurea receptor 1/inwardly rectifying K+ channel 6.2 (SUR/Kir6.2) selective potassium channel opener in human islets. Diabetes 53:1706-1713

79. Hammar E, Parnaud G, Bosco D, Perriraz N, Maedler K, Donath M, Rouiller DG, Halban PA 2004 Extracellular matrix protects pancreatic beta-cells against apoptosis: role of short- and long-term signaling pathways. Diabetes 53:2034-2041

80. Fei H, Zhao B, Zhao S, Wang Q 2008 Requirements of calcium fluxes and ERK kinase activation for glucose- and interleukin-1beta-induced beta-cell apoptosis. Mol Cell Biochem 315:75-84

81. Zhuang S, Schnellmann RG 2006 A death-promoting role for extracellular signal-regulated kinase. J Pharmacol Exp Ther 319:991-997

82. Cheung EC, Slack RS 2004 Emerging role for ERK as a key regulator of neuronal apoptosis. Sci STKE 2004:PE45

83. Marshall CJ 1995 Specificity of receptor tyrosine kinase signaling: transient versus sustained extracellular signal-regulated kinase activation. Cell 80:179-185

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

85. Ueki K, Okada T, Hu J, Liew CW, Assmann A, Dahlgren GM, Peters JL, Shackman JG, Zhang M, Artner I, Satin LS, Stein R, Holzenberger M, Kennedy RT, Kahn CR, Kulkarni RN 2006 Total insulin and IGF-I resistance in pancreatic beta cells causes overt diabetes. Nat Genet 38:583-588

86. Okada T, Liew CW, Hu J, Hinault C, Michael MD, Krtzfeldt J, Yin C, Holzenberger M, Stoffel M, Kulkarni RN 2007 Insulin receptors in beta-cells are critical for islet compensatory growth response to insulin resistance. Proc Natl Acad Sci U S A 104:8977-8982

87. Withers DJ, Gutierrez JS, Towery H, Burks DJ, Ren JM, Previs S, Zhang Y, Bernal D, Pons S, Shulman GI, Bonner-Weir S, White MF 1998 Disruption of IRS-2 causes type 2 diabetes in mice. Nature 391:900-904

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

89. Kushner JA, Ye J, Schubert M, Burks DJ, Dow MA, Flint CL, Dutta S, Wright CV, Montminy MR, White MF 2002 Pdx1 restores beta cell function in Irs2 knockout mice. J Clin Invest 109:1193-1201

90. Kulkarni RN, Bruning JC, Winnay JN, Postic C, Magnuson MA, Kahn CR 1999 Tissue-specific knockout of the insulin receptor in pancreatic beta cells creates an insulin secretory defect similar to that in type 2 diabetes. Cell 96:329-339

91. Vojtek AB, Hollenberg SM, Cooper JA 1993 Mammalian Ras interacts directly with the serine/threonine kinase Raf. Cell 74:205-214

92. Brtva TR, Drugan JK, Ghosh S, Terrell RS, Campbell-Burk S, Bell RM, Der CJ 1995 Two distinct Raf domains mediate interaction with Ras. J Biol Chem 270:9809-9812

93. Dhillon AS, Meikle S, Yazici Z, Eulitz M, Kolch W 2002 Regulation of Raf-1 activation and signalling by dephosphorylation. Embo J 21:64-71

94. Zimmermann S, Moelling K 1999 Phosphorylation and regulation of Raf by Akt (protein kinase B). Science 286:1741-1744

95. Abraham D, Podar K, Pacher M, Kubicek M, Welzel N, Hemmings BA, Dilworth SM, Mischak H, Kolch W, Baccarini M 2000 Raf-1-associated protein phosphatase 2A as a positive regulator of kinase activation. J Biol Chem 275:22300-22304

96. Jaumot M, Hancock JF 2001 Protein phosphatases 1 and 2A promote Raf-1 activation by regulating 14-3-3 interactions. Oncogene 20:3949-3958

97. King AJ, Sun H, Diaz B, Barnard D, Miao W, Bagrodia S, Marshall MS 1998 The protein kinase Pak3 positively regulates Raf-1 activity through phosphorylation of serine 338. Nature 396:180-183

98. Chaudhary A, King WG, Mattaliano MD, Frost JA, Diaz B, Morrison DK, Cobb MH, Marshall MS, Brugge JS 2000 Phosphatidylinositol 3-kinase regulates Raf1 through Pak phosphorylation of serine 338. Curr Biol 10:551-554

99. Tran NH, Frost JA 2003 Phosphorylation of Raf-1 by p21-activated kinase 1 and Src regulates Raf-1 autoinhibition. J Biol Chem 278:11221-11226

100. Sozeri O, Vollmer K, Liyanage M, Frith D, Kour G, Mark GE, 3rd, Stabel S 1992 Activation of the c-Raf protein kinase by protein kinase C phosphorylation. Oncogene 7:2259-2262

101. Kolch W, Heidecker G, Kochs G, Hummel R, Vahidi H, Mischak H, Finkenzeller G, Marme D, Rapp UR 1993 Protein kinase C alpha activates RAF-1 by direct phosphorylation. Nature 364:249-252

