UNIVERSITY OF CALIFORNIA, SAN DIEGO

Regulation of pancreatic beta cell aging and proliferation

A dissertation submitted in partial satisfaction of the requirements for the degree Doctor of Philosophy

in

Biomedical Sciences

by

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2016

The Dissertation of Jacqueline Rae Benthuysen is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

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University of California, San Diego

2016

DEDICATION

I would like to dedicate this dissertation to my husband, Matt, who has been a source of unwavering support and encouragement. He has believed in me and been by my side every step of the way, and I could not have accomplished this without him. And to my mom, dad, and sister whom have all been there for me to get me to this place. And finally to my friends, especially Stephanie, who have lent an ear when I needed it most.

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LIST OF ABBREVIATIONS

Adh1	Alcohol dehydrogenase 1
Adk	Adenosine kinase
Akt	Serine/Threonine kinase aka Protein Kinase B
Angptl7	Angiopoietin-related protein 7
Angptl8	Angiopoietin-related protein 8
ANOVA	Analysis of variance
BrdU	Bromodeoxyuridine
Ca2+	Calcium
cAMP	cyclic AMP
Ccnd1	Cyclin-dependent kinase 1
Ccnd2	Cyclin-dependent kinase 2
Cdkn2a	Cyclin-dependent kinase inhibitor 1
ChIP-seq	Chromatin immunoprecipitation followed by massively paralleled sequencing
CN	Calcineurin
Cre	Cre recombinase, tyrosine recombinase enzyme
db/db	Leptin-receptor deficient diabetic mouse
DE	Definitive endoderm
dL	Deciliter
Dlk1	Protein delta homolog 1
DMSO	Dimethyl sulfoxide
Dyrk1a	Dual specificity tyrosine-phosphorylation-regulated kinase 1A
EdU	5-ethynyl-2'-deoxyuridine
Egfr	Epidermal growth factor receptor
Erk1/2	Extracellular-signal-regulated kinase 1/2
ES	Embryonic stem cell
Ezh2	Enhancer of zeste homolog 2
FDR	False discovery rate
FE	Functional endocrine cells
FG	Posterier foregut
G1/S	Gap 1/Synethesis phase, cell cycle
Gck	Glucokinase
Glp-1	Glucagon-like peptide 1
Glp1r	Glucagon-like peptide receptor 1
Glut2	Glucose transporter 2
GO	Gene Ontology
Gsk-3beta	Glycogen synthase kinase 3 beta
Gstm2	Glutathione S-transferase Mu 2

GT	Primitive gut tube
HBSS	Hank's Balanced Salt Solution
hESC	Human embryonic stem cell
HI	Human Islet
IGF	Insulin-like growth factor
IIDP	Integrated Islet Distribution Program
Ins	Insulin
IPGTT	Intraperitoneal glucose tolerance test
Irs2	Insulin receptor substrate 2
kg	killigram
KRB	Krebs Ringer Bicarbonate Buffer
MafA	v-maf avian musculoaponeurotic fibrosarcoma oncogene homolog A
MAPK	Mitogen-activated protein kinase
Mek	MAP kinase-erk kinase
mg	milligram
Min	Minutes
MIPCreER	Mouse insulin promoter Cre recombinase-Estrogen receptor
mM	milliMolar
Mo	Mouse
MOM	Mouse on mouse kit
mRNA	Messenger ribonucleic acid
MS	Mass spectrometry
mTOR	Mammalian target of rapamycin
MudPIT	Multidimensional protein identification technology
NAD+	Nicotinamide adenine dinucleotide
Nampt	Nicotinamide phosphoribosyltransferase
Nfat	Nuclear factor of activated T-cells
Nkx6.1	NKX6 homeobox 1
$Nkx6.1^{\Delta\beta}$	Beta cell specific NKX6 homeobox 1 deficient mice
NMN	Nicotinamide mononucleotide
ob/ob	Leptin-deficient obese mouse
P4	Postnatal day 4
PBS	Phosphate-buffered saline
PcG	Polycomb-group proteins
Pdgf	Platelet-derived growth factor
Pdx1	Pancreatic and duodenal homeobox 1
PE	Pancreatic endoderm
PH	Polyhormonal cells

Pi3k	Phosphoinositide 3-kinase
Pka	Protein kinase A
Pkczeta	Protein kinase C zeta
qRT-PCR	Quantitative reverse transcriptase - Polymerase chain reaction
R26	Rosa 26 locus
Raf	Raf oncogene serine/threonine kinase
Ras	Ras viral oncogene homolog
RIP	Rat insulin promoter
RNA	Ribonucleic acid
RNA-seq	Ribonucleic acid sequencing
RPKM	Reads per kilobase per million
SEM	Standard error of the mean
SILAM	Stable isotope labeling of amino acids in mammals
Sirt1	Sirtuin 1
Sirt2	Sirtuin 2
$Sirt2^{\Delta\beta}$	Beta cell specific Sirtuin 2 deficient mice
STZ	Streptozotocin
T1D	Type 1 diabetes mellitus
T2D	Type 2 diabetes mellitus
TUNEL	Terminal deoxynucleotidyl transferase dUTP nick end labeling
Ucn	Urocortin 3
Wko	Weeks old
Wks	Weeks
YFP	Yellow fluorescent protein
Yo	Years old
Zyx	Zyxin

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ACKNOWLEDGEMENTS

I would like to thank my thesis advisor Maike Sander for her guidance throughout my Ph.D. studies. And I would also like to extend gratitude to all the members of the Sander lab, past and present, who have helped me on my projects. In particular, Fenfen Liu, Brandon Taylor, Tiffany Guan, Nancy Rosenblatt, and Andrea Carrano have been invaluable support for the past six years. I would also like to thank my committee (Larry Goldstein, Mark Mercola, John Chang, and Miles Wilkinson) for their insightful discussions of my thesis work during our committee meetings. And finally I would like to thank the Biomedical Sciences Program and its wonderful administrators, Leanne Nordeman and Gina Butcher, as well as the Genetics Training Program headed by Bruce Hamilton.

Chapter 1 and 5 include material, in part, currently being prepared for submission as a review article. Benthuysen, Jacqueline R; Carrano, A; Sander, M. “Advances in Beta Cell Regeneration-Strategies for the Treatment of Diabetes Mellitus.” The dissertation author was the primary investigator and author of this manuscript. J.B., A.C., and M.S. wrote the manuscript. J.B. prepared figures.

Chapter 2, in full, is a reprint of material as it appears in Taylor, Brandon L; Benthuysen, Jacqueline R; Sander, M. “Postnatal β-cell proliferation and mass expansion is dependent on the transcription factor Nkx6.1.” Diabetes. 2015 Mar;64(3):897-903. The dissertation author was co-first author of this paper. We thank S. Heller (Novo Nordisk) for anti-Glp1r, C. Kioussi (Oregon State University) for anti-GFP, and Mark Huising (UC Davis) for anti-Ucn3 antibody. We are grateful to N. Rosenblatt and F. Liu for technical assistance. This work was supported by the NIH/NIDDK grant R01-DK068471 to M.S. and the NIH training grant T32GM008666-15 to J.B. The authors have declared that no conflict of interest exists. M.S. is the guarantor of this work and, as such, has full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis. B.L.T. and J.B. designed and performed experiments, analyzed data and prepared figures. B.L.T.,

J.B., and M.S. wrote the manuscript. American Diabetes Association [Postnatal β-cell proliferation and mass expansion is dependent on the transcription factor Nkx6.1, [2015]. Copyright and all rights reserved. Material from this publication has been used with the permission of American Diabetes Association.

Chapter 3 includes material that is currently being prepared for submission as a manuscript. Benthuysen, Jacqueline R; Savas, Jeffrey N; Mulas, Francesca; Wortham, M; Divakaruni, A; Taylor, B.L.; Murphy, A; Yates, JR 3rd; Sander, M. (in preparation) "Comparative Analysis of Age-Dependent Changes in the Islet Proteome and Transcriptome." The dissertation author was the primary investigator and author of this study. The authors would like to thank D. Calzolari for assistance with Medusa programming, A. Carrano for critical reading and feedback, Y. Sui for processing RNA-sequencing data, F. Liu for islet isolations, and N. Rosenblatt for assistance in the mouse facility. RNA-sequencing was conducted at the IGM Genomics Center, University of California, San Diego, La Jolla, CA. J.B. designed and performed the experiments. J.N.S. with J.R.Y.III performed the mass spectrometry. J.B. analyzed the data and prepared figures. F.M. performed the Spearman's rank correlation and GSEA analysis. B.L.T. performed a pilot mass spectrometry analysis. J.B., M.W., A.D., with A.M. performed and analyzed mitochondrial experiments, data not shown in thesis. J.B. and M.S. wrote the manuscript.

Chapter 4 includes material that is currently being prepared for submission as a manuscript. Benthuysen, Jacqueline R; Sander, M. "Sirtuin 2 is a novel regulator of human beta cell proliferation." The dissertation author was the primary investigator and author of this study. The authors would like to thank J. Auwerx for the *Sirt2*^{flox} mice, and acknowledge A. Carrano for critical reading and feedback, Y. Sui for processing RNA-seq data, F. Liu and T. Guan for supportive technical roles, and N. Rosenblatt for assistance in the mouse facility. J.B. designed and performed the experiments, analyzed the data, and prepared figures. J.B. and M.S. wrote the manuscript. The authors would also like to acknowledge the Integrative Islet Distribution Program for providing human islet samples for this study.

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Benthuysen, J.R.; Sander, M**.** Sirtuin 2 is a novel regulator of human beta cell proliferation. (in preparation)

Benthuysen, J.R.; Savas, J.N.; Mulas, F.; Wortham, M.; Divakaruni, A.; Taylor, B.L.; Murphy, A.; Yates, J.R. 3rd; Sander, M. Comparative Analysis of Age-Dependent Changes in the Islet Proteome and Transcriptome. (in preparation)

Benthuysen, J.R.; Carrano, A.; Sander, M. Advances in Beta Cell Regeneration-Strategies for the Treatment of Diabetes Mellitus. (in preparation)

Taylor, B.L.*, **Benthuysen, J.R.***, Sander, M. Postnatal beta cell proliferation and mass expansion is dependent on the transcription factor Nkx6.1. Diabetes. 2015 Mar;64(3):897-903. *These authors contributed equally to this work.

Schaffer, A.E., Taylor, B.L., **Benthuysen, J.R.**, Liu, J., Thorel, F., Yuan, W., Jiao, Y., Kaestner, K.H., Herrera, P.L., Magnuson, M.A., May, C.L., Sander, M. (2013) Nkx6.1 controls a gene regulatory network required for establishing and maintaining pancreatic beta cell identity. PLoS Genetics, 9(1):e1003274.

ABSTRACT OF THE DISSERTATION

Regulation of pancreatic beta cell aging and proliferation

by

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Doctor of Philosophy in Biomedical Sciences

University of California, San Diego, 2016

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The replicative capacity of insulin-producing pancreatic beta cells is dynamically regulated during development, maturation, and aging. Early in life, beta cells proliferate rapidly to expand beta cell mass but quickly become quiescent with age. While this decline is well documented, the mechanisms that underlie this age-dependent beta cell replicative senescence are still poorly understood. Using mouse genetics and *in vivo* quantitative proteomics approaches, we found that nutrient sensing plays an important role in controlling the proliferation of beta cells. We show that the transcription factor Nkx6.1 is required for expanding beta cell mass during the early wave of rapid postnatal beta cell proliferation by regulating the expression of the nutrient sensing receptors Glut2 and Glp1r. Furthermore, by

quantitatively comparing the proteome of islets from young and aged mice, we found dynamic regulation of not only cell cycle proteins, but also proteins critical for beta cell function. Proteins important for insulin secretion and metabolic regulation increased with age, while proteins involved in expanding cell number declined with age. From our proteomic screen, we identified the NAD^+-dependent deacetylase Sirtuin2 as a protein that is upregulated during aging. Pharmacologic inhibition of Sirtuin2 promoted both rodent and human beta cell proliferation *ex vivo,* indicating Sirtuin2 is a repressor of beta cell proliferation. Beta cell-specific deletion of Sirtuin2 increased rodent beta cell proliferation and mass in diabetic mice *in vivo.* Importantly, inhibition of Sirtuin2 in human islets *ex vivo* did not negatively affect beta cell function or survival. Finally, we show that Sirtuin2 specifically regulates beta cell proliferation in conditions of elevated blood glucose through modulating the glucose-dependent MAPK pathway. Overall, our studies have uncovered dynamic regulation of beta cell proliferation from birth to advanced age and identified a viable therapeutic target for enhancing beta cell mass for the treatment of diabetes.

CHAPTER 1

Introduction

Diabetes mellitus is a chronic condition affecting an estimated 422 million people worldwide in 2014 (1). Characterized by high blood sugar levels, diabetes occurs in two major forms, type 1 (T1D) and type 2 diabetes (T2D). T1D results from autoimmune destruction of insulin producing pancreatic beta cells, while T2D is characterized initially by beta cell dysfunction from insulin resistance that eventually results in reduced beta cell mass. Because both types of diabetes lead to beta cell loss, research has focused on developing beta cell replacement strategies to address insulin insufficiency. Islet transplantation has proven to be a successful therapy, but its clinical application has been limited due to the shortage of donor cadaveric islets and the requirement of lifelong immune suppression. In the past decade, there have been intense efforts to identify alternative sources of beta cells. Beta cell replacement therapies based on the differentiation of embryonic stem cells into glucose-responsive, insulin-producing cells *in vitro* are currently being tested in human clinical trials. In addition, there have been exciting advances in *in vivo* regeneration approaches to replenish beta cell mass either through conversion of related cell types to beta cells, or by targeting natural beta cell replication to promote expansion of residual beta cells in diabetic patients. Here, we focus on strategies to uncover targets of beta cell proliferation for the treatment of diabetes.

1.1 AGING AND BETA CELL REPLICATION

Beta cell replication is the predominant mechanism that ensures the rapid expansion of beta cell mass early in life; however, the regenerative capacity of beta cells rapidly declines with advancing age (2-6). This age-dependent decline in beta cell proliferation is regulated by $p16^{Ink4a}$, a cyclin-dependent kinase inhibitor encoded by the *Cdkn2a* gene (7). Multiple age-dependently regulated factors, including p38MAPK and PcG/Trithorax group proteins, have been shown to epigenetically modify the *Cdkn2a* locus and to repress $p16^{Ink4a}$ expression (8-12). In young beta cells, Pdgf receptor signaling increases abundance of the PcG/Trithorax group protein Ezh2, thereby repressing $p16^{Ink4a}$ expression. However in aged beta cells,

decline of Pdgf receptor expression leads to $p16^{Ink4a}$ de-repression and beta cell cycle arrest (8).

To more globally define age-associated changes in the beta cell epigenome, Kaestner and colleagues carried out a genome-wide analysis of beta cells from young and old mice and found that the proliferative capacity of beta cells correlated with increased *de novo* promoter methylation and decreased expression of cell cycle regulators (13). This suggests that manipulation of epigenetic regulators could reverse beta cell senescence and promote regeneration. Interestingly, this group also observed upregulation of genes involved in beta cell function, such as critical beta cell transcription factor Nkx6.1 (14), and improved insulin secretory function with age (13). While these results contradict early studies showing a decline of beta cell function with age (15-18), it aligns with more recent work demonstrating sustained or improved beta cell secretory function in older animals (19; 20). Because beta cell proliferation and beta cell function are tightly linked metabolically, additional studies are needed to determine whether beta cell proliferation can be safely increased without compromising function.

While there is an overall decline in beta cell replicative capacity with age, beta cell replication and mass have been shown to increase in the face of metabolic challenges, such as pregnancy or obesity. With the hope of identifying targets for therapeutic intervention, much effort has been devoted to understand the mechanisms that trigger beta cell replication under conditions of increased metabolic demand.

1.2 BETA CELL REPLICATION IN PREGNANCY

During pregnancy, beta cell mass expands in order to adapt the organism to increasing insulin demand (21-24). Multiple factors, including lactogens, serotonin, and components of the EGFR signaling pathway have been shown to increase beta cell replication in pregnant rodents (21; 25-28). There is, however, controversy as to whether adaptive beta

cell proliferation during pregnancy occurs to the same extent in humans (22). Moreover, there is conflicting evidence that the molecular pathways regulating beta cell mass expansion in pregnant rodents are conserved in humans. While one study found beta cell proliferation induced by prolactin and placental lactogen in two human islet samples (25), more recently Chen and colleagues were unable to induce human beta cell proliferation with prolactin in up to six independent human islet samples (29). This could, at least in part, be explained by the lack of prolactin receptor expression on human beta cells (29). While prolactin receptor agonists may not be effective for stimulating human beta cell proliferation, downstream signaling pathways may be conserved and could provide insight into therapeutic targets. In support of this idea, Vasavada and colleagues found that treatment of human islets with recombinant osteoprotegerin, a lactogen target, induced human beta cell proliferation (30). Importantly, Denosumab, an FDA-approved osteoporosis drug, mimicked the activity of osteoprotegerin and enhanced human beta cell replication *in vitro* and after engraftment of human islets into mice. The pro-proliferative effect of Denosumab suggests that there is potential for repurposing this drug for treatment of diabetes.

With the goal of discovering novel targets for enhancing beta cell proliferation, Ahnfelt-Rønne and colleagues took a proteomic approach to identify proteins that change in abundance during pregnancy in mice (31). This analysis not only confirmed regulation of targets previously shown to be regulated at the mRNA level (27; 32-34), but also identified proteins not previously associated with pregnancy-induced beta cell expansion. Two examples are Stathmin 1 and the nuclear chloride ion channel 1, which have known roles in the regulation of cell proliferation and are being evaluated as drug targets in cancer (35-38). While follow up studies will be necessary, this study highlights the importance of global approaches to identify novel molecular targets.

1.3 BETA CELL REPLICATION IN HYPERGLYCEMIA AND INSULIN RESISTANCE

Apart from pregnancy, beta cell proliferation is also regulated by diet and changes in metabolic state. A recent study suggests that nutritional cues have immediate effects on the capacity of beta cells to mount a regenerative response. When mice were prematurely weaned from fat-rich milk to carbohydrate-rich chow, the potential of beta cells for compensatory proliferation increased (39). Although it remains to be studied whether a similar mechanism operates during adulthood, the finding suggests that diet composition could have effects on beta cell mass. Metabolic regulation of beta cell proliferation is also evident during a state of insulin resistance, which is known to trigger compensatory beta cell proliferation. This has been demonstrated in multiple rodent models of diabetes, including *ob/ob* mice (40), *db/db* mice (41), zucker fatty rats (42), and in high fat diet feeding (43). Increased beta cell mass is also observed in hyperinsulinemic humans with obesity and insulin resistance (44-46); however, whether proliferation is increased is less clear.

Glucose and insulin have been identified as inducers of beta cell replication. Multiple studies have demonstrated increased proliferation of rodent and human beta cells following glucose infusion (47-53). Glucose metabolism is required for beta cell proliferation, as lack of glucokinase, a key enzyme in glycolysis that converts glucose to glucose-6-phosphate, blunts beta cell proliferation and treatment with a small molecule glucokinase activator can stimulate proliferation (54). However, long-term glucose exposure can cause glucotoxicity, resulting in DNA damage and apoptosis, as also seen in beta cells from T2D patients (55). Therefore, there is a need to better understand where the mitogenic and DNA damage pathways diverge before the glucose-induced mitogenic pathway can be considered for therapeutic intervention.

While glucose can increase beta cell replication, beta cell hyperplasia occurs in *ob/ob* and db/db mice prior to the onset of hyperglycemia and is also observed in mouse models of insulin resistance in the absence of hyperglycemia (41; 56-58). These observations suggest that factors other than glucose contribute to beta cell mass expansion in the face of insulin

resistance. Insulin levels are highly elevated in the insulin resistant state, and insulin signaling has been shown to account for compensatory beta cell growth during insulin resistance. Ablation of the insulin receptor in an insulin resistant mouse model impaired beta cell proliferation and rendered mice prematurely diabetic (59). In contrast, deletion of the insulin-like growth factor (IGF) receptor had little effect on beta cell growth, suggesting that compensatory beta cell mass expansion predominantly depends on insulin rather than IGF signaling.