102. Johnson JD, Misler S 2002 Nicotinic acid-adenine dinucleotide phosphate-sensitive calcium stores initiate insulin signaling in human beta cells. Proc Natl Acad Sci U S A 99:14566-14571

103. Alejandro EU, Kalynyak TB, Taghizadeh F, Gwiazda KS, Rawstron EK, Jacob KJ, Johnson JD Acute insulin signaling in pancreatic beta-cells is mediated by multiple Raf-1 dependent pathways. Endocrinology 151:502-512

104. Monaco S, Illario M, Rusciano MR, Gragnaniello G, Di Spigna G, Leggiero E, Pastore L, Fenzi G, Rossi G, Vitale M 2009 Insulin stimulates fibroblast

proliferation through calcium-calmodulin-dependent kinase II. Cell Cycle 8:2024-2030

105. Illario M, Monaco S, Cavallo AL, Esposito I, Formisano P, D'Andrea L, Cipolletta E, Trimarco B, Fenzi G, Rossi G, Vitale M 2009 Calcium-calmodulin-dependent kinase II (CaMKII) mediates insulin-stimulated proliferation and glucose uptake. Cell Signal 21:786-792

106. Tsakiridis T, Taha C, Grinstein S, Klip A 1996 Insulin activates a p21-activated kinase in muscle cells via phosphatidylinositol 3-kinase. J Biol Chem 271:19664-19667

107. Sun J, Khalid S, Rozakis-Adcock M, Fantus IG, Jin T 2009 P-21-activated protein kinase-1 functions as a linker between insulin and Wnt signaling pathways in the intestine. Oncogene 28:3132-3144

108. Finegood DT, Scaglia L, Bonner-Weir S 1995 Dynamics of beta-cell mass in the growing rat pancreas. Estimation with a simple mathematical model. Diabetes 44:249-256

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

110. Teta M, Long SY, Wartschow LM, Rankin MM, Kushner JA 2005 Very slow turnover of beta-cells in aged adult mice. Diabetes 54:2557-2567

111. Weber CK, Slupsky JR, Kalmes HA, Rapp UR 2001 Active Ras induces heterodimerization of cRaf and BRaf. Cancer Res 61:3595-3598

112. Gupta RK, Gao N, Gorski RK, White P, Hardy OT, Rafiq K, Brestelli JE, Chen G, Stoeckert CJ, Jr., Kaestner KH 2007 Expansion of adult beta-cell mass in

response to increased metabolic demand is dependent on HNF-4alpha. Genes Dev 21:756-769

113. Schuppin GT, Pons S, Hugl S, Aiello LP, King GL, White M, Rhodes CJ 1998 A specific increased expression of insulin receptor substrate 2 in pancreatic beta-cell lines is involved in mediating serum-stimulated beta-cell growth. Diabetes 47:1074-1085

114. Hussain MA, Porras DL, Rowe MH, West JR, Song WJ, Schreiber WE, Wondisford FE 2006 Increased pancreatic beta-cell proliferation mediated by CREB binding protein gene activation. Mol Cell Biol 26:7747-7759

115. Bernal-Mizrachi E, Wen W, Srinivasan S, Klenk A, Cohen D, Permutt MA 2001 Activation of Elk-1, an Ets transcription factor, by glucose and EGF treatment of insulinoma cells. Am J Physiol Endocrinol Metab 281:E1286-1299

116. Laybutt DR, Weir GC, Kaneto H, Lebet J, Palmiter RD, Sharma A, Bonner-Weir S 2002 Overexpression of c-Myc in beta-cells of transgenic mice causes proliferation and apoptosis, downregulation of insulin gene expression, and diabetes. Diabetes 51:1793-1804

117. Wang S, Ghosh RN, Chellappan SP 1998 Raf-1 physically interacts with Rb and regulates its function: a link between mitogenic signaling and cell cycle regulation. Mol Cell Biol 18:7487-7498

118. Kinkade R, Dasgupta P, Carie A, Pernazza D, Carless M, Pillai S, Lawrence N, Sebti SM, Chellappan S 2008 A small molecule disruptor of Rb/Raf-1 interaction inhibits cell proliferation, angiogenesis, and growth of human tumor xenografts in nude mice. Cancer Res 68:3810-3818

119. Lee M, Yoo HS 2007 Human Raf-1 proteins associate with Rad24 and Cdc25 in cell-cycle checkpoint pathway of fission yeast, Schizosaccharomyces pombe. J Cell Biochem 101:488-497

120. Cozar-Castellano I, Fiaschi-Taesch N, Bigatel TA, Takane KK, Garcia-Ocana A, Vasavada R, Stewart AF 2006 Molecular control of cell cycle progression in the pancreatic beta-cell. Endocr Rev 27:356-370

121. Vasavada RC, Cozar-Castellano I, Sipula D, Stewart AF 2007 Tissue-specific deletion of the retinoblastoma protein in the pancreatic beta-cell has limited effects on beta-cell replication, mass, and function. Diabetes 56:57-64

122. Harb G, Vasavada RC, Cobrinik D, Stewart AF 2009 The retinoblastoma protein and its homolog p130 regulate the G1/S transition in pancreatic beta-cells. Diabetes 58:1852-1862

123. Cozar-Castellano I, Harb G, Selk K, Takane K, Vasavada R, Sicari B, Law B, Zhang P, Scott DK, Fiaschi-Taesch N, Stewart AF 2008 Lessons from the first comprehensive molecular characterization of cell cycle control in rodent insulinoma cell lines. Diabetes 57:3056-3068

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

125. Oyadomari S, Koizumi A, Takeda K, Gotoh T, Akira S, Araki E, Mori M 2002 Targeted disruption of the Chop gene delays endoplasmic reticulum stress-mediated diabetes. J Clin Invest 109:525-532

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

127. Nakatani Y, Kaneto H, Kawamori D, Yoshiuchi K, Hatazaki M, Matsuoka TA, Ozawa K, Ogawa S, Hori M, Yamasaki Y, Matsuhisa M 2005 Involvement of endoplasmic reticulum stress in insulin resistance and diabetes. J Biol Chem 280:847-851

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

129. Jeffrey KD, Alejandro EU, Luciani DS, Kalynyak TB, Hu X, Li H, Lin Y, Townsend RR, Polonsky KS, Johnson JD 2008 Carboxypeptidase E mediates palmitate-induced beta-cell ER stress and apoptosis. Proc Natl Acad Sci U S A 105:8452-8457

130. Jiang CC, Chen LH, Gillespie S, Wang YF, Kiejda KA, Zhang XD, Hersey P 2007 Inhibition of MEK sensitizes human melanoma cells to endoplasmic reticulum stress-induced apoptosis. Cancer Res 67:9750-9761

131. Shu CW, Sun FC, Cho JH, Lin CC, Liu PF, Chen PY, Chang MD, Fu HW, Lai YK 2008 GRP78 and Raf-1 cooperatively confer resistance to endoplasmic reticulum stress-induced apoptosis. J Cell Physiol 215:627-635

132. Scheffler NK, Miller SW, Carroll AK, Anderson C, Davis RE, Ghosh SS, Gibson BW 2001 Two-dimensional electrophoresis and mass spectrometric identification

of mitochondrial proteins from an SH-SY5Y neuroblastoma cell line. Mitochondrion 1:161-179

133. Ruiz-Romero C, Lopez-Armada MJ, Blanco FJ 2005 Proteomic characterization of human normal articular chondrocytes: a novel tool for the study of osteoarthritis and other rheumatic diseases. Proteomics 5:3048-3059

134. Sun FC, Wei S, Li CW, Chang YS, Chao CC, Lai YK 2006 Localization of GRP78 to mitochondria under the unfolded protein response. Biochem J 396:31-39

135. Zinszner H, Kuroda M, Wang X, Batchvarova N, Lightfoot RT, Remotti H, Stevens JL, Ron D 1998 CHOP is implicated in programmed cell death in response to impaired function of the endoplasmic reticulum. Genes Dev 12:982-995

136. Nozaki S, Sledge Jr GW, Nakshatri H 2001 Repression of GADD153/CHOP by NF-kappaB: a possible cellular defense against endoplasmic reticulum stress-induced cell death. Oncogene 20:2178-2185

137. Yamaguchi H, Wang HG 2004 CHOP is involved in endoplasmic reticulum stress-induced apoptosis by enhancing DR5 expression in human carcinoma cells. J Biol Chem 279:45495-45502

138. Song B, Scheuner D, Ron D, Pennathur S, Kaufman RJ 2008 Chop deletion reduces oxidative stress, improves beta cell function, and promotes cell survival in multiple mouse models of diabetes. J Clin Invest 118:3378-3389

139. Lawrence MC, McGlynn K, Naziruddin B, Levy MF, Cobb MH 2007 Differential regulation of CHOP-10/GADD153 gene expression by MAPK signaling in pancreatic beta-cells. Proc Natl Acad Sci U S A 104:11518-11525

140. Mikula M, Schreiber M, Husak Z, Kucerova L, Ruth J, Wieser R, Zatloukal K, Beug H, Wagner EF, Baccarini M 2001 Embryonic lethality and fetal liver apoptosis in mice lacking the c-raf-1 gene. Embo J 20:1952-1962

141. Yamaguchi O, Watanabe T, Nishida K, Kashiwase K, Higuchi Y, Takeda T, Hikoso S, Hirotani S, Asahi M, Taniike M, Nakai A, Tsujimoto I, Matsumura Y, Miyazaki J, Chien KR, Matsuzawa A, Sadamitsu C, Ichijo H, Baccarini M, Hori M, Otsu K 2004 Cardiac-specific disruption of the c-raf-1 gene induces cardiac dysfunction and apoptosis. J Clin Invest 114:937-943