1.4 CIRCULATING FACTORS AND BETA CELL REPLICATION

In recent years, significant effort has been put forth into identifying systemic regulators of beta cell proliferation in the context of aging, pregnancy, and metabolic challenge. Circulating factors that are regulated during metabolic adaptation are particularly attractive therapeutic targets, as manipulating their activity might mitigate the risk for inducing tumors in other tissues. Experiments testing aged islets in a young systemic environment have shown that circulating factors from young mice improve beta cell regeneration in aged mice (19; 60). Likewise, beta cell replication increases when islets from normal mice are grafted under the kidney capsule of insulin-resistant mice (61). While these experiments clearly illustrate the importance of circulating factors in beta cell regeneration, the specific factor(s) that account for the effects have remained elusive. However, multiple circulating factors, including glucagon-like peptide-1 (GLP-1), secreted by the intestinal L-cells (62-64); thyroid hormone (65; 66); the osteoblast-derived hormone osteocalcin (67-70); liver-derived Angiopoietin-like protein 8 (Angptl8/Betatrophin) (71); and recently SerpinB1, a liver-secreted protease inhibitor (72), have been identified as potentially pro-proliferative for beta cells, at least in rodents. It is less clear whether these circulating factors can also stimulate human beta cell growth. Controversial reports exist regarding effects of GLP-1 analogs on human beta cell growth with one study finding no effect (73) and another reporting stimulation (74). Illustrating the difficulty

of controlled studies using human cells, a subsequent report showed that the age of the islet donor might be a contributing factor to responsiveness of beta cells to GLP-1 analogs (75). While the specific role of GLP-1 in adaptive proliferation of human beta cells is still unclear, further studies on Angptl8/Betatrophin have called its role in beta cell mass expansion into question. Genetic loss- and gain-of-function experiments for *Angptl8/Betatrophin* revealed no effect on beta cell mass in insulin resistant mice (76; 77), showing that Angptl8/Betatrophin is not the long thought-after liver-derived factor, which stimulates beta cell growth. Consistent with the findings by Gusarova et al., Angptl8/Betatrophin also failed to exert a pro-proliferative effect on transplanted human beta cells (78). More promising are recent findings for osteocalcin (70) and SerpinB1 (72), which indicate that these hormones could be effective in stimulating human beta cell proliferation. Beta cell proliferation was increased after treatment of human islets with decarboxylated osteocalcin or small molecules mimicking SerpinB1 activity both *ex vivo* and after transplantation into mice. Preliminary analysis of mice treated with small molecule mimics of SerpinB1 suggests that the effects on proliferation of extra-pancreatic tissues are limited, which raises hope that it might be possible to identify growth-stimulating agents that are selective to beta cells.

1.5 INTRACELLULAR SIGNALING PATHWAYS REGULATING BETA CELL REPLICATION

Many groups have utilized high throughput screening methods to discover novel molecules and pathways that could stimulate beta cell mass expansion (79-89). Some of these screens have led to the discovery of novel compounds with therapeutic potential. For example, aminopyrazine compounds, harmine, and INDY were identified from a high throughput chemical screen for inducers of beta cell proliferation using rodent beta cell lines, and shown to also augment human beta cell proliferation (87; 88). Interestingly, all three molecules inhibit the kinase Dyrk1a, which blocks nuclear localization of NFAT, a transcription

factor that activates expression of cell cycle genes in beta cells (90; 91). Aminopyrazine compounds have a larger effect on beta cell proliferation than harmine, which is explained by additional inhibition of glycogen synthase kinase-3 beta (Gsk-3β) (88). Like Dyrk1a, Gsk-3β prevents nuclear localization of NFAT (92) and inhibits beta cell proliferation (93-96). Interestingly, osteoprotegerin and SerpinB1 have been shown to inhibit Gsk-3β activity (30; 72), suggesting that their effect on beta cell proliferation may, at least in part, be mediated by Gsk-3β. A significant hurdle for advancing beta cell therapeutics for these pathways is the unclear specificity of many of the small molecules as well as effects on multiple tissues.

Additional potentially druggable intracellular regulators of beta cell proliferation have been identified through candidate genetic approaches. The literature on intracellular signaling in beta cell proliferation has recently been comprehensively reviewed (97-100), and we refer to these reviews for details. From the numerous studies, the MAP kinase (MAPK) and PI3 kinase (PI3K)/AKT pathways have emerged as critical regulators of beta cell proliferation, also in humans. The MAPK pathway via Erk1/2 phosphorylation is the key mitogenic pathway that separates metabolic regulation of beta cell function and proliferation, as Erk1/2 phosphorylation is not required for glucose stimulated insulin secretion (101). The MAPK pathway mediates the beta cell mitogenic effect of multiple growth factors, hormones and nutrients, including Pdgf, GLP-1, prolactin, insulin, and glucose (8; 102-109). The second major pathway responsible for transducing beta cell proliferative signals is the PI3K/AKT/mTOR pathway, which is activated by insulin, GLP-1 and glucose (103; 109-112). AKT activation is an important component that links growth signals to its downstream target mTOR, which coordinates a cell growth response directly through its effect on cell cycle regulators (93; 113). Numerous studies have demonstrated a role of this pathway in promoting beta cell proliferation *in vitro* and increasing beta cell mass *in vivo* (93; 110; 113-117). Further illustrating its pro-proliferative role, AKT-mTOR signaling is active in pancreatic endocrine tumors (118). Notably, PI3K signaling can induce beta cell proliferation not only by activating

AKT, but also through AKT-independent PKCζ, which mediates the proliferative effect of glucose on human beta cells(119-122).

It is important to consider that significant cross talk exists between the signaling pathways. For example, high glucose and GLP-1 activate both mTOR and MAPK signaling (103; 108; 110). A recent study nicely illustrates how the balance between different signaling arms determines the beta cell response to insulin (123). Knockdown of PI3K resulted in rerouting of the insulin signal from PI3K-mediated metabolic signaling to ERK-mediated mitogenic signaling, which induced a switch of beta cells from highly glucose-responsive to proliferative. Extensive feedback inhibition and amplification constitutes a further layer of complexity, exemplified by mTOR negatively feeding back on insulin signaling via IRS2 (124).

All of these intracellular signals converge to regulate the core G1/S cell cycle machinery (97-100). Successful targeting of beta cell proliferation will hinge on the downregulation of cell cycle inhibitors and upregulation of cell cycle activators. The example of aminopyrazine compounds, which target Dyrk1a and Gsk-3β, illustrates that targeting more than one pathway will likely have a more robust effect on beta cell proliferation than targeting one pathway alone. Given the extremely low proliferation rate of human beta cells (4), hitting multiple targets might be necessary to produce clinically relevant effects. Furthermore, as regenerative and oncogenic pathways share similar effector proteins, a major challenge will be to enhance beta cell proliferation without inducing aberrant growth of other tissues.

1.6 OBJECTIVE OF THE DISSERTATION

The work presented in this dissertation is centered on understanding how beta cell proliferation is regulated. To this end, the objectives were to (1) characterize transcription factor Nkx6.1 in regulating postnatal beta cell proliferation, (2) gain a comprehensive understanding of pancreatic islet aging using quantitative proteomics to identify novel beta cell proliferation regulators, (3) characterize a druggable candidate protein, Sirtuin2 in regulation of

human beta cell proliferation. Chapter 2 focuses on a candidate approach studying the requirement of Nkx6.1 during postnatal beta cell mass expansion and its importance for glucose sensing of the beta cell during a critical time window of beta cell maturation. Chapter 3 addresses, on a more global scale, the proteins that change in beta cells from juvenile to adult rodents, and finally Chapter 4 characterizes one of the age-dependently regulated proteins, Sirtuin2, in controlling beta cell proliferation.

1.7 ACKNOWLEDGEMENTS

Chapter 1 includes material, in part, currently being prepared for submission as a review article. Benthuysen, Jacqueline R; Carrano, A; Sander, M. “Advances in Beta Cell Regeneration-Strategies for the Treatment of Diabetes Mellitus.” The dissertation author was the primary investigator and author of this manuscript. J.B., A.C., and M.S. wrote the manuscript. J.B. prepared figures.

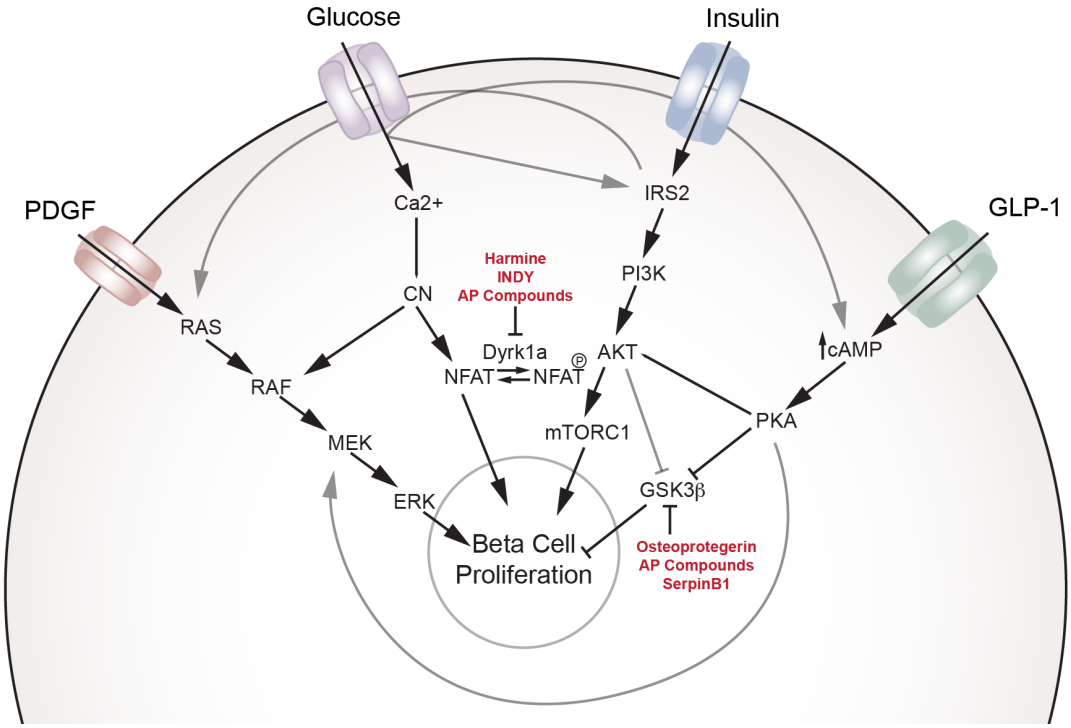

Figure 1.1 Major signaling pathways known to regulate beta cell proliferation. Lighter lines indicate non-canonical signaling crosstalk and red words indicate recent targets known to induce human beta cell replication. See "List of Abbreviations" for protein names.

CHAPTER 2

Postnatal Beta Cell Proliferation and Mass Expansion is Dependent on the Transcription Factor Nkx6.1

2.1 INTRODUCTION

The establishment of sufficient beta cell mass depends on the rapid expansion of beta cell numbers during early postnatal life (4; 6; 125-127). The extent of this early postnatal beta cell growth is postulated to influence later susceptibility to type 2 diabetes (128). Postnatal beta cell mass expansion is driven by beta cell proliferation (129), which is controlled by the cell cycle regulators *Cyclin D1* or *Cyclin D2* (encoded by *Ccnd1* and *Ccnd2*, respectively) (125; 126). It has been shown that beta cells are highly proliferative in the perinatal period and that this early proliferation is necessary to establish sufficient beta cell mass for maintaining glucose homeostasis (4; 6; 125-127). However, the cell extrinsic and intrinsic factors that drive beta cell proliferation and mass expansion during the perinatal period are still poorly defined.

Glucose has been identified as a systemic factor that stimulates beta cell proliferation (47; 48), and recent studies suggest that glucose is a significant driver of early postnatal beta cell proliferation (130). Furthermore, it has been shown that glucose metabolism in beta cells produces signals that increase *Cyclin D2* expression and beta cell proliferation (54; 131; 132). Independent of glucose, beta cell proliferation is also stimulated by gut-derived hormone glucagon-like peptide 1 (Glp1), which is secreted by intestinal enteroendocrine cells in response to food intake (62; 119). Thus, there is an established link between feeding, increases in blood glucose levels, and beta cell proliferation. However, beta cells also exhibit significant proliferation during fetal life, when blood glucose concentrations are low and glucose has little effect on beta cell proliferation (133). The distinct mechanisms employed in prenatal and postnatal beta cells to regulate proliferation remain unclear.

The beta cell-restricted transcription factor Nkx6.1 is essential for maintaining the functional state of beta cells during adulthood (134). Both *in vitro* and *in vivo* experiments have suggested a role for Nkx6.1 in beta cell proliferation (134-136), but whether it is required for beta cell growth *in vivo* is unknown. To reveal a possible role for Nkx6.1 beta cell mass

expansion, we inactivated *Nkx6.1* in newly-formed beta cells of the embryo and examined the effects on beta cell proliferation and mass during the prenatal and postnatal period.

2.2 RESULTS

2.2.1 *Nkx6.1* inactivation in embryonic beta cells causes hyperglycemia and reduced beta cell mass.

To investigate the role of Nkx6.1 in perinatal beta cell development, we intercrossed mice to generate progeny carrying a *Nkx6.1* null allele (*Nkx6.1*$^{-}$), a *Nkx6.1* conditional loss of function allele (*Nkx6.1*^{flox}), and the *rat insulin2-Cre* transgene (*RIP-Cre*). Additionally, the mice carried a conditional *YFP* reporter gene targeted to the *Rosa-26* locus (*R26-YFP*), resulting in heritable YFP expression upon *RIP-Cre*-mediated recombination of a translational stop signal. Thus, in *RIP-Cre;Nkx6.1*$^{flox/-}$*;R26-YFP* (hereafter referred to as *Nkx6.1*$^{\Delta\beta}$) mice, YFP labels all cells in which *Nkx6.1* has been inactivated (Figure 1A).

Nkx6.1$^{\Delta\beta}$ mice were born with the expected mendelian frequency (data not shown). Consistent with previous reports showing incomplete targeting of beta cells by the *RIP-Cre* transgene (137), most but not all beta cells were devoid of Nkx6.1 at birth (Figure 2.1B,C). At six weeks of age, *Nkx6.1*$^{\Delta\beta}$ mice exhibited significantly elevated blood glucose levels (Figure 2.1D) and impaired glucose tolerance after intraperitoneal injection of a glucose bolus (Figure 2.1E). To investigate whether Nkx6.1 deficiency affects postnatal beta cell growth, we examined beta cell mass in *Nkx6.1*$^{\Delta\beta}$ mice. Compared to littermate controls, six-week-old *Nkx6.1*$^{\Delta\beta}$ mice exhibited a 40% reduction in beta cell mass (1.26±0.05 mg in *Nkx6.1*$^{\Delta\beta}$ mice versus 2.13±0.29 mg in controls) (Figure 2.1F). Thus, Nkx6.1 is necessary to establish appropriate beta cell mass.

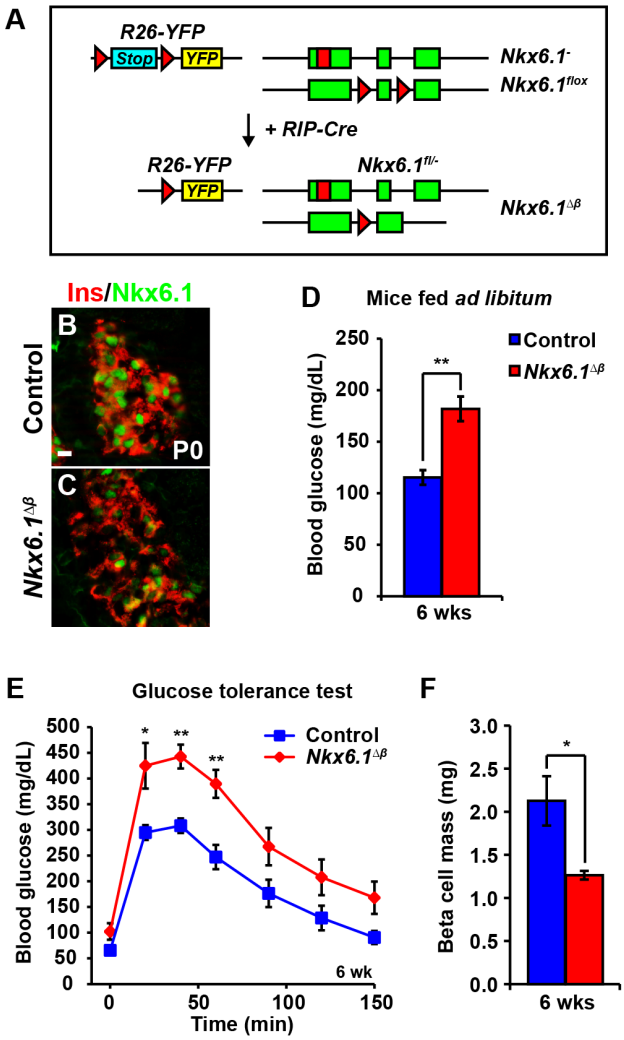

Figure 2.1 *Nkx6.1* deletion in newly-formed beta cells leads to glucose intolerance and reduced beta cell mass. (**A**) Schematic of alleles and transgenes utilized to inactivate *Nkx6.1* in fetal beta cells. Rectangles: coding sequences; triangles: *loxP* sites; red rectangle: *DsRed* coding sequence. (**B, C**) Immunofluorescence staining for Nkx6.1 and insulin reveals loss of Nkx6.1 in most beta cells of *Nkx6.1*$^{\Delta\beta}$ mice at postnatal day (P) 0. (**D**) Blood glucose levels in 6-week-old *Nkx6.1*$^{\Delta\beta}$ mice fed *ad libitum* compared to control mice (n=6). (**E**) Intraperitoneal glucose tolerance test shows glucose intolerance in 6-week-old *Nkx6.1*$^{\Delta\beta}$ mice as compared to control mice (n=6). (**F**) Quantification of beta cell mass reveals decreased beta cell mass in *Nkx6.1*$^{\Delta\beta}$ mice at 6 weeks of age (n=3). Scale bars = 20 μm. Ins, insulin; YFP, yellow fluorescent protein; wks, weeks. Data shown as mean ± SEM. *p<0.05, **p<0.01.

2.2.2 *Nkx6.1* is required for postnatal, but not prenatal beta cell mass expansion.

To determine when beta cell mass is first affected in *Nkx6.1*$^{\Delta\beta}$ mice, we measured the relative insulin^{+} area in *Nkx6.1*$^{\Delta\beta}$ mice immediately after birth. In contrast to six-week-old mice,beta cell mass in neonatal *Nkx6.1*$^{\Delta\beta}$ mice was indistinguishable from control mice (Figure 2.2A), showing that Nkx6.1 is required for postnatal expansion but not for establishing prenatal beta cell mass.

Because *RIP-Cre*-mediated recombination of the *Nkx6.1*^{flox} allele is mosaic and did not delete *Nkx6.1* in all beta cells (Figure 2.1C), both unrecombined Nkx6.1^{+} and recombined Nkx6.1-deficient beta cells can contribute to beta cell growth in *Nkx6.1*$^{\Delta\beta}$ mice. To investigate the contribution of recombined beta cells to postnatal beta cell mass expansion, we quantified the percentage of recombined beta cells in *Nkx6.1*$^{\Delta\beta}$ and control mice. In line with our observation that Nkx6.1 is dispensable for prenatal beta cell growth (Figure 2.2A), the percentage of recombined YFP^{+} beta cells was similar in newborn *Nkx6.1*$^{\Delta\beta}$ and control mice (73±1.4% in *Nkx6.1*$^{\Delta\beta}$ mice versus 76±9.5% in control mice) (Figure 2.2B-C",H). Consistent with a slight decrease in overall beta cell mass in *Nkx6.1*$^{\Delta\beta}$ mice at postnatal (P) day 4 (Figure 2.2A), a reduction in the percentage of recombined beta cells was discernable in *Nkx6.1*$^{\Delta\beta}$ mice by P4 (Figure 2.2D-E",H). At six weeks of age the reduction of YFP^{+} beta cells was highly significant (15±2.02% of beta cells in *Nkx6.1*$^{\Delta\beta}$ mice versus 83±1.72% in control mice) (Figure 2.2F-H). The decrease of YFP^{+} beta cells in *Nkx6.1*$^{\Delta\beta}$ mice was accompanied by an age-dependent increase in the percentage of beta cells expressing Nkx6.1 (Figure 2.2B-G",I). Closely mirroring the reported 82% recombination efficiency of the *RIP-Cre* transgene (137), 27±1.41% of beta cells expressed Nkx6.1 in newborn *Nkx6.1*$^{\Delta\beta}$ mice (Figure 2.2I). This percentage increased significantly to 40±3.75% at P4 (Figure 2.2I). These findings indicate that a selective disadvantage becomes apparent for Nkx6.1-deficient beta cells shortly after birth.

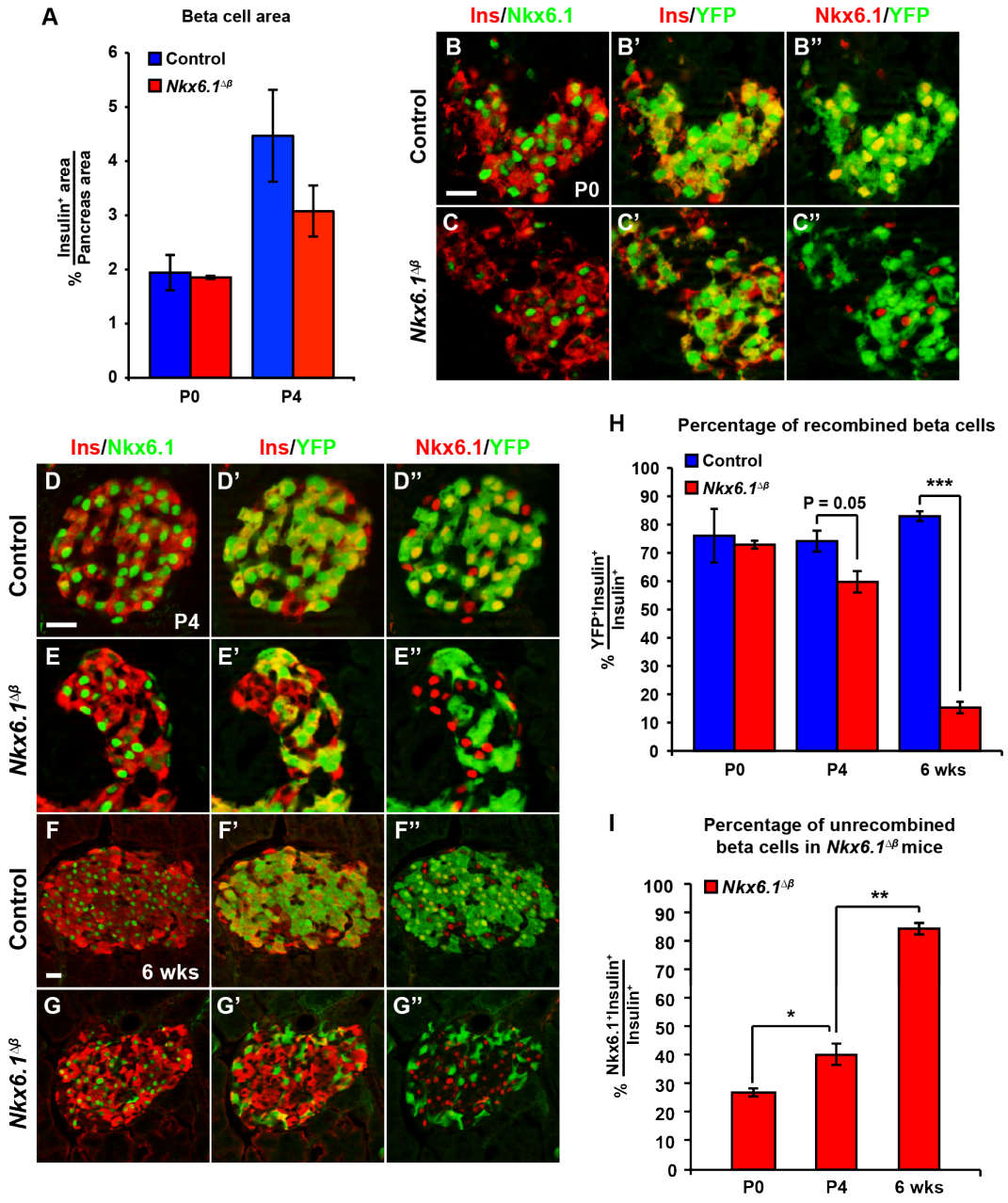

Figure 2.2 Nkx6.1 is required for postnatal beta cell mass expansion. (**A**) Quantification of the insulin immunofluorescent area relative to total pancreatic area reveals no difference in beta cell mass between *Nkx6.1*$^{\Delta\beta}$ and control mice at postnatal day (P) 0 and a slight but not significant decrease at P4 (n=3). (**B-G"**) Immunofluorescence staining for insulin, Nkx6.1, and YFP at P0 (B-C"), P4 (D-E"), and 6 weeks of age (F-G"). (**H**) Quantification of insulin^{+} cells expressing YFP at P0, P4, and 6 weeks shows a progressive decrease of YFP^{+} recombined beta cells in *Nkx6.1*$^{\Delta\beta}$ mice postnatally (n=3). (**I**) Quantification of insulin^{+} cells expressing Nkx6.1 reveals a progressive increase of Nkx6.1-expressing unrecombined beta cells in *Nkx6.1*$^{\Delta\beta}$ mice between P0 and 6 weeks (n=3). Scale bar = 20 μm. Ins, insulin; YFP, yellow fluorescent protein; wks, weeks. Data shown as mean ± SEM. *, p<0.05; **, p<0.01; ***, p<0.001.

2.2.3 Postnatal, but not prenatal beta cell proliferation depends on Nkx6.1.

We next investigated whether the postnatal beta cell growth defect in *Nkx6.1*$^{\Delta\beta}$ mice is caused by reduced beta cell proliferation and/or survival. First, we examined the possibility that Nkx6.1 deficiency causes increased beta cell apoptosis by performing TUNEL assays on pancreatic sections. Virtually no TUNEL^{+} beta cells were detected in either *Nkx6.1*$^{\Delta\beta}$ or control mice at P4 (Figure 2.3A-C), indicating that apoptosis does not account for the negative selection of Nkx6.1-deficient beta cells. By contrast, analysis of beta cell proliferation by immunofluorescence staining for Ki67, insulin, and YFP in *Nkx6.1*$^{\Delta\beta}$ mice at P4 revealed reduced numbers of Ki67^{+} beta cells (Figure 2.3F-H). Quantification of Ki67^{+}YFP^{+} beta cells showed a 3-fold decrease in beta cell proliferation in four-day-old *Nkx6.1*$^{\Delta\beta}$ compared to control mice (4.48±1.01% in *Nkx6.1*$^{\Delta\beta}$ mice versus 13.00±1.58% in control mice) (Figure 3H). Consistent with our finding that *Nkx6.1* inactivation does not affect prenatal beta cell growth (Figure 2.2A), the frequency of Ki67^{+} beta cells did not differ between *Nkx6.1*$^{\Delta\beta}$ and control mice at P0 (2.67±1.14% in *Nkx6.1*$^{\Delta\beta}$ mice versus 1.78±1.05% in control mice) (Figure 2.3D-E"',H). Thus, Nkx6.1 is required for beta cell proliferation and expansion during early postnatal life but is dispensable prenatally. Furthermore, the effect of *Nkx6.1* deletion on beta cell proliferation is cell autonomous, as revealed by comparing proliferation rates between recombined and unrecombined beta cells in *Nkx6.1*$^{\Delta\beta}$ mice at P4 (4.48±1.01% YFP^{+}insulin^{+} cells versus 11.0±0.93% YFP^{-}insulin^{+} cells expressed Ki67) (Figure 2.3I).

2.2.4 *Nkx6.1* deletion causes a cell-autonomous loss of markers for beta cell maturation and nutrient sensing.

To determine whether loss of Nkx6.1 affects other beta cell markers, we performed immunofluorescence staining for Pdx1 and MafA. While Pdx1 was unaffected, MafA was lost in recombined Nkx6.1-deficient beta cells (Figure 2.4A-F"'). We further assessed whether Nkx6.1 regulates beta cell maturation markers. To this end, we selected genes found to be significantly changed between immature and mature postnatal beta cells (138), and performed

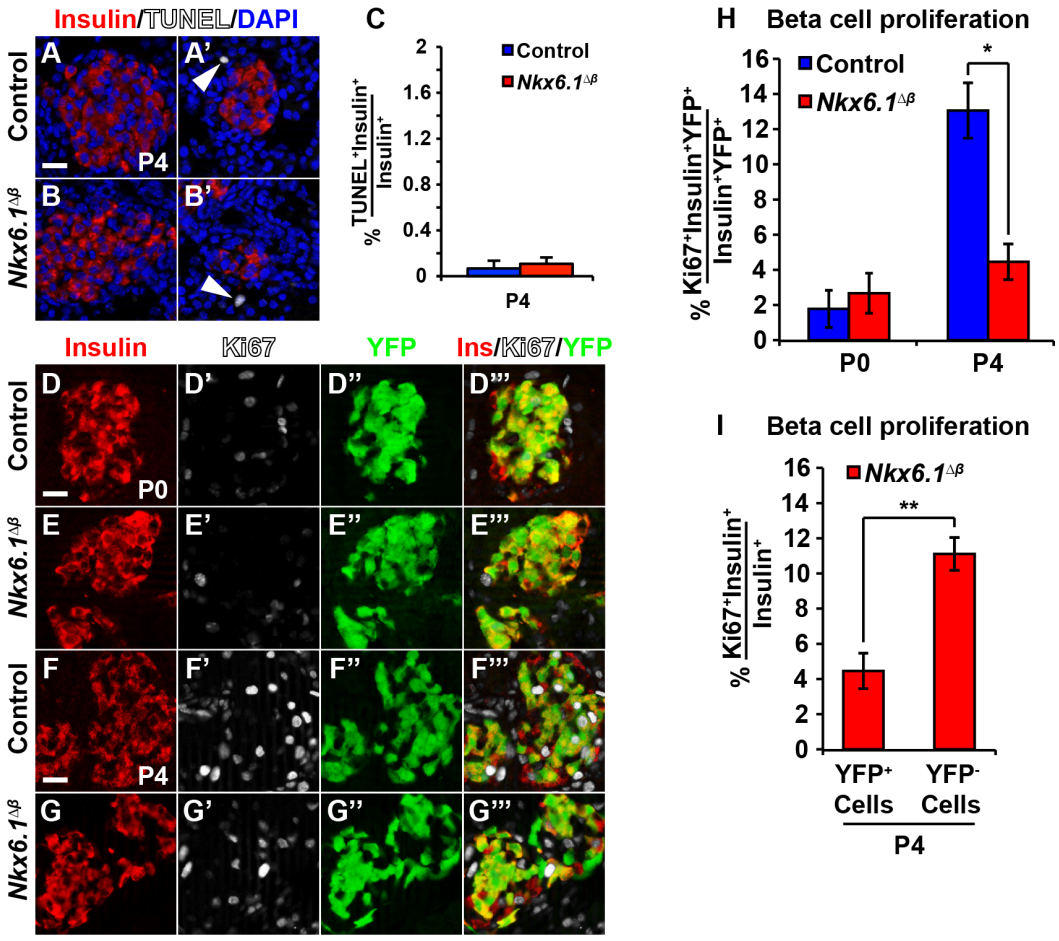

Figure 2.3 Nkx6.1 is required for postnatal beta cell proliferation. (**A-C**) Beta cells are not apoptotic at postnatal day (P) 4 in *Nkx6.1*$^{\Delta\beta}$ or control mice based on TUNEL combined with immunofluorescence staining for insulin and DAPI. TUNEL^{+} cells in the pancreas are shown as a positive control (arrowheads) and TUNEL^{+}insulin^{+} cells were quantified. (**D-G'''**) Immunofluorescence staining for insulin, Ki67, and YFP at P0 and P4. (**H**) Quantification of the percentage of insulin^{+}YFP^{+} cells expressing Ki67 shows decreased beta cell proliferation in *Nkx6.1*$^{\Delta\beta}$ mice at P4, but not at P0 (n=3). (**I**) Quantification of Ki67-expressing YFP^{+}insulin^{+} cells and YFP^{-}insulin^{+} cells in *Nkx6.1*$^{\Delta\beta}$ mice at P4 reveals a selective decrease in proliferation of recombined compared to unrecombined beta cells within the same animal (n=3). Scale bar = 20 µm. Ins, insulin; YFP, yellow fluorescent protein. Data shown as mean ± SEM. *, $p<0.05$; **, $p<0.01$.

qRT-PCR analysis on pancreata from control and *Nkx6.1*$^{\Delta\beta}$ mice at P2, when beta cell mass is similar between *Nkx6.1*$^{\Delta\beta}$ and control mice (Figure 2.2A). Of these genes, *Ucn3, Adh1*, *Gstm2*, and *Zyx* were expressed at significantly lower levels in *Nkx6.1*$^{\Delta\beta}$ mice, while *Angptl7* and *Dlk1* were unchanged (Figure 2.4G-J"'). These results suggest that Nkx6.1 regulates a subset of genes associated with beta cell maturation. Given the postnatal onset of the beta cell proliferation defect in *Nkx6.1*$^{\Delta\beta}$ mice, we next investigated whether Nkx6.1-deficient beta cells are able to receive feeding-induced signals that stimulate beta cell proliferation. We analyzed the expression of glucose transporter 2 (Glut2) and the Glp1 receptor (Glp1r), which are known to have a role in the regulation of postnatal beta cell growth (62; 133). In accordance with *Glut2* being a direct Nkx6.1 target gene (134), *Nkx6.1*$^{\Delta\beta}$ mice exhibited a selective loss of Glut2 expression only in recombined beta cells (Figure 2.4K-M"'). Similarly, recombined beta cells displayed a cell autonomous reduction in Glp1r expression (Figure 2.4N-P"'). The cell autonomous role of Nkx6.1 in regulating beta cell proliferation, Glut2, and Glp1r expression argues against an Nkx6.1-dependent paracrine or systemic factor affecting beta cell proliferation in *Nkx6.1*$^{\Delta\beta}$ mice. These findings demonstrate that Nkx6.1-deficient beta cells lack key sensors for extrinsic stimuli of postnatal beta cell growth.

2.3 DISCUSSION

The role of Nkx6.1 in beta cell proliferation has been controversial. While *in vitro* studies have suggested a direct role of Nkx6.1 in stimulating beta cell proliferation through the regulation of *Cyclin* gene expression (135), *in vivo* overexpression of Nkx6.1 in beta cells showed no effect on beta cell proliferation or mass (136). Moreover, we have recently reported that beta cell-specific inactivation of *Nkx6.1* in adult mice has no overt effect on beta cell mass (134). However, due to the extremely low proliferation rate of beta cells in adult animals (6), the role of Nkx6.1 in beta cell mass expansion could not be rigorously tested in this model. By ablating *Nkx6.1* in newly-formed beta cells of the embryo, we here show that postnatal beta

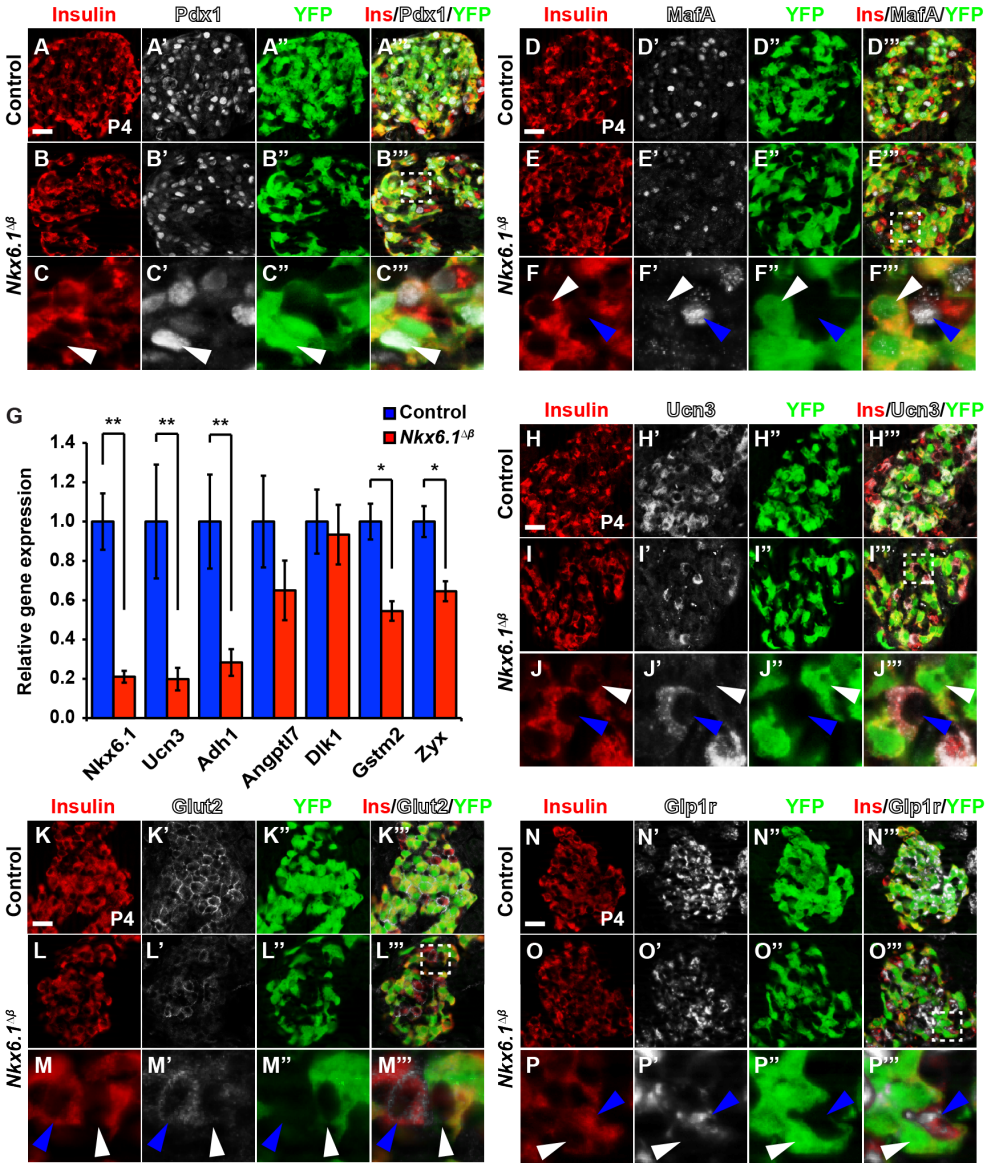

Figure 2.4 *Nkx6.1* inactivation leads to a cell autonomous loss of beta cell maturation and nutrient sensing markers. Immunofluorescence staining for insulin, Pdx1, and YFP (**A-C'''**) or insulin, MafA, and YFP (**D-F'''**) shows Pdx1 but not MafA expression in recombined YFP^{+}insulin^{+} cells of *Nkx6.1*$^{\Delta\beta}$ mice at postnatal day (P) 4. Unrecombined YFP^{-}insulin^{+} cells express Pdx1 and MafA in *Nkx6.1*$^{\Delta\beta}$ mice. (**G**) qRT-PCR analysis of pancreata from *Nkx6.1*$^{\Delta\beta}$ and control mice at P2 for genes associated with beta cell maturation (n=3). Immunofluorescence staining for insulin, Ucn3, and YFP (**H-J'''**), insulin, Glut2, and YFP (**K-M'''**), or insulin, Glp1r, and YFP (**N-P'''**) shows loss of Ucn3, Glut2, and Glp1r expression in recombined YFP^{+}insulin^{+} cells but not in unrecombined YFP^{-}insulin^{+} cells of *Nkx6.1*$^{\Delta\beta}$ mice at P4. For each marker, representative areas are shown in lower panels for *Nkx6.1*$^{\Delta\beta}$ mice, as indicated by a dashed box in the merged middle panel. White arrowheads point to recombined YFP^{+}insulin^{+} cells and blue arrowheads to unrecombined YFP^{-}insulin^{+} cells. Scale bar = 20 μm. Ins, insulin; YFP, yellow fluorescent protein. Data shown as mean ± SEM. *, p<0.05; **, p<0.01.

cell proliferation and mass expansion depends on Nkx6.1 activity. We found that Nkx6.1-deficient beta cells begin to exhibit reduced proliferation between P0 and P4, which manifests in a measurable decrease in the contribution of Nkx6.1-deficient beta cells to beta cell mass as early as P4. We have previously reported that Nkx6.1 deficiency leads to a loss of beta cell identity and ultimately their conversion into delta cells (139). It is important to note that this fate conversion occurs later and is not yet observed at P4 (see Fig. 2E-E"; all YFP^+ cells express Thus, the reduced contribution of Nkx6.1-deficient beta cells to beta cell mass is caused by the proliferation defect and cannot be attributed to a beta-to-delta cell fate conversion.