142. Wang HG, Miyashita T, Takayama S, Sato T, Torigoe T, Krajewski S, Tanaka S, Hovey L, 3rd, Troppmair J, Rapp UR, et al. 1994 Apoptosis regulation by interaction of Bcl-2 protein and Raf-1 kinase. Oncogene 9:2751-2756

143. Wang HG, Rapp UR, Reed JC 1996 Bcl-2 targets the protein kinase Raf-1 to mitochondria. Cell 87:629-638

144. Jin S, Zhuo Y, Guo W, Field J 2005 p21-activated Kinase 1 (Pak1)-dependent phosphorylation of Raf-1 regulates its mitochondrial localization, phosphorylation of BAD, and Bcl-2 association. J Biol Chem 280:24698-24705

145. Fueller J, Becker M, Sienerth AR, Fischer A, Hotz C, Galmiche A 2008 C-RAF activation promotes BAD poly-ubiquitylation and turn-over by the proteasome. Biochem Biophys Res Commun 370:552-556

146. Zhong J, Troppmair J, Rapp UR 2001 Independent control of cell survival by Raf-1 and Bcl-2 at the mitochondria. Oncogene 20:4807-4816

147. Danial NN, Walensky LD, Zhang CY, Choi CS, Fisher JK, Molina AJ, Datta SR, Pitter KL, Bird GH, Wikstrom JD, Deeney JT, Robertson K, Morash J, Kulkarni A,

Neschen S, Kim S, Greenberg ME, Corkey BE, Shirihai OS, Shulman GI, Lowell BB, Korsmeyer SJ 2008 Dual role of proapoptotic BAD in insulin secretion and beta cell survival. Nat Med 14:144-153

148. Le Mellay V, Troppmair J, Benz R, Rapp UR 2002 Negative regulation of mitochondrial VDAC channels by C-Raf kinase. BMC Cell Biol 3:14

149. Ichijo H, Nishida E, Irie K, ten Dijke P, Saitoh M, Moriguchi T, Takagi M, Matsumoto K, Miyazono K, Gotoh Y 1997 Induction of apoptosis by ASK1, a mammalian MAPKKK that activates SAPK/JNK and p38 signaling pathways. Science 275:90-94

150. Ou D, Wang X, Metzger DL, Robbins M, Huang J, Jobin C, Chantler JK, James RF, Pozzilli P, Tingle AJ 2005 Regulation of TNF-related apoptosis-inducing ligand-mediated death-signal pathway in human beta cells by Fas-associated death domain and nuclear factor kappaB. Hum Immunol 66:799-809

151. Allison J, Thomas HE, Catterall T, Kay TW, Strasser A 2005 Transgenic expression of dominant-negative Fas-associated death domain protein in beta cells protects against Fas ligand-induced apoptosis and reduces spontaneous diabetes in nonobese diabetic mice. J Immunol 175:293-301

152. Zhang S, Liu H, Liu J, Tse CA, Dragunow M, Cooper GJ 2006 Activation of activating transcription factor 2 by p38 MAP kinase during apoptosis induced by human amylin in cultured pancreatic beta-cells. FEBS J 273:3779-3791

153. O'Neill E, Rushworth L, Baccarini M, Kolch W 2004 Role of the kinase MST2 in suppression of apoptosis by the proto-oncogene product Raf-1. Science 306:2267-2270

154. Matsumoto K, Ebihara K, Yamamoto H, Tabuchi H, Fukunaga K, Yasunami M, Ohkubo H, Shichiri M, Miyamoto E 1999 Cloning from insulinoma cells of synapsin I associated with insulin secretory granules. J Biol Chem 274:2053-2059

155. Longuet C, Broca C, Costes S, Hani el H, Bataille D, Dalle S 2005 Extracellularly regulated kinases 1/2 (p44/42 mitogen-activated protein kinases) phosphorylate synapsin I and regulate insulin secretion in the MIN6 beta-cell line and islets of Langerhans. Endocrinology 146:643-654

156. Danial NN, Gramm CF, Scorrano L, Zhang CY, Krauss S, Ranger AM, Datta SR, Greenberg ME, Licklider LJ, Lowell BB, Gygi SP, Korsmeyer SJ 2003 BAD and glucokinase reside in a mitochondrial complex that integrates glycolysis and apoptosis. Nature 424:952-956

157. Liu S, Okada T, Assmann A, Soto J, Liew CW, Bugger H, Shirihai OS, Abel ED, Kulkarni RN 2009 Insulin signaling regulates mitochondrial function in pancreatic beta-cells. PLoS One 4:e7983

158. Edwards LA, Verreault M, Thiessen B, Dragowska WH, Hu Y, Yeung JH, Dedhar S, Bally MB 2006 Combined inhibition of the phosphatidylinositol 3-kinase/Akt and Ras/mitogen-activated protein kinase pathways results in synergistic effects in glioblastoma cells. Mol Cancer Ther 5:645-654

159. Naylor E, Arredouani A, Vasudevan SR, Lewis AM, Parkesh R, Mizote A, Rosen D, Thomas JM, Izumi M, Ganesan A, Galione A, Churchill GC 2009 Identification of a chemical probe for NAADP by virtual screening. Nat Chem Biol 5:220-226

160. Miyazaki J, Araki K, Yamato E, Ikegami H, Asano T, Shibasaki Y, Oka Y, 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

161. Salvalaggio PR, Deng S, Ariyan CE, Millet I, Zawalich WS, Basadonna GP, Rothstein DM 2002 Islet filtration: a simple and rapid new purification procedure that avoids ficoll and improves islet mass and function. Transplantation 74:877-879

162. Rossner M, Yamada KM 2004 What's in a picture? The temptation of image manipulation. J Cell Biol 166:11-15

163. Dror V, Nguyen V, Walia P, Kalynyak TB, Hill JA, Johnson JD 2007 Notch signalling suppresses apoptosis in adult human and mouse pancreatic islet cells. Diabetologia

164. Bondeva T, Balla A, Varnai P, Balla T 2002 Structural determinants of Ras-Raf interaction analyzed in live cells. Mol Biol Cell 13:2323-2333

165. Saperstein R, Vicario PP, Strout HV, Brady E, Slater EE, Greenlee WJ, Ondeyka DL, Patchett AA, Hangauer DG 1989 Design of a selective insulin receptor tyrosine kinase inhibitor and its effect on glucose uptake and metabolism in intact cells. Biochemistry 28:5694-5701

166. Baltensperger K, Lewis RE, Woon CW, Vissavajjhala P, Ross AH, Czech MP 1992 Catalysis of serine and tyrosine autophosphorylation by the human insulin receptor. Proc Natl Acad Sci U S A 89:7885-7889

167. Diaz LE, Chuan YC, Lewitt M, Fernandez-Perez L, Carrasco-Rodriguez S, Sanchez-Gomez M, Flores-Morales A 2007 IGF-II regulates metastatic properties of choriocarcinoma cells through the activation of the insulin receptor. Mol Hum Reprod 13:567-576

168. Jonsson J, Carlsson L, Edlund T, Edlund H 1994 Insulin-promoter-factor 1 is required for pancreas development in mice. Nature 371:606-609

169. Li Y, Cao X, Li LX, Brubaker PL, Edlund H, Drucker DJ 2005 beta-Cell Pdx1 expression is essential for the glucoregulatory, proliferative, and cytoprotective actions of glucagon-like peptide-1. Diabetes 54:482-491

170. Johnson JD, Ahmed NT, Luciani DS, Han Z, Tran H, Fujita J, Misler S, Edlund H, Polonsky KS 2003 Increased islet apoptosis in Pdx1+/- mice. J Clin Invest 111:1147-1160

171. Macfarlane WM, McKinnon CM, Felton-Edkins ZA, Cragg H, James RF, Docherty K 1999 Glucose stimulates translocation of the homeodomain transcription factor PDX1 from the cytoplasm to the nucleus in pancreatic beta-cells. J Biol Chem 274:1011-1016

172. Wu H, MacFarlane WM, Tadayyon M, Arch JR, James RF, Docherty K 1999 Insulin stimulates pancreatic-duodenal homoeobox factor-1 (PDX1) DNA-binding activity and insulin promoter activity in pancreatic beta cells. Biochem J 344 Pt 3:813-818

173. Stanojevic V, Yao KM, Thomas MK 2005 The coactivator Bridge-1 increases transcriptional activation by pancreas duodenum homeobox-1 (PDX-1). Mol Cell Endocrinol 237:67-74

174. Volinic JL, Lee JH, Eto K, Kaur V, Thomas MK 2006 Overexpression of the coactivator bridge-1 results in insulin deficiency and diabetes. Mol Endocrinol 20:167-182

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

176. Kitamura T, Nakae J, Kitamura Y, Kido Y, Biggs WH, 3rd, Wright CV, White MF, Arden KC, Accili D 2002 The forkhead transcription factor Foxo1 links insulin signaling to Pdx1 regulation of pancreatic beta cell growth. J Clin Invest 110:1839-1847

177. De Meyts P, Whittaker J 2002 Structural biology of insulin and IGF1 receptors: implications for drug design. Nat Rev Drug Discov 1:769-783

178. Johnson JD, Alejandro EU 2008 Control of pancreatic beta-cell fate by insulin signaling: The sweet spot hypothesis. Cell Cycle 7:1343-1347

179. Kiselyov VV, Versteyhe S, Gauguin L, De Meyts P 2009 Harmonic oscillator model of the insulin and IGF1 receptors' allosteric binding and activation. Mol Syst Biol 5:243

180. Diaz B, Barnard D, Filson A, MacDonald S, King A, Marshall M 1997 Phosphorylation of Raf-1 serine 338-serine 339 is an essential regulatory event for Ras-dependent activation and biological signaling. Mol Cell Biol 17:4509-4516

181. Bain J, Plater L, Elliott M, Shpiro N, Hastie CJ, McLauchlan H, Klevernic I, Arthur JS, Alessi DR, Cohen P 2007 The selectivity of protein kinase inhibitors: a further update. Biochem J 408:297-315