Employing ChIP-seq analysis, we have recently shown that Nkx6.1 does not bind to *Cyclin* gene regulatory regions (134). Therefore, Nkx6.1 is likely an indirect regulator of beta cell proliferation. Consistent with this idea, our current work shows that prenatal beta cell proliferation is unaffected in *Nkx6.1*$^{\Delta\beta}$ mice. Interestingly, we found that the onset of reduced beta cell proliferation in *Nkx6.1*$^{\Delta\beta}$ mice coincides with birth and thus the beginning of food intake, suggesting that Nkx6.1 could enable beta cells to respond to nutrient-dependent inducers of beta cell proliferation. Supporting this notion, *Nkx6.1*-deleted beta cells fail to express two important nutrient sensors, Glut2 and Glp1r. At the transition from prenatal to postnatal life, glucose becomes an important stimulus of beta cell proliferation (133) and similar to *Nkx6.1*$^{\Delta\beta}$ mice, Glut2-deficient mice exhibit reduced beta cell proliferation during the early postnatal period (140). Since Glp1 regulates beta cell proliferation independent of glucose (119), loss of Glut2 and Glp1r in *Nkx6.1*$^{\Delta\beta}$ mice likely have additive effects on beta cell proliferation. In addition to regulating nutrient sensors, we found that Nkx6.1 also regulates several markers associated with postnatal beta cell maturation (138). It is still largely unclear whether and how these genes affect beta cell maturation, but the regulation of several of these genes by Nkx6.1 suggests a role for Nkx6.1 in beta cell maturation. Collectively, our results demonstrate that Nkx6.1 controls multiple relevant pathways for postnatal beta cell development.

2.4 MATERIALS AND METHODS

2.4.1 Mice

RIP-Cre (137), *Nkx6.1*^{flox} (139), *Nkx6.1* null (141), and *R26-YFP* mice (142) have been described. *RIP-Cre;Nkx6.1*$^{flox/+}$*;R26-YFP* mice served as control mice in all experiments. All experiments were approved by the Institutional Animal Care and Use Committee of the University of California.

2.4.2 Tissue Preparation and Immunofluorescence

Methods for tissue preparation, immunofluorescence staining, and terminal deoxynucleotidyl transferase dUTP nicked end labeling (TUNEL) have been previously described (139). The following primary antibodies were used: guinea pig anti-insulin (Dako), 1:2000; mouse anti-Nkx6.1, (BCBC #2023), 1:500; rabbit anti-Glut2 (Millipore), 1:1000; rabbit anti-Glp1r (S. Heller, Novo Nordisk), 1:2000; rat anti-GFP (C. Kioussi, Oregon State University), 1:1000; rabbit anti-Ki67 (Lab Vision), 1:500; rabbit anti-Ucn3 (M. Huising, UC Davis), 1:500; rabbit anti-MafA (Bethyl), 1:200; rabbit anti-Pdx1 (Abcam), 1:500. Staining with antibodies raised in mice was performed using the M.O.M. Kit (Vector Labs). When necessary, nuclei were counterstained with DAPI (Sigma) at 0.1 µg/ml. Primary antibodies were detected with donkey-raised secondary antibodies conjugated to Cy3, Cy5, or Alexa 488 (Jackson ImmunoResearch). Beta cell mass and marker^{+} area were determined as described (139). Images were captured on a Zeiss Axio Observer Z1 microscope with an Apotome module and processed with Zeiss Axiovision 4.8 software. All images were processed in accordance with *Diabetes* journal guidelines.

2.4.3 qRT-PCR

The qRT-PCR analysis was performed as previously described (134) on total RNA isolated from postnatal day 2 pancreata from individual mice. Primers used are as follows: Nkx6.1 (f-CTTCTGGCCCGGAGTGATG; r-GGGTCTGGTGTGTTTTCTCTTC), Ucn3 (f-

GCTGTGCCCCTCGACCT; r-TGGGCATCAGCATCGCT), Adh1 (f-GCAAAGCTGCGGTGCTATG; r-TCACACAAGTCACCCCTTCTC), Angptl7 (f-TGACTGTTCTTCCCTGTACCA; r-CAAGGCCACTCTTACGTCTCT), Dlk1 (f-CCCAGGTGAGCTTCGAGT; r-GGAGAGGGGTACTCTTGTTGAG), Gstm2 (f-ACACCCGCATACAGTTGGC; r-TGCTTGCCCAGAAACTCAGAG), Zyx (f-TCCCACCGCAGGTATCATC; r-GGAGCTAGAAGGGGTCTTCCA), Gapdh (f-CATGTTCCAGTATGACTCCACTC; r-GGCCTCACCCCATTTGATGT).

2.4.4 Glucose Tolerance Tests

Glucose tolerance tests and blood glucose measurements were performed as described (134). For glucose tolerance tests, a 1.5 g/kg body weight intraperitoneal injection of glucose was administered after overnight fasting.

2.4.5 Statistics

All values are shown as mean ± standard error of the mean (SEM); p-values were calculated using a two-tailed student's t-test in Microsoft Excel. $P<0.05$ was considered significant.

2.5 ACKNOWLEDGEMENTS

Chapter 2, in full, is a reprint of material as it appears in Taylor, Brandon L; Benthuysen, Jacqueline R; Sander, M. "Postnatal β-cell proliferation and mass expansion is dependent on the transcription factor Nkx6.1." Diabetes. 2015 Mar;64(3):897-903. The dissertation author was co-first author of this paper. We thank S. Heller (Novo Nordisk) for anti-Glp1r, C. Kioussi (Oregon State University) for anti-GFP, and Mark Huising (UC Davis) for anti-Ucn3 antibody. We are grateful to N. Rosenblatt and F. Liu for technical assistance. This work was supported by the NIH/NIDDK grant R01-DK068471 to M.S. and the NIH training grant T32GM008666-15 to J.B. The authors have declared that no conflict of interest exists. M.S. is the guarantor of this work and, as such, has full access to all the data in the study and

takes responsibility for the integrity of the data and the accuracy of the data analysis. B.L.T. and J.B. designed and performed experiments, analyzed data and prepared figures. B.L.T., J.B., and M.S. wrote the manuscript. American Diabetes Association [Postnatal β-cell proliferation and mass expansion is dependent on the transcription factor Nkx6.1, [2015]. Copyright and all rights reserved. Material from this publication has been used with the permission of American Diabetes Association.

CHAPTER 3

Comparative Analysis of Age-Dependent Changes in the Islet Proteome and Transcriptome

3.1 INTRODUCTION

The regenerative capacity of beta cells declines rapidly with age (2; 3; 6). In humans, beta cells proliferate until age five with a peak proliferation index of 2% at one year of age (3; 4). This age-dependent decline in proliferative capacity is also observed in rodents when proliferation is nearly halted by ten weeks of age (6). Understanding the pathways that are regulated during this process will be key to regenerative therapies aimed at expanding endogenous beta cell mass.

Recent work by Avrahami et al. described changes in genome-wide methylation and RNA transcripts during beta cell aging. They found that several key cell cycle gene promoters become *de novo* methylated during aging and concordantly decrease in expression with age (13). This may provide insight into why aged beta cells replicate so poorly, and point to a need to target epigenetic regulators in conjunction with mitogenic stimuli. Surprisingly, they also found that beta cell function increases with age. This may be reflective of a juvenile maturation process from four-weeks-old to sixteen-months-old as compared to previous literature comparing 2-months-old to 24-months-old mice (18). Interestingly it has also been demonstrated that inducing beta cell senescence can improve beta cell function (20). This corroborates the inverse relationship between beta cell function and proliferation (13), suggesting that an increase in beta cell proliferation could in fact be detrimental to insulin secretion. It is still unclear, though, what causes the improvements in beta cell function during aging, and what changes occur for a beta cell to switch from a mitogenic to a secretory pathway and vice versa.

While comparative gene expression studies have provided some insight into the molecular changes associated with beta cell aging, many biologically relevant age-dependent pathways remain elusive. One underlying limitation of profiling gene expression is the assumption that changes in RNA abundance correlate with changes at the protein level. It is clear that significant regulation occurs post-transcriptionally to affect protein abundance,

including rate of translation and protein degradation. In fact, one study analyzed the relationships of transcripts, proteins, and clinically measured traits in 97 strains of mice, and found that only 15% of transcripts that correlated with traits exhibited corresponding protein-trait correlations (143). This highlights the need to study protein changes in order to obtain a more comprehensive understanding of biologically meaningful pathways.

Therefore, to gain further insight into the molecular changes that occur during beta cell aging we performed *in vivo* quantitative proteomics and RNA-sequencing analyses of four-week-old and 1-year-old pancreatic islets. We identified key signatures of regulated proteins, many of which were not found changed at the RNA level. While cell cycle and RNA splicing proteins decreased with age, proteins important to metabolism and secretion increased with age. Interestingly, we observed specific signatures functionally relevant to the beta cell changed only at the protein level. Together, our data suggests that between 4 weeks and 1 year of age, beta cells upregulate proteins involved in beta cell function correlating with the known increase in secretory capacity, while downregulating proteins involved in cell proliferation.

3.2 RESULTS

3.2.1 *In vivo* quantitative proteomics captures a diverse and comprehensive set of proteins in mouse islets.

To identify age-regulated proteins in pancreatic islets, we performed ^{15}N stable isotope labeling of amino acids in mammals (SILAM) coupled with Multidimensional Protein Identification Technology (MudPIT) mass spectrometry (144). We metabolically labeled C57BL/6 mice by administering chow containing ^{15}N-labeled amino acids for 10-11 weeks at the onset of weaning (3-weeks-old). Islets were then isolated and pooled from these SILAM mice, and total protein lysates were mixed 1:1 with protein from either 4-week-old or 1-year-old “light” ^{14}N-non-labeled islets (Figure 3.1A). Next, proteins were proteolytically digested,

loaded onto a two dimensional column, and analyzed by multidimensional liquid chromatography coupled to an electro-spray ionization tandem mass spectrometer. The mass spectrometer distinguished “heavy” ^{15}N-labeled proteins from “light” ^{14}N-non-labeled proteins and, through subsequent ratio-of-ratios analyses, measured relative protein abundance between young and old islets.

With this approach, we achieved ^{15}N enrichment of greater than 95.6% (Figure 3.1B). We identified 37,721 peptides (9058 proteins) in 4-week-old islets and 38,657 peptides (9000 proteins) in 1-year-old islets at a false discovery rate (FDR) <1%, and quantified 10,245 total proteins based on confidence measurements (see *Materials and Methods*). To identify statistically significant differentially expressed proteins, we generated ^{14}N-4-week-old/^{15}N-14-week-old or ^{14}N-1-year-old/^{15}N-14-week-old peptide ratios to calculate a final ^{14}N-1-year-old/^{14}N-4-week-old “ratio of ratios” (Figure 3.1A). We compared four young with four old biological islet replicates to calculate $\log_2$(old/young) and ANOVA p-values (Figure 3.1C). Consolidating isoforms, 551 uniquely named proteins were significantly enriched in 1-year-old islets and 393 proteins were significantly enriched in 4-week-old islets by at least 1.2-fold (Table A1). Gene Ontology analysis of the entire complement of identified proteins revealed a broad representation of proteins in all cellular compartments and association with diverse cellular functions and biological processes (Figure 3.1D,E). Therefore, this quantitative proteomics approach captures a comprehensive, unbiased, and diverse set of proteins.

Interestingly, among the proteins enriched in old islets, Urocortin 3 (Ucn3) was found significantly increased with age (Figure 3.1C). Ucn3 is a beta cell maturation marker that mediates control of insulin secretion (138; 145). The increase in Ucn3 protein expression from 4-weeks-old to 1-year-old is consistent with previous findings showing that as beta cells functionally mature from P1 to P15 in rodents, Ucn3 expression increases (138). Interestingly, two known regulators of beta cell proliferation, Glucokinase (Gck) and Adenosine kinase (Adk), were found to be age-dependently regulated (Figure 3.1C). Glucokinase activation has

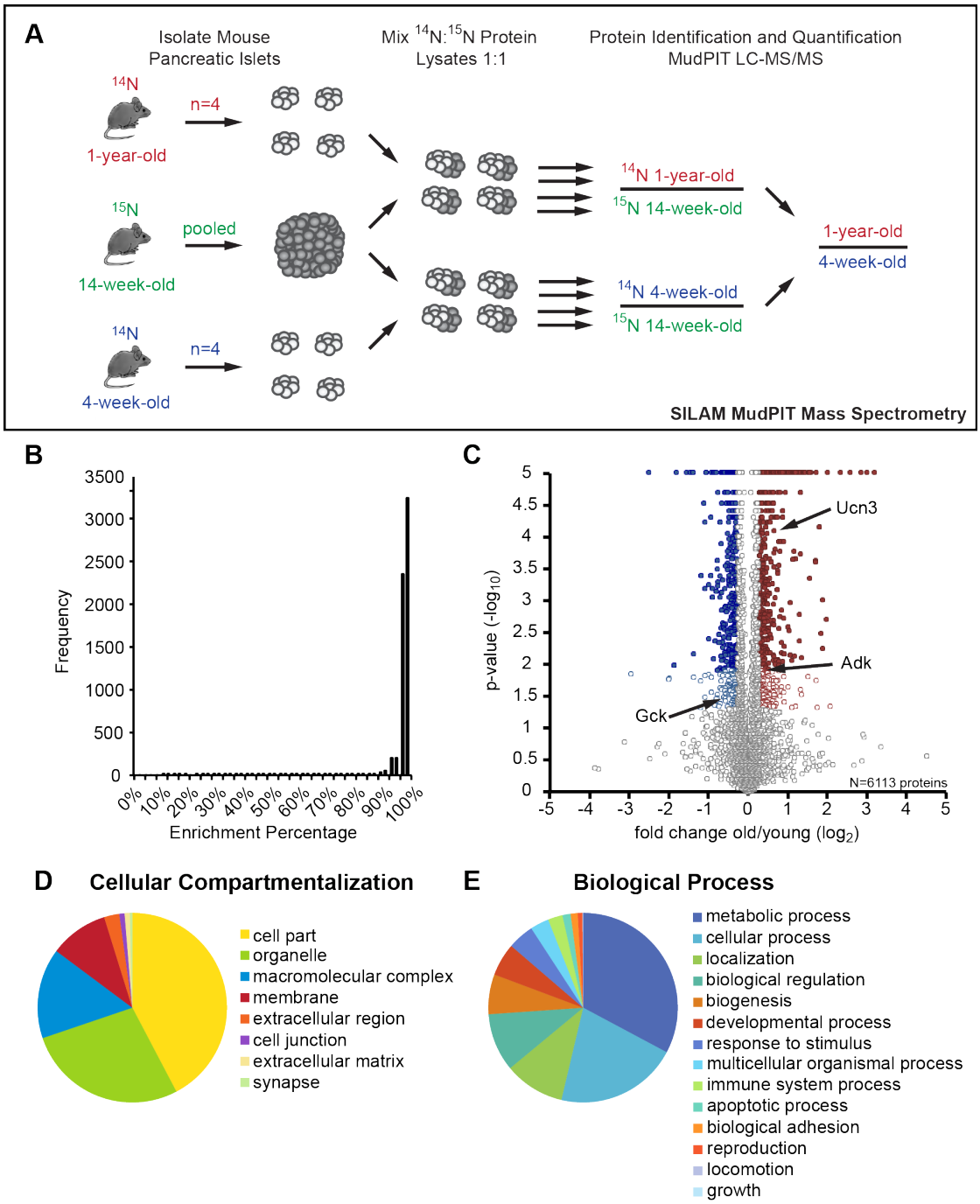

Figure 3.1 SILAM MudPIT Mass Spectrometry in young and aged pancreatic islets. Schematic showing the process for analyzing the islet proteome quantitatively in 4-week-old and 1-year-old mice (A). Greater than 95% enrichment of ^{15}N label was detected in the islet proteome (B). Volcano plot representing the age-dependent proteome; blue=proteins downregulated with age, red=proteins upregulated with age; arrows point to select proteins; n=4 (C). GO analysis of all quantified proteins reveals identification of diverse proteins from different cellular components (D) and association with a variety of biological processes (E).

been shown to promote beta cell proliferation (146) and, consistent with this role, was found lower in abundance in aged islets. Conversely, adenosine kinase inhibition has been shown to increase beta cell proliferation (80; 84) and accordingly was found to increase in abundance with age. These findings demonstrate the ability to uncover beta cell proliferation regulators with this novel quantitative proteomics approach.

3.2.2 Gene Ontology and network analyses reveal proteome dynamics during beta cell aging.

To identify biological processes that are regulated during beta cell aging, we next performed Gene Ontology (GO) and network analyses of the differentially expressed proteins. Proteins that decreased in abundance during beta cell aging were functionally associated with expansion of cell numbers, including proteins involved in cell cycle regulation, RNA splicing, and epigenetic regulation of gene expression (Figure 3.2A,C). This was consistent with the known decline in beta cell replication with age (2; 6). Conversely, proteins showing increased levels with age have roles in cell metabolism and secretion, including proteins involved in glycolysis, oxidative phosphorylation, and positive regulation of secretion (Figure 3.2B,D). This suggested that the previously observed increase in beta cell function with age might be attributed to an upregulation of secretory and respiratory machinery, including components of the mitochondrial respiratory chain.