182. Benes C, Roisin MP, Van Tan H, Creuzet C, Miyazaki J, Fagard R 1998 Rapid activation and nuclear translocation of mitogen-activated protein kinases in response to physiological concentration of glucose in the MIN6 pancreatic β-cell line. J Biol Chem 273:15507-15513

183. Aplin AE, Stewart SA, Assoian RK, Juliano RL 2001 Integrin-mediated adhesion regulates ERK nuclear translocation and phosphorylation of Elk-1. J Cell Biol 153:273-282

184. Nishimoto S, Nishida E 2006 MAPK signalling: ERK5 versus ERK1/2. EMBO Rep 7:782-786

185. Arnette D, Gibson TB, Lawrence MC, January B, Khoo S, McGlynn K, Vanderbilt CA, Cobb MH 2003 Regulation of ERK1 and ERK2 by glucose and peptide hormones in pancreatic beta cells. J Biol Chem 278:32517-32525

186. Duvillie B, Currie C, Chrones T, Bucchini D, Jami J, Joshi RL, Hill DJ 2002 Increased islet cell proliferation, decreased apoptosis, and greater vascularization leading to beta-cell hyperplasia in mutant mice lacking insulin. Endocrinology 143:1530-1537

187. Babaya N, Nakayama M, Moriyama H, Gianani R, Still T, Miao D, Yu L, Hutton JC, Eisenbarth GS 2006 A new model of insulin-deficient diabetes: male NOD mice with a single copy of Ins1 and no Ins2. Diabetologia 49:1222-1228

188. Zha J, Harada H, Yang E, Jockel J, Korsmeyer SJ 1996 Serine phosphorylation of death agonist BAD in response to survival factor results in binding to 14-3-3 not BCL-X(L). Cell 87:619-628

189. Aspinwall CA, Qian WJ, Roper MG, Kulkarni RN, Kahn CR, Kennedy RT 2000 Roles of insulin receptor substrate-1, phosphatidylinositol 3-kinase, and release of intracellular Ca2+ stores in insulin-stimulated insulin secretion in beta -cells. J Biol Chem 275:22331-22338

190. Johnson JD, Ford EL, Bernal-Mizrachi E, Kusser KL, Luciani DS, Han Z, Tran H, Randall TD, Lund FE, Polonsky KS 2006 Suppressed insulin signaling and increased apoptosis in CD38-null islets. Diabetes 55:2737-2746

191. Mitchell KJ, Lai FA, Rutter GA 2003 Ryanodine receptor type I and nicotinic acid adenine dinucleotide phosphate receptors mediate Ca2+ release from insulin-containing vesicles in living pancreatic beta-cells (MIN6). J Biol Chem 278:11057-11064

192. Paschen W, Hotop S, Aufenberg C 2003 Loading neurons with BAPTA-AM activates xbp1 processing indicative of induction of endoplasmic reticulum stress. Cell Calcium 33:83-89

193. Hu P, Han Z, Couvillon AD, Exton JH 2004 Critical role of endogenous Akt/IAPs and MEK1/ERK pathways in counteracting endoplasmic reticulum stress-induced cell death. J Biol Chem 279:49420-49429

194. Illario M, Cavallo AL, Bayer KU, Di Matola T, Fenzi G, Rossi G, Vitale M 2003 Calcium/calmodulin-dependent protein kinase II binds to Raf-1 and modulates integrin-stimulated ERK activation. J Biol Chem 278:45101-45108

195. Lackey K, Cory M, Davis R, Frye SV, Harris PA, Hunter RN, Jung DK, McDonald OB, McNutt RW, Peel MR, Rutkowske RD, Veal JM, Wood ER 2000 The discovery of potent cRaf1 kinase inhibitors. Bioorg Med Chem Lett 10:223-226

196. Zha J, Harada H, Osipov K, Jockel J, Waksman G, Korsmeyer SJ 1997 BH3 domain of BAD is required for heterodimerization with BCL-XL and pro-apoptotic activity. J Biol Chem 272:24101-24104

197. Wei MC, Zong WX, Cheng EH, Lindsten T, Panoutsakopoulou V, Ross AJ, Roth KA, MacGregor GR, Thompson CB, Korsmeyer SJ 2001 Proapoptotic BAX and BAK: a requisite gateway to mitochondrial dysfunction and death. Science 292:727-730

198. Certo M, Del Gaizo Moore V, Nishino M, Wei G, Korsmeyer S, Armstrong SA, Letai A 2006 Mitochondria primed by death signals determine cellular addiction to antiapoptotic BCL-2 family members. Cancer Cell 9:351-365

199. Stiles BL, Kuralwalla-Martinez C, Guo W, Gregorian C, Wang Y, Tian J, Magnuson MA, Wu H 2006 Selective deletion of Pten in pancreatic beta cells leads to increased islet mass and resistance to STZ-induced diabetes. Mol Cell Biol 26:2772-2781

200. Harding HP, Ron D 2002 Endoplasmic reticulum stress and the development of diabetes: a review. Diabetes 51 Suppl 3:S455-461