3.2.3 RNA-sequencing reveals poor correlation of associated mRNAs with proteins that change during beta cell aging.

Recent work characterizing the age-associated methylation changes that occur during beta cell aging suggested that chromatin state is important for regulating age-dependent transcriptional changes that occur in the beta cell (13). However, it is unknown whether the changes in RNA with age are reflected at the protein level. In order to determine how well the

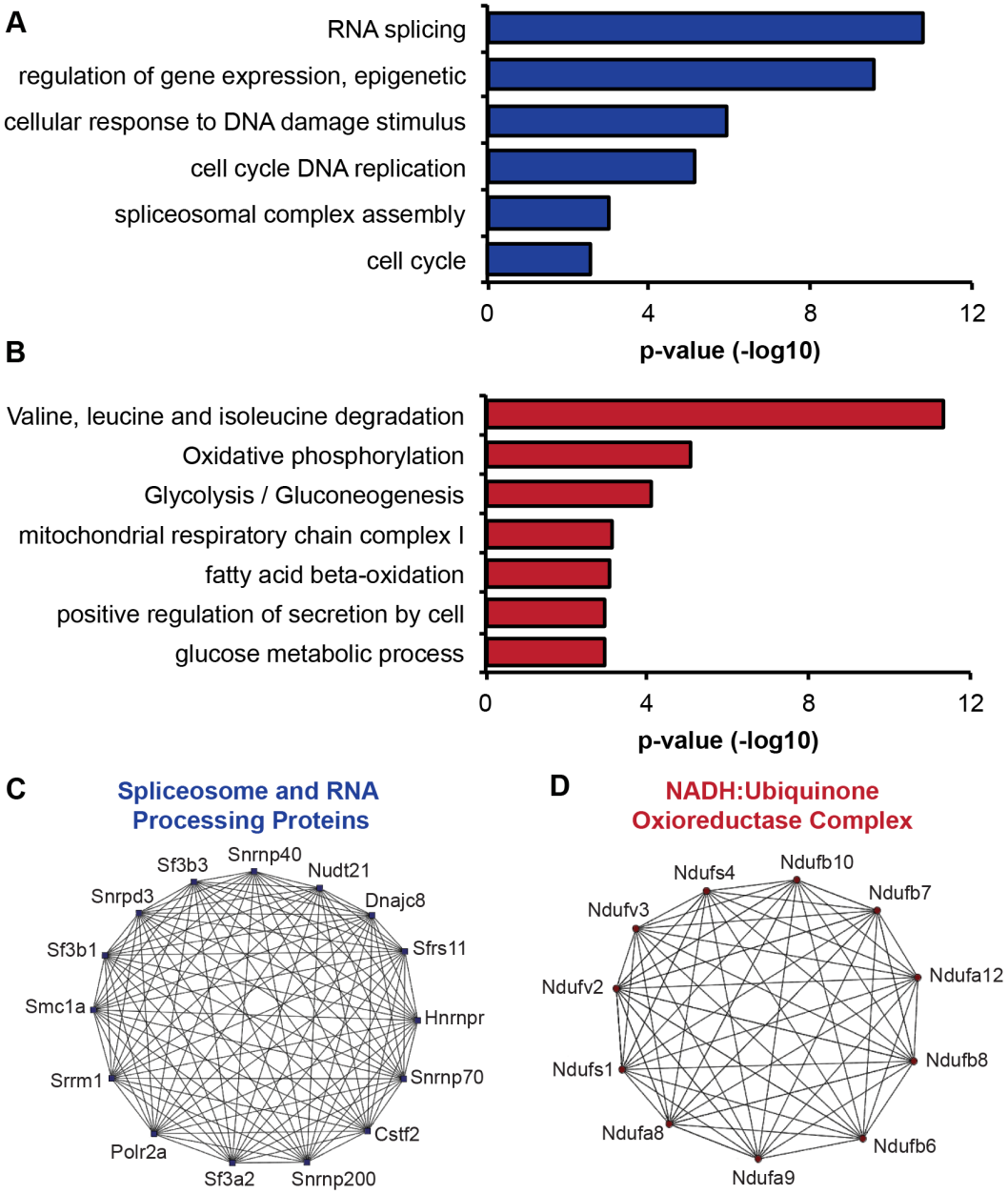

Figure 3.2 Gene Ontology and network analysis of the age-dependent islet proteome. GO analysis of proteins that decreased in blue (A), and increased in abundance with age in red (B). Network analysis shows complexes of proteins that decreased with age in blue (C) and increased with age in red (D).

age-regulated proteome correlates with the age-regulated transcriptome, we sequenced mRNA isolated from pancreatic islets of 4-week-old and 1- year-old mice. We identified 1349 genes significantly increased in expression with age and 1545 genes significantly decreased in expression with age (1.2 fold change, $p \leq 0.05$). Consistent with the changes observed at the protein level, we found *Ucn3* significantly regulated at the mRNA level (Figure 3.3A). Interestingly, we did not find *Gck* or *Adk* changed at the mRNA level. We next analyzed the correlation between protein and mRNA changes with age and found only a modest correlation (ρ=0.4, $p=2.2x10^{-16}$, Figure 3.3B). In fact, of the proteins that were changed with age, only 11.6% were significantly regulated at the mRNA level (Figure 3.3C). Interestingly, the proteins not regulated at the mRNA level were enriched in GO categories oxidative phosphorylation, fatty acid beta-oxidation, lysosome, and spliceosome. Gene set enrichment analysis (GSEA) demonstrated that these functional categories were unique to proteins that were not regulated at the mRNA level, as proteins also regulated at the mRNA level were grouped in categories such as post-translational modification and biological oxidation (Figure 3.4). This suggests that key age-dependent signatures are dynamically regulated only at the protein level and would not be otherwise identified had only mRNA been profiled. Therefore it is important to consider protein regulation in making biologically meaningful conclusions about a given paradigm.

3.3 DISCUSSION

It is becoming increasingly clear that young beta cells with higher proliferative capacity switch to a senescent, more functional state with age. This process is thought to be triggered in rodents by a metabolic shift during weaning from high-fat milk to a high-carbohydrate chow diet (39). While transcriptional and methylation analyses have provided some insight into the regulatory mechanisms that underlie beta cell maturation with age (13), our study is the first comprehensive analysis of the *in vivo* islet proteome during islet aging. With this dataset,

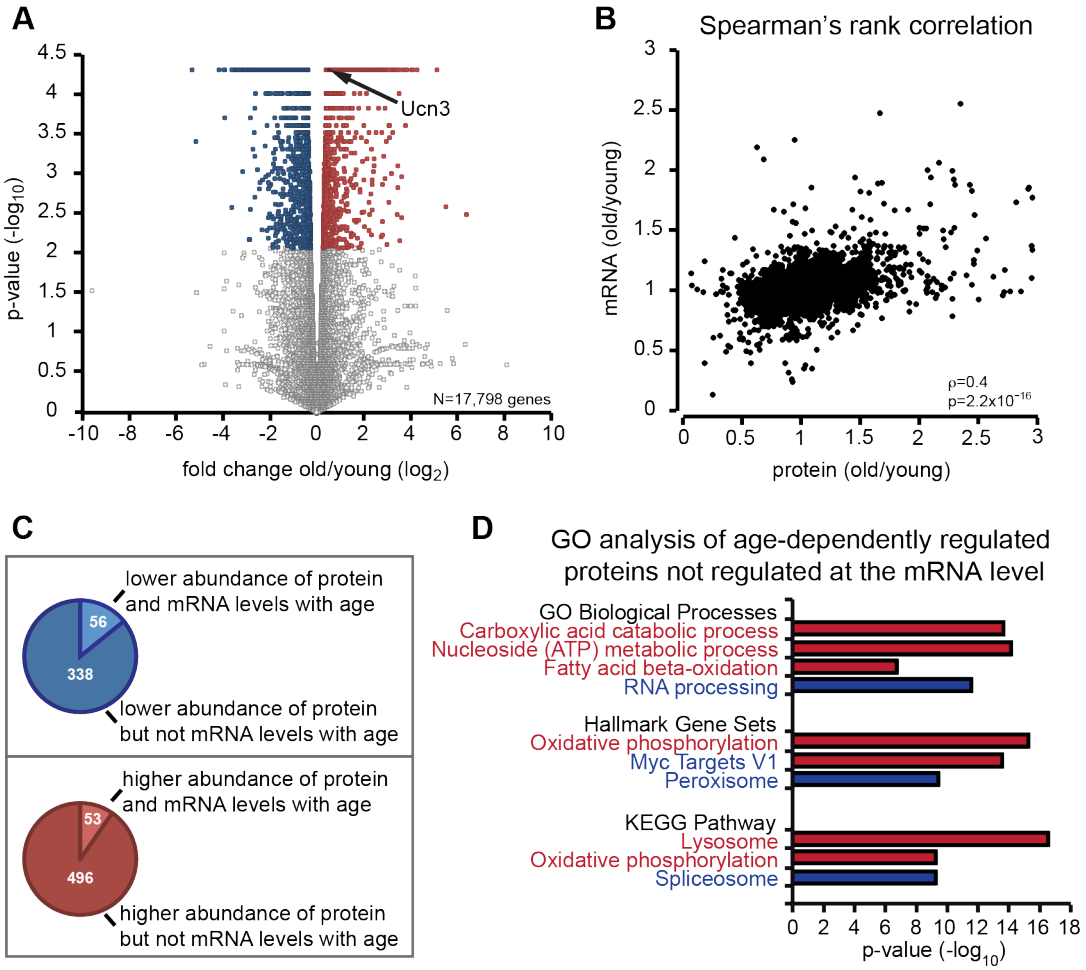

Figure 3.3 Quantitative proteomics comparison with RNA-sequencing of the pancreatic islet during aging. Summary volcano plot of mRNA from 3 biological replicates of mouse pancreatic islets by RNA-seq; x axis = log2 fold change (old/young), y axis = -log10 p-value, N = 17,798 total genes represented. 2896 (16.3%) regulated mRNAs (p-value < 0.05 and > 1.2 fold change; blue open circles=down with age, red open circles=up with age). Arrow indicates data point for *Ucn3* (A). Spearman's rank correlation between mRNA and proteins from young and old mouse islet samples shows a correlation of ρ=0.4, $p=2.2x10^{-16}$ (B). Pie graphs representing the number of unique proteins found differentially expressed during islet aging with and without coordinate mRNA regulation (C). Unique molecular signatures from GO analysis of proteins only changed at the protein level with age; blue=down with age, red=up with age (D).

we identified key functionally linked groups of proteins that change during aging. The decline in proliferative capacity corresponds with the downregulation of proteins important for cell proliferation and also includes proteins involved in the regulation of gene expression, such as RNA splicing and chromatin remodeling factors. The decline in proteins regulating gene expression could suggest altered or aberrant regulation of transcripts and chromatin accessibility during islet aging. Consistent with this, Avrahami et al. observed changes in chromatin accessibility in their genome-wide methylation analysis of islet aging (13). However, it would be interesting to analyze how splicing is altered during this timeframe and determine what effects this may have on the beta cell.

Our results also show that beta cell function proteins are upregulated during aging, including those critical for secretion and mitochondrial respiration, which is consistent with previous findings that show insulin secretion in beta cells increases with age (13; 19; 20). This enhanced beta cell function with age is partly due to senescence itself, as the overexpression of senescence-inducing factor p16 in beta cells increases respiratory capacity *in vivo* (20). Whether beta cells during normal aging have increased respiratory capacity still remains to be functionally tested. Furthermore, it remains unknown exactly how the beta cell maturation process is triggered and whether there are factors that can be targeted to selectively increase beta cell proliferation without negatively affecting beta cell function. These factors would be ideal targets for regenerative strategies aimed at expanding endogenous beta cell mass in diabetic patients. Further work to characterize potential targets from our dataset will be necessary to identify beta cell proliferation regulators that can be drug targets for beta cell mass expansion.

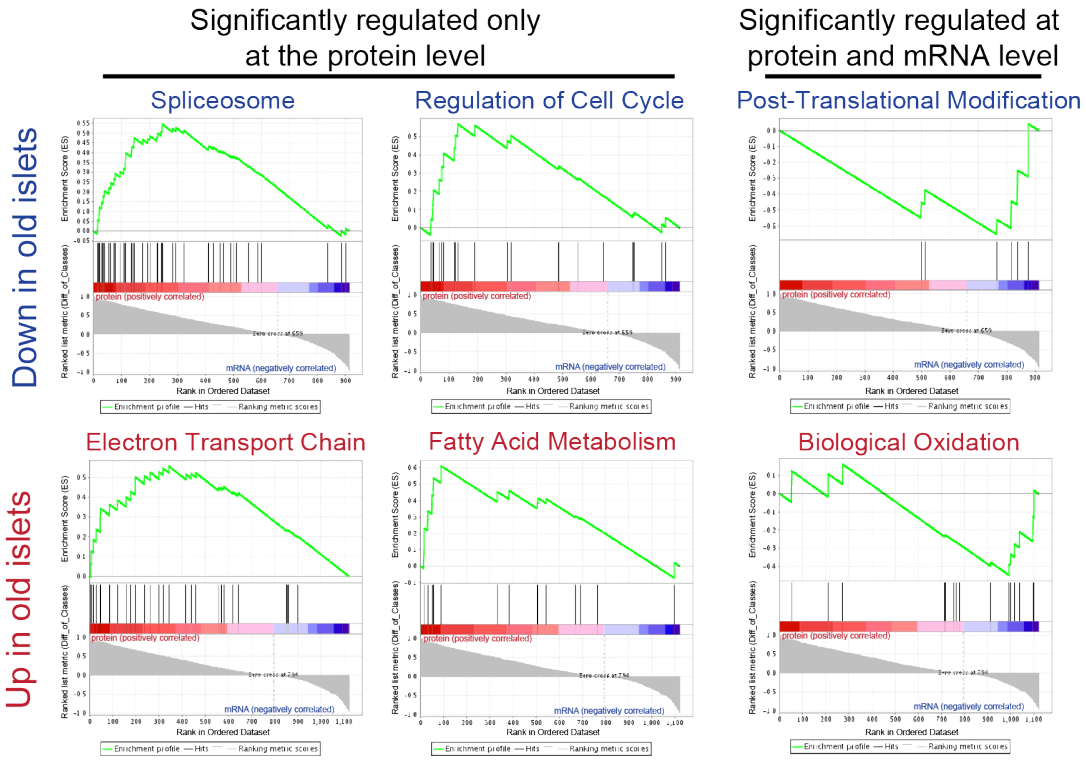

Figure 3.4 GSEA analysis of differentially expressed proteins and genes. Functional signatures of proteins that changed only at the protein level were proteins involved in spliceosome, mitotic cell cycle (down with age) and electron transport, fatty acid metabolism (up with age). Proteins that were also changed at the mRNA level are proteins involved in post-translational modification (down with age) and biological oxidation (up with age).

3.4 MATERIALS AND METHODS

3.4.1 Mice and tissue preparation

Cohorts of mice utilized for this study comprised equal numbers of female and male C57BL/6N mice. 56 mice were weaned at 3 weeks of age and fed a ^{15}N-rich, Spirulina-based diet (Cambridge Isotopes) for 10-11 weeks. Islets were isolated and pooled from these SILAM mice using a standard islet isolation protocol as previously described (136). Briefly, Liberase TL (Roche) was perfused into pancreata at a working concentration of 0.655 units/mL through the common hepatic bile duct. Pancreata were then removed and dissociated at 37^{o}C for roughly 15 minutes (dissociation time depends on age and size of pancreas). Islets were separated onto a gradient composed of HBSS (Cellgro) and Histopaque (Sigma) layers. Purified islets were then handpicked under a dissection microscope to minimize acinar cell contamination. Islets were also isolated from 62 4-week-old and 38 1-year-old ^{14}N non-labeled mice and pooled to produce 4 biological replicate samples per age group. Islets were lysed in a buffer containing 10 mM Tris-HCl pH8, 10 mM NaCl, 3 mM $MgCl_2$, 1% NP-40, 1% SDS, 0.5% Sodium Deoxycholate with 1X protease inhibitor cocktail (Roche) and 1 mM PMSF. 40 µg of ^{15}N protein was mixed with 40 µg of ^{14}N protein for 4 young biological replicates and 3 old biological replicate samples. A fourth old biological replicate sample was composed of 20 µg of ^{15}N and 20 µg of ^{14}N protein. All animal experiments were approved by the Institutional Animal Care and Use Committees of the University of California, San Diego.

3.4.2 MudPIT and LTQ Velos Orbitrap mass spectrometry

MudPIT mass spectrometry was performed as previously described (147).

3.4.3 Analysis of tandem mass spectra

Analysis of tandem mass spectra was performed as previously described (147). For ANOVA P-values to be calculated, each protein had to be quantified in at least two biological replicates. To show how many measurements our quantitative peptide ratios were drawn from,

we have provided peptide counts for each protein listed in Table A1 (see the columns headed "Total # of $^{14}N/^{15}N$ pairs").

3.4.4 Network Analysis

A network visualizer was used from open source Medusa viewer (148), which accesses protein interaction data from STRING (149). Differentially expressed proteins were color-coded as blue for downregulated with age and red for upregulated with age. Connections were filtered for type of interaction including co-expression, co-occurrence, experimental, database, text mining, gene fusion, and neighborhood, and the strength of the interaction was filtered by confidence score. Protein nodes were manually moved into clusters based on connectivity to identify protein complexes and network formation.

3.4.5 Gene Ontology analysis

Gene Ontology analysis was performed using the Panther Classification System (150) for the analysis of the entire identified proteome and using Metascape (151) for the analysis of the significantly changed proteins during islet aging.

3.4.6 RNA-sequencing

Isolated islets were lysed in RLT Buffer and total RNA was extracted using the RNeasy Micro Kit (Qiagen) per manufacturer's instructions. TruSeq stranded mRNA libraries were prepared by the UCSD Institute for Genomic Medicine Genomics Center and sequenced using HiSeq2500 Highoutput Run V4 (Illumina). The single-end 50 base pair reads were mapped to the UCSC mouse transcriptome (mm9) by STAR, allowing for up to 10 mismatches. Only the reads aligned uniquely to one genomic location were retained for subsequent analysis. Expression levels of all genes were estimated by Cufflink using only the reads with exact matches.

3.4.7 Spearman's rank correlation and GSEA analysis

Computational analyses were performed based on custom scripts developed using the programming languages Python and R, with several Bioconductor packages (152). In order to compare differential regulation observed in mRNA and proteins, fold changes (FC) representing differences of old compared to young islet samples were considered for each gene gi, with i ranging from one to the number of genes with encoded proteins found in the proteomic dataset. The two sets of fold changes available SFP=(FCiP) and SFR=(FCiR), accounting for expression changes at the level of proteins and RNA, respectively, were subsequently scored for global concordance by computing their Spearman rank correlation.

A second comparison of the two data sets aimed at going beyond the perspective of single genes and focused on gene sets, i.e. groups of genes participating in known biological pathways. A similar approach to the one described above was adopted to collect measures of the differential changes in all genes found in both the data sets, this time by taking into account p-values and performing a separate analysis for up and downregulated genes. Considering only genes upregulated in old compared to young islets, two quantitative signatures SPP-UP=(pkP) and SPR-UP=(pkR) were obtained, with pk representing the significance (p-value) of the k-th upregulated gene and the two sets SPP-UP and SPR-UP accounting for changes at the level of proteins and RNA, respectively. Genes in the signatures were first mapped to Human Ensembl gene identifiers using Biomart (153) and subsequently analyzed with Gene Set Enrichment Analysys (GSEA) (154). In detail, the first step of GSEA analysis allowed ordering all upregulated genes according to the differences of their p-values in SPP-UP and SPP-UP, thus obtaining a ranked list with the top-ranked elements being genes for which a more significant change (p-value) in proteins, compared to RNA, was observed. Finally, for each canonical pathway from collection C2 of the MSigDB database (155), the Enrichment Score (ES) and corresponding Enrichment p-values were computed with GSEA to identify pathways for which gene members were found at the top (positive ES) or at the bottom (negative ES) of the ranked list. Pathways ($p < 0.01$) enriched for upregulated

genes with more significant changes in proteins (positive ES) or in RNA (negative ES), were considered for further analysis and interpretation. Analogous signatures SPP-DOWN and SPR-DOWN were derived for downregulated genes and the same approach described above was used to compare their changes in proteins and RNA.