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

202. Scheuner D, Kaufman RJ 2008 The unfolded protein response: a pathway that links insulin demand with beta-cell failure and diabetes. Endocr Rev 29:317-333

203. Srinivasan S, Ohsugi M, Liu Z, Fatrai S, Bernal-Mizrachi E, Permutt MA 2005 Endoplasmic Reticulum Stress-Induced Apoptosis Is Partly Mediated by Reduced Insulin Signaling Through Phosphatidylinositol 3-Kinase/Akt and

Increased Glycogen Synthase Kinase-3{beta} in Mouse Insulinoma Cells. Diabetes 54:968-975

204. Jun T, Gjoerup O, Roberts TM 1999 Tangled webs: evidence of cross-talk between c-Raf-1 and Akt. Sci STKE 1999:PE1

205. Zimmermann S, Rommel C, Ziogas A, Lovric J, Moelling K, Radziwill G 1997 MEK1 mediates a positive feedback on Raf-1 activity independently of Ras and Src. Oncogene 15:1503-1511

206. Hindley A, Kolch W 2002 Extracellular signal regulated kinase (ERK)/mitogen activated protein kinase (MAPK)-independent functions of Raf kinases. J Cell Sci 115:1575-1581

207. Chen J, Fujii K, Zhang L, Roberts T, Fu H 2001 Raf-1 promotes cell survival by antagonizing apoptosis signal-regulating kinase 1 through a MEK-ERK independent mechanism. Proc Natl Acad Sci U S A 98:7783-7788

208. Tobiume K, Saitoh M, Ichijo H 2002 Activation of apoptosis signal-regulating kinase 1 by the stress-induced activating phosphorylation of pre-formed oligomer. J Cell Physiol 191:95-104

209. Herrera PL 2000 Adult insulin- and glucagon-producing cells differentiate from two independent cell lineages. Development 127:2317-2322

210. Lee JY, Ristow M, Lin X, White MF, Magnuson MA, Hennighausen L 2006 RIP-Cre revisited, evidence for impairments of pancreatic beta-cell function. J Biol Chem 281:2649-2653

211. Havrankova J, Brownstein M, Roth J 1981 Insulin and insulin receptors in rodent brain. Diabetologia 20 Suppl:268-273

212. Gittes GK, Rutter WJ 1992 Onset of cell-specific gene expression in the developing mouse pancreas. Proc Natl Acad Sci U S A 89:1128-1132

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

214. Song SH, McIntyre SS, Shah H, Veldhuis JD, Hayes PC, Butler PC 2000 Direct measurement of pulsatile insulin secretion from the portal vein in human subjects. J Clin Endocrinol Metab 85:4491-4499

215. Li J, Johnson JD 2009 Mathematical models of subcutaneous injection of insulin analogues: A mini-review. Discrete and Continuous Dynamical System- Series B

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

217. Stagner JI, Samols E, Bonner-Weir S 1988 beta----alpha----delta pancreatic islet cellular perfusion in dogs. Diabetes 37:1715-1721

218. Stagner JI, Samols E, Marks V 1989 The anterograde and retrograde infusion of glucagon antibodies suggests that A cells are vascularly perfused before D cells within the rat islet. Diabetologia 32:203-206

219. Guillen C, Bartolome A, Nevado C, Benito M 2008 Biphasic effect of insulin on beta cell apoptosis depending on glucose deprivation. FEBS Lett 582:3855-3860

220. Jimenez-Feltstrom J, Lundquist I, Obermuller S, Salehi A 2004 Insulin feedback actions: complex effects involving isoforms of islet nitric oxide synthase. Regul Pept 122:109-118

221. Muller D, Jones PM, Persaud SJ 2006 Autocrine anti-apoptotic and proliferative effects of insulin in pancreatic beta-cells. FEBS Lett 580:6977-6980

222. Evert M, Sun J, Pichler S, Slavova N, Schneider-Stock R, Dombrowski F 2004 Insulin receptor, insulin receptor substrate-1, Raf-1, and Mek-1 during hormonal hepatocarcinogenesis by intrahepatic pancreatic islet transplantation in diabetic rats. Cancer Res 64:8093-8100

223. Frasca F, Pandini G, Sciacca L, Pezzino V, Squatrito S, Belfiore A, Vigneri R 2008 The role of insulin receptors and IGF-I receptors in cancer and other diseases. Arch Physiol Biochem 114:23-37

224. Khoo S, Griffen SC, Xia Y, Baer RJ, German MS, Cobb MH 2003 Regulation of insulin gene transcription by ERK1 and ERK2 in pancreatic beta cells. J Biol Chem 278:32969-32977

225. Ohsugi M, Cras-Meneur C, Zhou Y, Bernal-Mizrachi E, Johnson JD, Luciani DS, Polonsky KS, Permutt MA 2005 Reduced expression of the insulin receptor in mouse insulinoma (MIN6) cells reveals multiple roles of insulin signaling in gene expression, proliferation, insulin content, and secretion. Journal of Biological Chemistry 280:4992-5003