3.5 ACKNOWLEDGEMENTS

Chapter 3 includes material that is currently being prepared for submission as a manuscript. Benthuysen, Jacqueline R; Savas, Jeffrey N; Mulas, Francesca; Wortham, M; Divakaruni, A; Taylor, B.L.; Murphy, A; Yates, JR 3rd; Sander, M. (in preparation) “Comparative Analysis of Age-Dependent Changes in the Islet Proteome and Transcriptome.” The dissertation author was the primary investigator and author of this study. The authors would like to thank D. Calzolari for assistance with Medusa programming, A. Carrano for critical reading and feedback, Y. Sui for processing RNA-sequencing data, F. Liu for islet isolations, and N. Rosenblatt for assistance in the mouse facility. RNA-sequencing was conducted at the IGM Genomics Center, University of California, San Diego, La Jolla, CA. J.B. designed and performed the experiments. J.N.S. with J.R.Y.III performed the mass spectrometry. J.B. analyzed the data and prepared figures. F.M. performed the Spearman’s rank correlation and GSEA analysis. B.L.T. performed a pilot mass spectrometry analysis. J.B., M.W., A.D., with A.M. performed and analyzed mitochondrial experiments, data not shown in thesis. J.B. and M.S. wrote the manuscript.

CHAPTER 4

Sirtuin2 is a novel regulator of human beta cell proliferation

4.1 INTRODUCTION

Diabetes is a chronic condition characterized by the loss or dysfunction of insulin-producing pancreatic beta cells. It is well established that loss of beta cell numbers or mass causes Type 1 diabetes, although residual beta cells remain even years after diagnosis in patients with Type 1 diabetes (156). In Type 2 diabetes, beta cell loss is preceded by an initial phase of beta cell compensation by hyper-proliferation and hyper-secretion ,(46). While islet transplantation has been proven as a successful therapy, there is a lack of sufficient donor cadaveric islets, which has prevented more widespread use of islet transplantation. Therefore, there have been intense efforts to find alternative approaches to regenerate beta cells in order to increase beta cell mass. Because beta cell mass expansion is predominantly driven by beta cell replication (129; 157), one method to regenerate beta cells could be to target factors that stimulate beta cell proliferation *in vivo*. Human beta cells replicate very slowly, with a peak replication index of 2% in the first year of life dropping to less than 0.1% by about age 5 (3; 4). While various growth factors, hormones, and small molecules have been shown to stimulate beta cell replication in rodents (98-100), very few factors have been found to stimulate human beta cells to enter the cell cycle. For example, lactogen is a hormone that induces beta cell replication during pregnancy in rodents but cannot do so in humans (29). Other pathways, however, are conserved such as stimulation by glucose, which can induce beta cell proliferation in both rodents and humans through the mitogenic-activated protein kinase (MAPK) pathway (47; 53; 103). Glucose has pro-proliferative functions during short-term administration, but has been shown to cause beta cell destruction, DNA damage and apoptosis during long-term exposure (55). Thus, in order to be therapeutically beneficial, molecules must be identified that can stimulate mitogenic pathway(s) without causing long-term damage to the beta cell.

Additionally, it has been recognized that some factors can stimulate beta cell proliferation only in young animals but not in aged animals (8). Because aged beta cells have

lower replication rates in both rodents and humans (2; 3), we performed an *in vivo* quantitative proteomics analysis during beta cell aging to identify novel regulators of beta cell replication (Chapter 3). From this analysis, we found two Sirtuin proteins, Sirtuin 2 (Sirt2) and Sirtuin 5 (Sirt5), to increase in abundance during beta cell aging. Sirtuins are a class of deacetylases that use nicotinamide adenine dinucleotide (NAD+) as a co-factor, and have been implicated in various cellular processes involved in aging, metabolism, and disease (158). Sirt1 and Sirt2 have been found to be upregulated in late-passage senescent human fibroblasts (159), and resveratrol, a sirtuin activator, has been shown to induce senescence in primary human fibroblasts (160). This implies that sirtuins may be responsible for inducing replicative senescence. Sirt2 is of particular interest because it has previously been shown to act as a tumor suppressor protein, as aged *Sirt2* null mutant mice develop spontaneous tumors (161). And of the seven Sirtuin-family proteins, which are primarily expressed in the mitochondria or nucleus, Sirt2 is the only primarily cytoplasmically localized sirtuin, which makes it an attractive drug target (162). Interestingly, Sirt2 has been previously shown to de-acetylate and inhibit Mek, one of the kinases that activates the MAPK cascade (163; 164). This suggests that the observed increase in Sirt2 abundance during beta cell aging, could contribute to the age-dependent decline in beta cell proliferation.

Therefore, to explore the role of Sirt2 in pancreatic beta cell proliferation, we pharmacologically inhibited Sirt2 activity in mouse and human islets and found increased beta cell proliferation. Furthermore, we genetically inactivated *Sirt2* in adult mouse beta cells and found an increase in beta cell proliferation and mass in mice rendered diabetic with streptozotocin (STZ). Finally, we show that Sirt2 inhibition or deletion does not have an effect on beta cell function and appears to only stimulate beta cell proliferation in conditions of elevated glucose levels. Mechanistically, we show that Sirt2 inhibition modulates Mek activity after glucose stimulation of the MAPK mitogenic pathway. Together, these results suggest that Sirt2 is a promising candidate for expanding beta cell mass in diabetic patients.

4.2 RESULTS

4.2.1 Sirtuin2 is expressed in pancreatic beta cells and controls beta cell proliferation.

To determine whether Sirt2 expression is specific to beta cells, we first queried our previously published RNA sequencing dataset of *in vitro* beta cell differentiation from human embryonic stem cells (165). Of all seven Sirtuin mRNAs, *Sirt2* is found most highly expressed in hESC-derived polyhormonal endocrine cells (PH), functional endocrine cells after *in vivo* transplantation (FE), and human islets (HI) (Figure 4.1A). This suggests that Sirt2 is a human endocrine-specific protein. We next performed co-immunofluorescence staining for Sirt2 protein together with islet hormones on sections of mouse pancreas and human islets and found Sirt2 to be expressed in the cytoplasm of both beta and alpha cells in mice, and beta cells in humans (Figure 4.1B).

To validate the finding from our quantitative proteomics analysis that Sirt2 protein levels increase with age in pancreatic islets, we performed a western blot analysis for Sirt2 and Beta-Tubulin. We verified that Sirt2 is increased at the protein level in islets from one-year-old mice as compared to islets from four-week-old mice, although mRNA transcript remains unchanged (Figure 4.1C-E). Because Sirt2 has been previously shown to act as a tumor suppressor protein (161), we hypothesized that its upregulation during aging may play a role in the age-dependent decline of beta cell proliferation.

To test whether Sirt2 controls beta cell proliferation, we inhibited its activity with a Sirt2-specific inhibitor AGK2 (166). After a two-day treatment of 9 to 12 month-old mouse islets, we found increased incorporation of thymidine analog 5–ethynyl–2–deoxyuridine (EdU) in insulin+ beta cells as compared to vehicle dimethyl sulfoxide (DMSO) treated beta cells (Figure 4.2A). Furthermore, we saw increased EdU incorporation and S-phase marker Ki67 in human beta cells treated with AGK2, suggesting that Sirt2 is a suppressor of beta cell proliferation (Figure 4.2B,C). To validate these findings, we treated human islets with two other Sirt2-specific inhibitors, SirReal2 and AK-1 (167; 168), and found a similar increase in

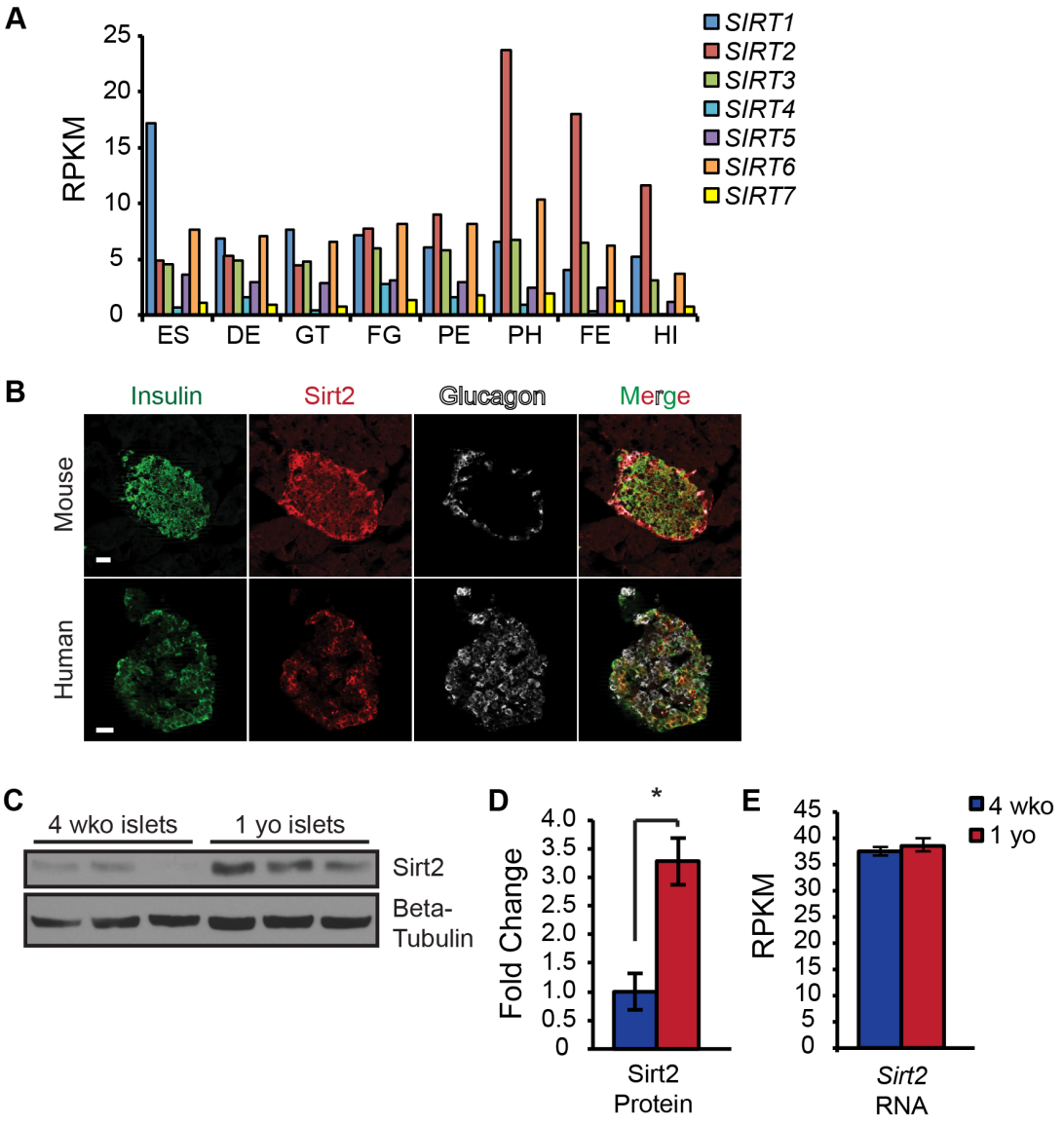

Figure 4.1 Sirt2 is a pancreatic endocrine-specific protein. *Sirtuin* mRNA expression (RPKM) of lineage intermediates during stepwise pancreatic differentiation of hESCs. ES=Embryonic stem cell, DE=Definitive endoderm, GT=Primitive gut tube, FG=Posterior foregut, PE=Pancreatic endoderm, PH=Polyhormonal cells, FE=Functional endocrine cells, HI=Human islet (A). Sirt2 protein expression in mouse pancreas (upper panel) and human islets (lower panel) co-localizes with insulin and glucagon expression (B). Western blot analysis shows Sirt2 protein expression in three biological replicates of 4-week-old and 1-year-old mouse islets (C), quantified by densitometry (n=3; D). *Sirt2* mRNA expression (RPKM) in 4-week-old and 1-year-old mouse islets as determined by RNA-sequencing (n=3, E). Scale bars=20 μm, error bars= standard error of the mean, * $p<0.05$.

beta cell proliferation (Figure 4.2D). While we did not detect significant increases in EdU incorporation in acinar cells after AGK2 treatment (Figure 4.2E), we did find an increase in EdU incorporation of alpha cells, albeit to a lesser extent (Figure 4.2F). This suggests that Sirt2 can suppress proliferation of beta cells, and, to a smaller degree, alpha cells.

4.2.2 Sirtuin2 inhibition does not impair beta cell function or cause cell death.

Previous studies have suggested that stimulating beta cell proliferation may result in DNA damage and cell cycle arrest or apoptosis (169). To test whether Sirt2 inhibition initiates a DNA damage response, we stained for the DNA damage marker, γH2AX, and quantified beta cells with γH2AX foci after Sirt2 inhibition in both mouse and human beta cells. We were not able to detect significant differences between DMSO- and AGK2-treated beta cells in DNA damage response (Figure 4.3A,B). Next, to determine whether Sirt2 inhibition causes beta cell apoptosis, we performed terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining and quantified TUNEL+ Nkx6.1+ beta cells. This revealed no difference in the number of human beta cells undergoing apoptosis *ex vivo* (Figure 4.3C), which suggests Sirt2 inhibition does not induce beta cell death.

To determine if AGK2 treatment affects beta cell function, we measured glucose stimulated insulin secretion (GSIS) and insulin content in human islets after a two day treatment with AGK2 and found that compared to DMSO-treated islets, Sirt2 inhibition has no negative effects beta cell function *ex vivo* (Figure 4.3D,E). To further corroborate this, we performed RNA-sequencing of human islets treated with AGK2 or DMSO for two days and compared global mRNA expression changes. Relative mRNA expression of critical beta cell function genes remained unchanged (Figure 4.3F). This was further supported by our immunohistochemistry analysis showing strong NKX6-1 and MAFA protein expression in both DMSO and AGK2-treated human islets (Figure 4.3G). Taken together, these findings indicate that Sirt2 inhibition does not impair beta cell function or cause beta cell death.

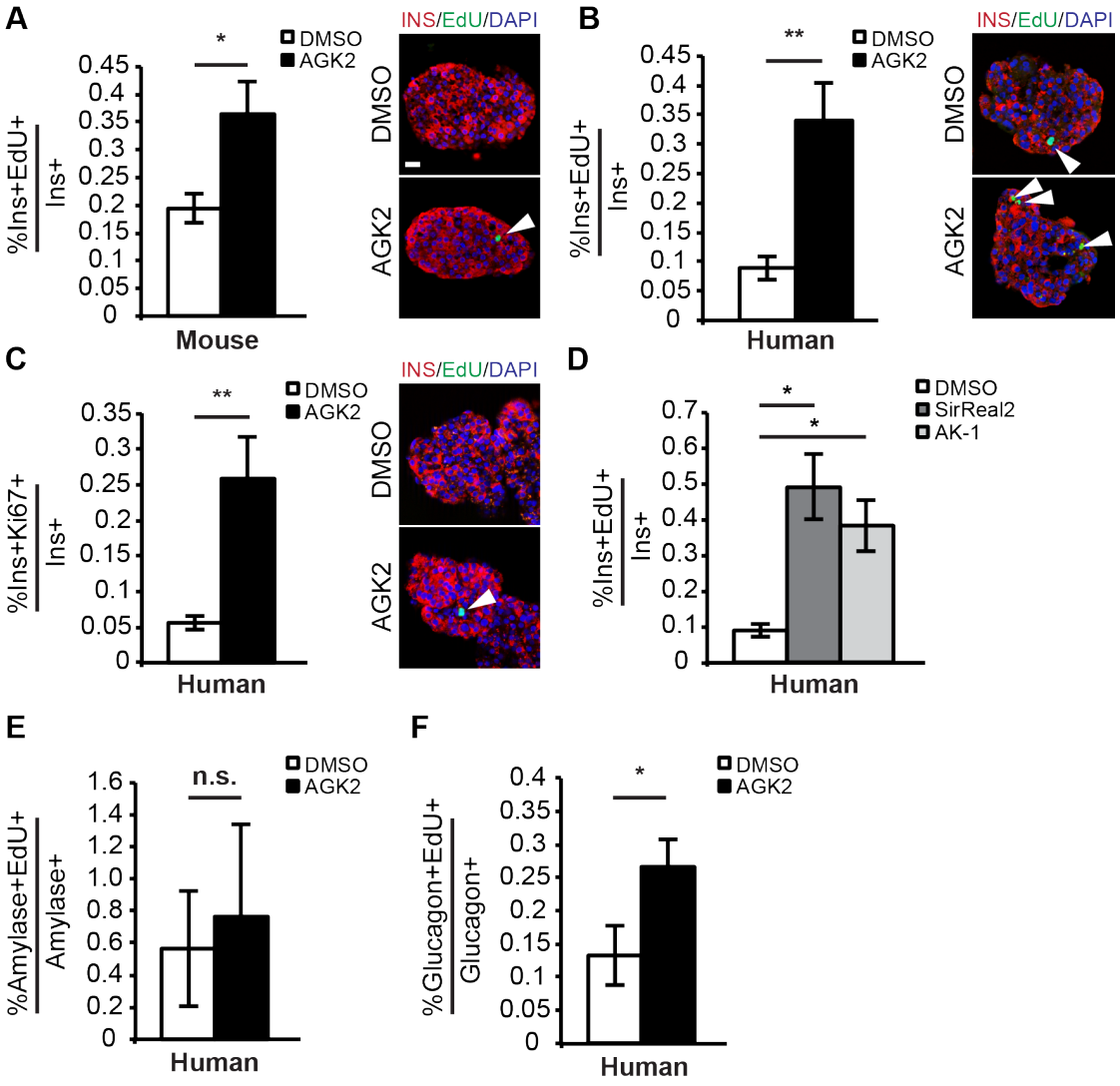

Figure 4.2 Sirt2 controls pancreatic beta cell proliferation. EdU incorporation in insulin+ beta cells from 9-12 month old mouse islets (n=4; A) and adult human islets (n=15; B) after treatment with AGK2 or vehicle control, DMSO. Quantification and representative images of Ki67+ insulin+ beta cells in adult human islets (n=6; C) after treatment with AGK2 or DMSO. Quantification of EdU incorporation in insulin+ beta cells from adult human islets after treatment with SirReal2, AK-1, or DMSO (n=3-6; D). Quantification of EdU incorporation in amylase+ acinar cells (n=7; E) or glucagon+ alpha cells (n=8, F) in adult human islets after treatment with AGK2 or DMSO. White arrowheads point to insulin+EdU+ cells, scale bar=20 μm, error bars= standard error of the mean, * $p<0.05$, ** $p<0.01$, n.s.=not significant.

4.2.3 Sirtuin2 inhibition requires elevated glucose levels for beta cell proliferation effects.

Next to test whether Sirt2 controls beta cell proliferation *in vivo*, we generated beta cell-specific *Sirt2* deleted mice (MIPCreER; Sirt2f/f), subsequently referred to as $Sirt2^{\Delta\beta}$ (170; 171). One week after tamoxifen administration, we observed diminished levels of Sirt2 transcript and protein in heterozygous $Sirt2^{\Delta\beta/+}$ (MIPCreER; Sirt2f/+) mice and near total loss of Sirt2 mRNA and protein in $Sirt2^{\Delta\beta}$ (MIPCreER; Sirt2f/f) mice as compared to control (MIPCreER; Sirt2+/+) mice (Figure 4.4A-C). We monitored these mice for thirty days and found no change in fed *ad libitum* blood glucose levels (Figure 4.4A,D). Additionally, an intraperitoneal glucose tolerance test (IPGTT) showed no difference between control and $Sirt2^{\Delta\beta}$ mice (Figure 4.4E). This demonstrates that beta cell-specific *Sirt2* deletion *in vivo* does not affect beta cell function, similar to findings with Sirt2 inhibition of human islets *ex vivo*.

To determine whether $Sirt2^{\Delta\beta}$ mice have increased beta cell proliferation, we quantified the number of Ki67+ beta cells and found no difference between control and $Sirt2^{\Delta\beta}$ mice (Figure 4.5A,B). Interestingly, after isolation of the islets from mice and two days of culture in 8 milliMolar (mM) glucose, $Sirt2^{\Delta\beta}$ beta cells exhibited increased EdU incorporation compared to controls (Figure 4.5C,D). The 8mM glucose concentration used in standard islet culture medium is elevated compared to the 5.5 mM glucose concentration observed in euglycemic mice. To determine whether glucose levels account for the different effect of Sirt2 inhibition on beta cell proliferation observed *in vitro* and *in vivo*, we cultured control and $Sirt2^{\Delta\beta}$ islets in 5.5 mM glucose. Consistent with the *in vivo* findings, we only observed a minor pro-proliferative effect of *Sirt2* deletion in 5.5 mM glucose (Figure 4.5C,D). This suggests that elevated glucose levels potentiate the ability to stimulate beta cell proliferation in Sirt2-deleted beta cells and may, in part, explain why Sirt2-deleted beta cells are not observed to proliferate *in vivo*.

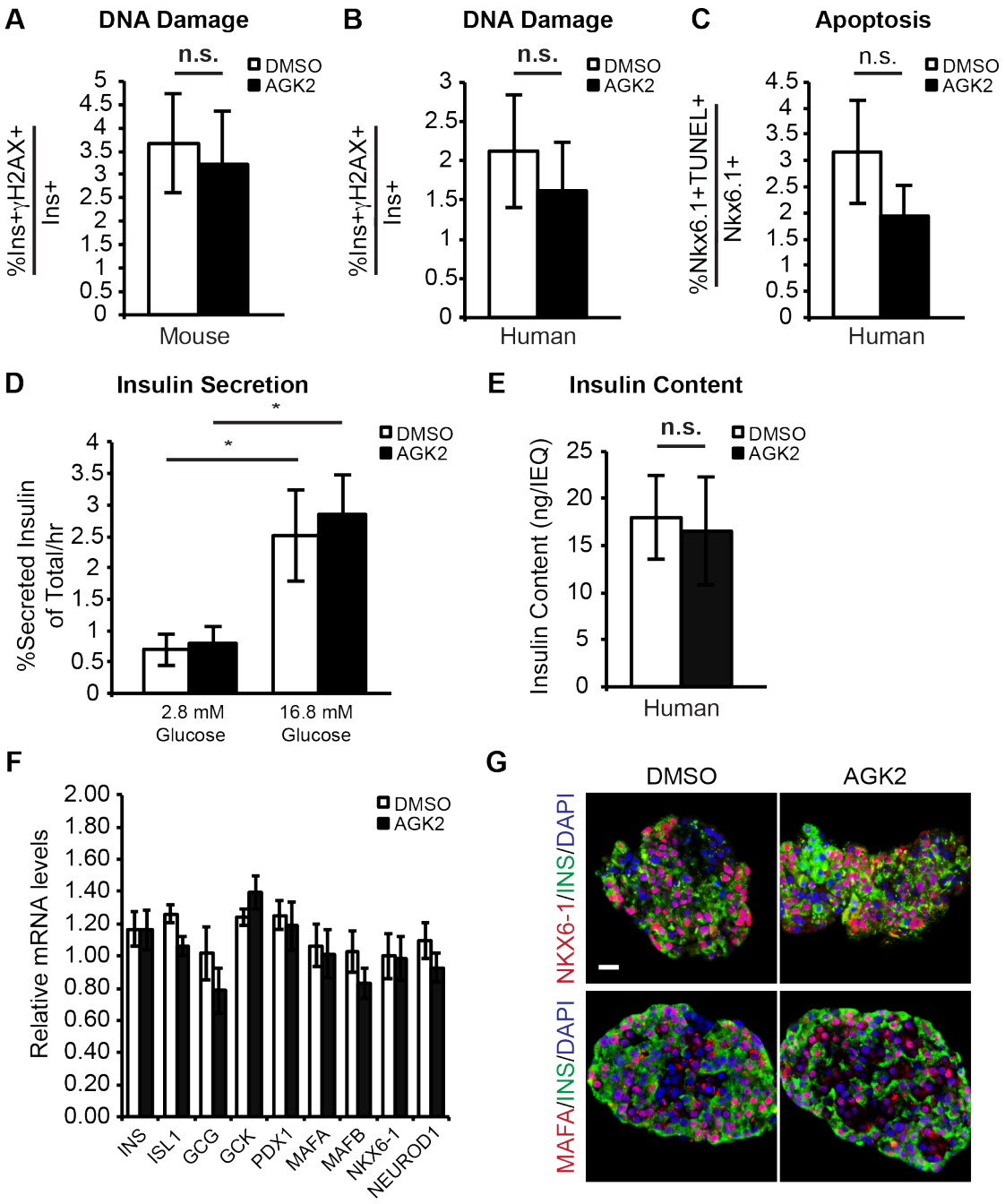

Figure 4.3 Sirt2 does not cause beta cell death or impair beta cell function. Quantification of insulin+ beta cells with γH2AX foci in adult mouse (n=4; A) and human (n=8; B) islets after AGK2 or DMSO treatment. Quantification of Nkx6.1+ beta cells that are co-localized with TUNEL signal (n=4; C) after AGK2 or DMSO treatment in human islets. Glucose stimulated insulin secretion (n=8; D) and insulin content (n=4; E) of human islets after AGK2 or DMSO treatment. Relative mRNA levels of beta cell identity genes after treatment of human islets with AGK2 or DMSO (n=3; F). Immunofluorescence for beta cell markers NKX6-1 and MAFA in human islets after treatment with AGK2 or DMSO (G). Scale bar=20 μm, error bars= standard error of the mean, * p<0.05, n.s=not significant.

To test whether Sirt2$^{\Delta\beta}$ beta cells exhibit increased proliferation under high blood glucose levels *in vivo*, we treated control and Sirt2$^{\Delta\beta}$ mice with one high dose (200 mg/kg) of streptozotocin (STZ) to specifically ablate the beta cells and induce hyperglycemia as has been previously used to assess compensatory beta cell proliferation (8). We monitored blood glucose levels in these mice for 21 days, and 7 days prior to harvesting pancreata we supplied BrdU to the drinking water (Figure 4.6A). Recent studies have shown that mice harboring the MIPCreER transgene are protected against STZ-induced diabetes due to the ectopic expression of human growth hormone driven by the transgene (172). Therefore, only 45% of the control and Sirt2$^{\Delta\beta}$ mice developed hyperglycemia, as defined by blood glucose levels of greater than 250 mg/dL three days post STZ treatment (Figure 4.6 B,C). In the hyperglycemic mice specifically designated as “responders”, we observed significant increases in BrdU incorporation in Sirt2$^{\Delta\beta}$ beta cells as compared to controls (Figure 4.6E). We saw no difference in BrdU incorporation between Sirt2$^{\Delta\beta}$ and control euglycemic mice (Figure 4.6D). To determine whether stimulated proliferation led to increased beta cell number, we quantified beta cell area and found a significant increase in Sirt2$^{\Delta\beta}$ mice as compared to control mice (Figure 4F). This shows that *Sirt2* deletion can stimulate beta cell proliferation and beta cell mass expansion *in vivo* when blood glucose levels are elevated.

4.2.4 Sirtuin2 inhibition activates the glucose-stimulated MAPK mitogenic pathway.

It has been well described that glucose increases Erk1/2 phosphorylation in beta cells, which is required for the stimulation of beta cell proliferation by platelet-derived growth factor (PDGF) and oncogenic K-Ras (8; 173). Additionally, Sirt2 has been found to deacetylate Mek, which inhibits the activity of Mek to phosphorylate Erk1/2 (163; 164). Therefore, we next sought to determine the convergence of glucose and Sirt2 mitogenic pathways in the beta cell. First, we tested whether *Sirt2* deletion increases phospho-Erk1/2 levels in mouse islets. Western blot analysis revealed a dose-dependent increase in phospho-Erk1/2 in isolated mouse islets from control, Sirt2$^{\Delta\beta/+}$, and Sirt2$^{\Delta\beta}$ mice, with lowest Erk1/2 phosphorylation in

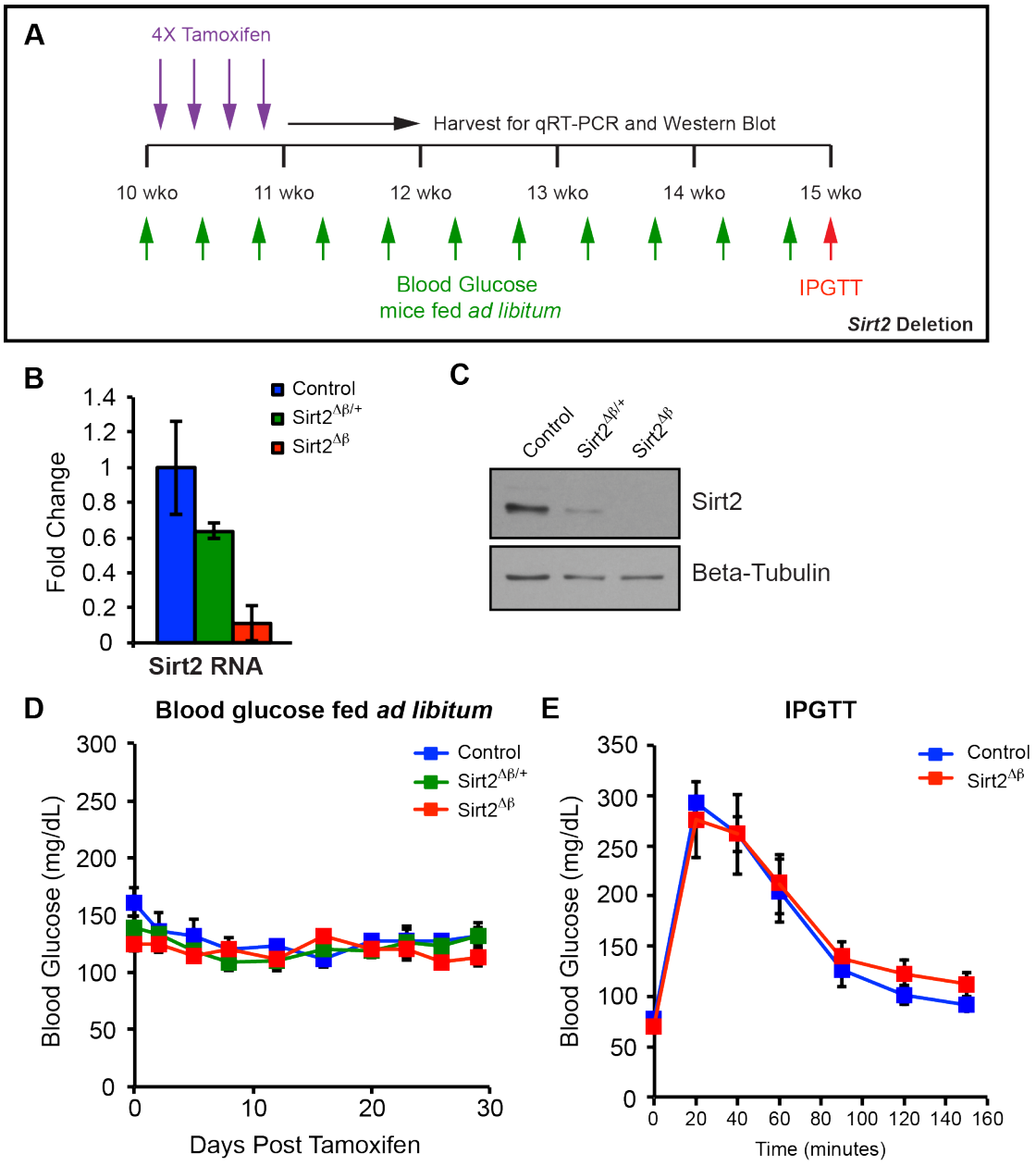

Figure 4.4 Beta cell-specific deletion of *Sirt2*. Schematic showing tamoxifen and blood glucose regimen to analyze control and Sirt2$^{\Delta\beta}$ mice (A). Relative *Sirt2* mRNA levels (B) and protein levels (C) of control, Sirt2$^{\Delta\beta/+}$, and Sirt2$^{\Delta\beta}$ mice; islets pooled from 3 mice per genotype, error bars=standard deviation of technical replicates. Blood glucose fed *ad libitum* in control, Sirt2$^{\Delta\beta/+}$, and Sirt2$^{\Delta\beta}$ mice (n=6-13; D). Intraperitoneal glucose tolerance test in control and Sirt2$^{\Delta\beta}$ mice (n=4-6; E), error bars= standard error of the mean.

control islets and greatest Erk1/2 phosphorylation in Sirt2$^{\Delta\beta}$ islets (Figure 4.7A). This was conserved in human beta cells, as human islets treated with AGK2 after two days had significantly increased phospho-Erk1/2 levels (Figure 4.7B,C). Next we tested whether Erk1/2 phosphorylation is required for the stimulation of human beta cell proliferation by Sirt2 inhibition. When we treated human islets with the Mek inhibitor U0126 (174) in conjunction with AGK2 for two days, we no longer saw the increase in BrdU incorporation into beta cells observed with AGK2 treatment alone (Figure 4.7D). This suggests that Sirt2 is controlling the same effectors as the glucose-stimulated MAPK mitogenic pathway.

Glucose stimulates the MAPK pathway through increased glycolysis and increased ATP which blocks the potassium channel, de-polarizing the cell and stimulating calcium influx (Figure 4.9) (103; 104; 175; 176). Therefore, to further test the dependency on glucose stimulation for the mitogenic effects of Sirt2 inhibition, we treated human islets for two days with AGK2 and the potassium channel activator diazoxide, which blocks calcium channel opening (177). This also blunted the proliferative effect of Sirt2 inhibition in beta cells (Figure 4.7E). Together, these results suggest that glucose stimulation of the MAPK pathway is required to potentiate the mitogenic effects of Sirt2 inhibition on the beta cell (Figure 4.9).

4.2.5 NAD+ levels regulate human beta cell proliferation.

Like all the sirtuins, Sirt2 requires the co-factor NAD+ for its activity (178). However unlike the other sirtuins, Sirt2 is primarily localized to the cytoplasm (162), which we also found in beta cells (Figure 4.1B). Therefore, Sirt2 activity is likely responsive to cytoplasmic NAD+ levels, which decline during glycolysis (179). It is probable that Sirt2 activity is diminished at very high glucose levels, which could also contribute to the proliferative effects of high glucose. Consistent with this, we did not see an added proliferative effect of Sirt2 inhibition or deletion at a high glucose concentration of 16.8 mM in mouse or human beta cells (Figure 4.8A,B).

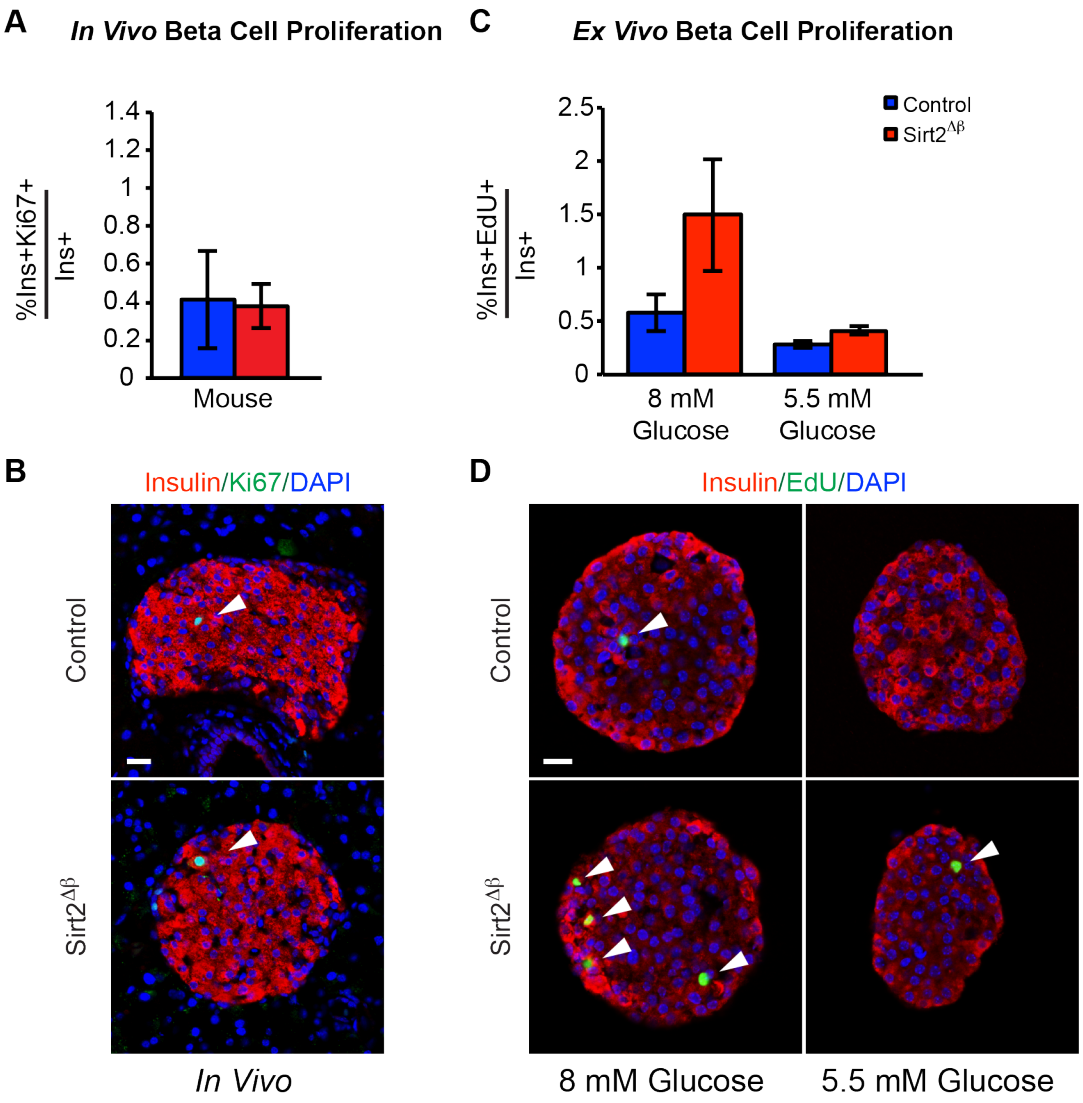

Figure 4.5 Elevated glucose levels are necessary for the stimulation of beta cell proliferation by *Sirt2* deletion. Quantification of Ki67+ insulin+ beta cells from pancreata of control and Sirt2$^{\Delta\beta}$ mice *in vivo* (n=3, A). Immunofluorescence of pancreata showing insulin, Ki67 and DAPI expression in control and Sirt2$^{\Delta\beta}$ mice *in vivo* (B). Quantification of EdU incorporation in insulin+ beta cells of isolated islets from control and Sirt2$^{\Delta\beta}$ mice cultured in 8 mM and 5.5 mM glucose *ex vivo* (n=4, C). Immunofluorescence of isolated islets showing insulin, Ki67 and DAPI staining in control and Sirt2$^{\Delta\beta}$ mice *ex vivo* (D). White arrowheads point to insulin+ EdU+ cells, scale bar= 20 μm, error bars= standard error of the mean.

We then tested whether NAD+ modulation, itself, could alter beta cell proliferation by altering cellular levels of NAD+ pharmacologically. Nicotinamide phosphoribosyltransferase (NAMPT) is an upstream component of the NAD+ biosynthesis pathway, which can be inhibited by the small molecule FK866 (180). Treatment of islets with FK866 has been demonstrated to deplete NAD+ levels, while supplementing with the NAD+ precursor nicotinamide mononucleotide (NMN) has been shown to increase NAD+ levels (Figure 4.8C) (181). Two-day treatment of human islets with FK866 revealed higher EdU incorporation in beta cells (Figure 4.8D), suggesting that NAD+ depletion alone can increase beta cell proliferation.