226. Da Silva Xavier G, Qian Q, Cullen PJ, Rutter GA 2004 Distinct roles for insulin and insulin-like growth factor-1 receptors in pancreatic beta-cell glucose sensing revealed by RNA silencing. Biochem J 377:149-158

227. Wicksteed B, Alarcon C, Briaud I, Lingohr MK, Rhodes CJ 2003 Glucose-induced translational control of proinsulin biosynthesis is proportional to preproinsulin

mRNA levels in islet beta-cells but not regulated via a positive feedback of secreted insulin. J Biol Chem 278:42080-42090

228. Assmann A, Ueki K, Winnay JN, Kadowaki T, Kulkarni RN 2009 Glucose effects on beta-cell growth and survival require activation of insulin receptors and insulin receptor substrate 2. Mol Cell Biol 29:3219-3228

229. Alejandro EU, Johnson JD 2008 Inhibition of Raf-1 alters multiple downstream pathways to induce pancreatic beta-cell apoptosis. J Biol Chem 283:2407-2417

230. Kowluru A, Veluthakal R, Rhodes CJ, Kamath V, Syed I, Koch BJ 2010 Protein farnesylation-dependent Raf/extracellular signal-related kinase signaling links to cytoskeletal remodeling to facilitate glucose-induced insulin secretion in pancreatic beta-cells. Diabetes 59:967-977

231. Loonstra A, Vooijs M, Beverloo HB, Allak BA, van Drunen E, Kanaar R, Berns A, Jonkers J 2001 Growth inhibition and DNA damage induced by Cre recombinase in mammalian cells. Proc Natl Acad Sci U S A 98:9209-9214

232. Silver DP, Livingston DM 2001 Self-excising retroviral vectors encoding the Cre recombinase overcome Cre-mediated cellular toxicity. Mol Cell 8:233-24

Appendix

THE UNIVERSITY OF BRITISH COLUMBIA

ANIMAL CARE CERTIFICATE

Application Number: A07-0442

Investigator or Course Director: James D. Johnson

Department: Cellular & Physiological Sc.

Animals:

Mice C57Bl/6 (50-150) and various sub-strains (50-100 each - depending on activity of study) (INS-Cre, Rip-Cre, CPE, Akita, RyR2flox, Raf1flox, gad, ins1null, ins2null, RIP-rtta) 480

Start Date: **September 30, 2007** **Approval Date:** **October 31, 2007**

Funding Sources:

Funding Agency:	Juvenile Diabetes Research Foundation International
Funding Title:	Regulation of the beta-cell life cycle by insulin
Funding Agency:	Natural Sciences and Engineering Research Council of Canada (NSERC)
Funding Title:	Mapping networks of intracellular calcium stores
Funding Agency:	Michael Smith Foundation for Health Research
Funding Title:	Intracellular calcium stores as master regulators of pancreatic beta-cell survival: Studies on transplantable human islets and knockout mice
Funding Agency:	Canadian Institutes of Health Research (CIHR)
Funding Title:	Calcium signalling pathways mediate anti-apoptotic effects of insulin on human and mouse beta-cells: Implications for transplantation, diabetes progession and islet

	neogenesis
Funding Agency:	Canadian Institutes of Health Research (CIHR)
Funding Title:	Intracellular calcium stores as master regulators of pancreatic beta-cell survival: Studies on transplantable human islets and knockout mice
Funding Agency:	Canadian Institutes of Health Research (CIHR)
Funding Title:	Intracellular calcium stores as master regulators of pancreatic beta-cell survival: Studies on transplantable human islets and knockout mice.
Funding Agency:	Juvenile Diabetes Research Foundation International
Funding Title:	Regulation of beta-cell precursors by insulin receptor and notch signaling
Funding Agency:	Canadian Diabetes Association
Funding Title:	Calcium-dependent pathways in fatty acid-induced b-cell death: gene-Environment interactions
Funding Agency:	Michael Smith Foundation for Health Research
Funding Title:	Intracellular calcium stores as master regulators of pancreatic beta-cell survival: Studies on transplantable human islets and knockout mice
Funding Agency:	Canadian Institutes of Health Research (CIHR)
Funding Title:	Calcium signalling pathways mediate anti-apoptotic effects of insulin on human and mouse beta-cells: Implications for transplantation, diabetes progression and islet neogenesis
Funding Agency:	Juvenile Diabetes Research Foundation International
Funding Title:	Raf-1 in beta-cell survival and insulin signaling
Funding Agency:	Michael Smith Foundation for Health Research
Funding Title:	Intracellular calcium stores as master regulators of pancreatic beta-cell survival: Studies on transplantable human islets and knockout mice
Funding Agency:	Canadian Diabetes Association
Funding Title:	Identifying and validating novel islet survival factors

Unfunded title: N/A

The Animal Care Committee has examined and approved the use of animals for the above experimental project.

This certificate is valid for one year from the above start or approval date (whichever is later) provided there is no change in the experimental procedures. Annual review is required by the CCAC and some granting agencies.

A copy of this certificate must be displayed in your animal facility.

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