Furthermore, NMN supplementation in high glucose diminished the mitogenic effects of high glucose (Figure 4.8D). This suggests that NAD+ reduction is important for the stimulation of beta cell proliferation by high glucose, and that reducing NAD+ alone can stimulate beta cells to proliferate. Altogether, our results have uncovered a novel role for NAD+ modulation in regulating beta cell proliferation.

4.3 DISCUSSION

Recent attention has been placed on identifying regulators of human beta cell proliferation for replenishing beta cell mass in diabetic patients. Here, we have discovered Sirt2 as a novel regulator of beta cell proliferation that utilizes known mitogenic pathways to specifically affect beta cell proliferation and mass without detrimental effects to beta cell function or identity. We show that Sirt2 is expressed in beta and alpha cells of the pancreas and suggest that upregulation of this protein during islet aging from a proliferative state to a senescent state may contribute to the age-dependent decline in beta cell proliferative capacity. Consistent with this hypothesis, we show that Sirt2 inhibition stimulates beta cell proliferation in not only islets from 9- to 12-month-old mice but also in islets from humans ranging from ages 24- to 68-years-old. Importantly, we did not see a significant increase in proliferation of human acinar cells and only observed a minor increase in proliferation of alpha

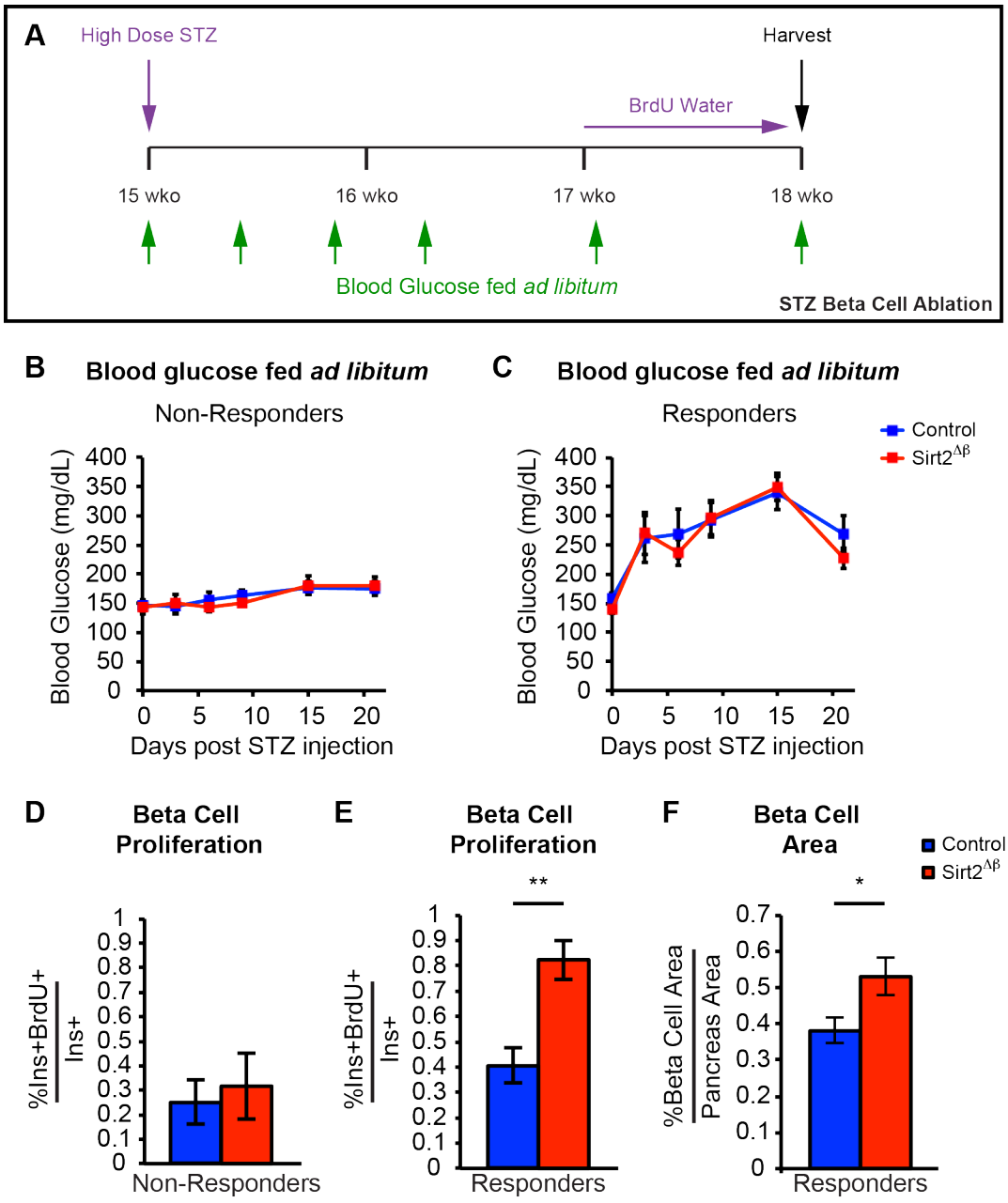

Figure 4.6 STZ-induced hyperglycemia triggers increased beta cell proliferation in Sirt2-deleted mice *in vivo*. Schematic showing streptozotocin (STZ) treatment, blood glucose measurements, and BrdU administration regimen for control and Sirt2$^{\Delta\beta}$ mice (A). Blood glucose levels in mice fed *ad libitum* for control and Sirt2$^{\Delta\beta}$ mice after STZ-mediated beta cell ablation that either did not become hyperglycemic (n=8-10; B) or exhibited blood glucose levels >250 mg/dL (n=9-13; C). Quantification of BrdU incorporation over 1 week in insulin+ beta cells *in vivo* after STZ treatment in non-responders (n=4-6; D) and diabetic responders (n=9-10; E). Beta cell area quantified as percent insulin+ area over total pancreas area in diabetic control and Sirt2$^{\Delta\beta}$ mice (n=4-7; F). Error bars= standard error of the mean, * p<0.05, ** p<0.01.

cells. This indicates that Sirt2 pharmacological inhibition mainly affects proliferation of beta cells within the pancreas.

Beta cell-specific deletion of closely related Sirtuin 1 (Sirt1) has been previously shown to impair beta cell function by acting on targets downstream of glycolysis (182; 183). However, unlike Sirt1, we did not see negative effects on beta cell function after Sirt2 inhibition in human islets or beta cell-specific *Sirt2* deletion in mice. This illustrates the difference in cellular function of these closely related proteins, and demonstrates that the proliferative effects observed from pharmacological inhibition of Sirt2 are not due to off target effects on Sirt1, which has yet to be assessed as a regulator of proliferation. Furthermore, the lack of effect on beta cell function suggests that targeting Sirt2 specifically could be a feasible method to induce beta cell mass expansion without negatively impacting blood glucose control. Recently, there have been intense efforts to develop potent Sirt2-specific inhibitors for *in vivo* usage due to their neuroprotective effect in Parkinson's disease (166). Therefore, it will be important to test these inhibitors as they become available in order to assess the therapeutic potential of utilizing Sirt2 inhibition for beta cell mass expansion.

Interestingly, elevated glucose levels were necessary for the mitogenic effect of *in vivo Sirt2* deletion. *Sirt2*-deleted beta cells cultured in 5.5 mM glucose *ex vivo*, which is physiologically euglycemic, had a minor increase in beta cell proliferation, while beta cell proliferation in islets cultured in 8 mM glucose media was stimulated to a much greater extent. Furthermore, beta cell proliferation *in vivo* was only stimulated in $Sirt2^{\Delta\beta}$ mice with blood glucose levels greater than 250 mg/dL from STZ-induced beta cell ablation, while euglycemic $Sirt2^{\Delta\beta}$ mice did not have increased proliferation. Experiments *ex vivo* showed that glucose-induced calcium influx and Mek activity is required for stimulation of beta cell proliferation by Sirt2 inhibition. This suggests that the mitogenic MAPK pathway must be activated in order for Sirt2 inhibition to exert effects on the beta cell. This could be because Sirt2 deacetylates Mek, inhibiting Mek activity (163; 164). Mek is activated by phosphorylation from Raf as part of the MAPK cascade (Figure 4.9) (184). Mek activation can be further enhanced by acetylation from

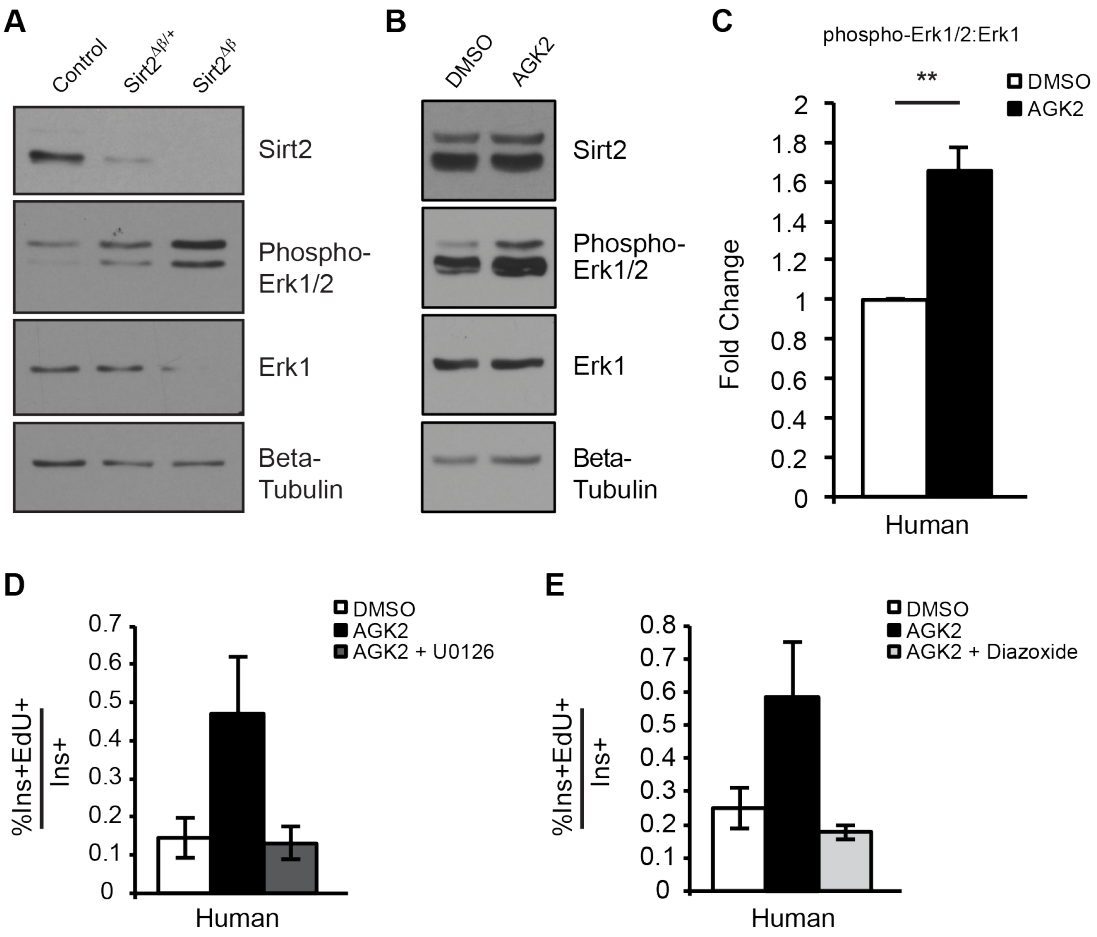

Figure 4.7 Sirt2 deletion or inhibition increases Erk1/2 phosphorylation, which is required for the stimulation of human beta cell proliferation. Sirt2, phosphorylated Erk1/2, Erk1, and beta-tubulin expression in isolated islets from control, $Sirt2^{\Delta\beta/+}$, and $Sirt2^{\Delta\beta}$ mice (A) and human islets treated with DMSO and AGK2 (B). Phosphorylated Erk1/2 to Erk1 ratios were quantified by densitometry from human islets treated with DMSO and AGK2 (n=3; C). Quantification of EdU incorporation in insulin+ beta cells after human islets were treated with DMSO, AGK2, or AGK2 and the Mek inhibitor, U0126 (n=6; $p<0.05$, One-way ANOVA; D). Quantification of EdU incorporation in insulin+ beta cells after human islets were treated with DMSO, AGK2, or AGK2 and the K^+ATP channel opener, Diazoxide (n=3; $p=0.06$, One-way ANOVA; D). Error bars= standard error of the mean, ** $p<0.01$.

P300, which is thought to enhance autophosphorylation (164). Therefore, in order for Sirt2 inhibition to help drive beta cell proliferation through Mek, Mek must first be phosphorylated by Raf (Figure 4.9).

During glycolysis, the Sirt2 co-factor NAD+ is consumed in the cytoplasm, likely inhibiting Sirt2 activity (179). This suggests that at very high levels of glucose, Sirt2 is already inhibited, maintaining Mek in an acetylated state to retain maximal MAPK activity. Consistent with this, we did not observe a further increase in beta cell proliferation when cells were cultured in 16.8 mM glucose with the Sirt2 inhibitor. Furthermore, we found that alterations of NAD+ levels could modulate human beta cell proliferation *ex vivo*. While glucose can also stimulate DNA damage pathways, we did not observe increased expression of the DNA damage marker γH2AX after Sirt2 inhibition. Additionally, we did not observe any effects on human beta cell apoptosis *ex vivo* after Sirt2 inhibition. It is still unclear, however, whether these results will be confirmed after long-term treatments with the Sirt2 inhibitor and further studies will be necessary to test this. It is also unknown whether other mitogenic signals that activate the MAPK cascade, such as insulin or GLP-1, exert similar effects on Sirt2-inhibited beta cells, and further studies will be needed to determine this.

Finally, we find that when we induced hyperglycemia in control and Sirt2$^{\Delta\beta}$ mice, there was a significant increase in beta cell area. This observation has important implications in identifying beta cell regenerative approaches in humans. While it is yet unknown whether human beta cell proliferation and mass increases *in vivo* after Sirt2 inhibition, this can be tested by pharmacological inhibition of Sirt2 in diabetic animals transplanted with human islets. This also has further implications for diabetes therapeutics, as Sirt2 inhibition potentially would only promote beta cell proliferation in patients with elevated blood glucose levels and would no longer promote proliferation once euglycemia is restored. This may alleviate concerns over long-term oncogenic effects. All together, our data identifies and characterizes Sirt2 as a novel beta cell proliferation regulator that specifically controls beta cell proliferation in the presence of elevated glucose concentrations to increase beta cell mass.

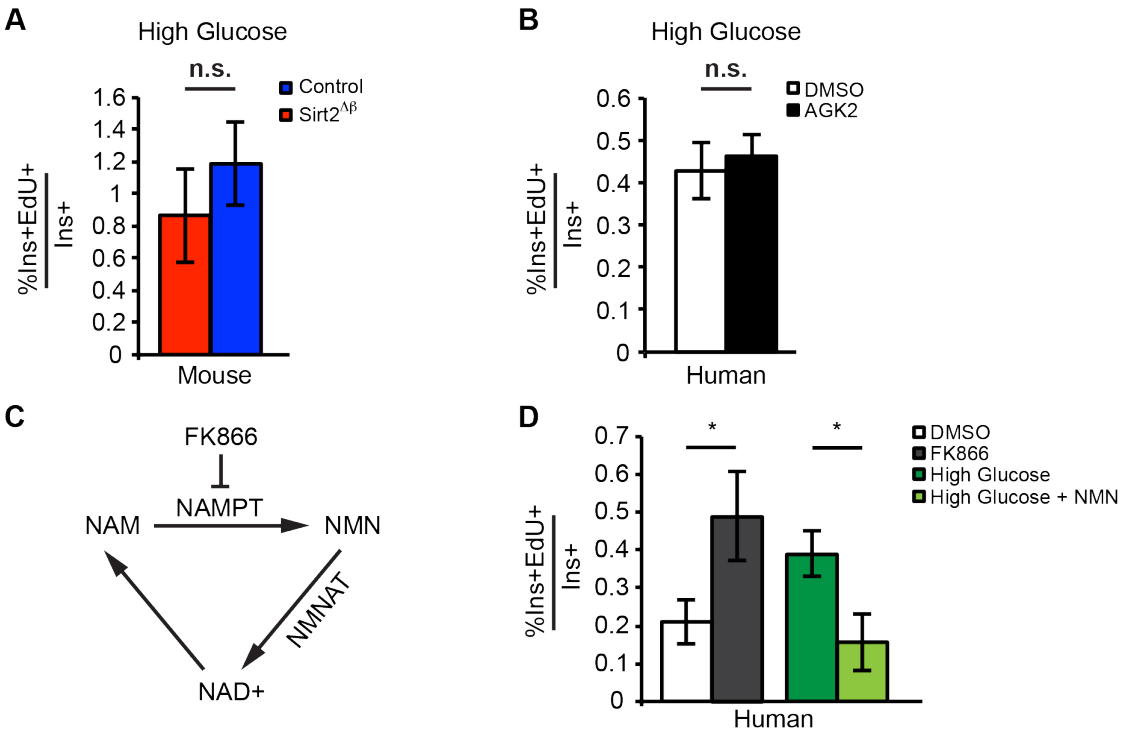

Figure 4.8 NAD+ levels modulate beta cell proliferation. Quantification of EdU incorporation in insulin+ beta cells from control and Sirt2$^{\Delta\beta}$ mouse islets cultured in 16.8 mM glucose (n=3; A) and human islets treated with DMSO or AGK2 cultured in high glucose media (n=4; B). Schematic of the NAD+ biosynthesis pathway; NAM= Nicotinamide, NAMPT= Nicotinamide phosphoribosyltransferase, NMN= Nicotinamide mononucleotide, NMNAT= Nicotinamide mononucleotide adenylyltransferase (C). Quantification of EdU incorporation in insulin+ beta cells treated with NAMPT inhibitor, FK866, high glucose, and high glucose with NMN (n=3, paired T-Test, D). Error bars= standard error of the mean, * p<0.05.

4.4 MATERIALS AND METHODS

4.4.1 Mice and STZ-induced diabetes

The following mouse strains were utilized in this study: *MIPCreER* (Wicksteed et al., 2010), *Sirt2*^{flox} mice (Beirwoski et al., 2011) were maintained on a C57Bl6/J (Jackson Laboratories) genetic background. Unless otherwise stated in the text, male mice were used for metabolic experiments and both male and female mice were used for proliferation experiments. Tamoxifen (Sigma) was dissolved in corn oil at 20mg/mL and 6 mg was injected subcutaneously four times, every other day. For the STZ experiment, mice were fasted for 4 hours before receiving an i.p. injection of 200 mg/kg body weight of STZ (Calbiochem) dissolved in citrate buffer (pH 4.5). Animals were given 10% sucrose water for 3 days and blood glucose was measured (Bayer Contour glucometer; Bayer) every 3 days for 21 days. For BrdU labeling, mice were given 0.8 mg/mL BrdU in drinking water for 7 days prior to harvesting pancreata. All animal experiments were approved by the Institutional Animal Care and Use Committees of the University of California, San Diego.

4.4.2 Tissue preparation, immunofluorescence, and morphometric analysis

Tissue preparation, immunofluorescence staining, TUNEL assays, and morphometry were performed as previously described (136; 139). Mouse pancreata were fixed in 4% paraformaldehyde (Fisher Scientific) at 4^{o}C overnight. Human islet samples were fixed in 4% paraformaldehyde at room temperature for 30 minutes. After fixation, samples were washed three times with PBS and then incubated in 30% sucrose at 4^{o}C overnight. Pancreata and islet samples were embedded with Optimal Cutting Temperature Compound (OCT) (Tissue-Tek), frozen in a 100% ethanol/dry-ice bath, and sectioned at 10 μm using a Cryostat (Leica). Sections were washed with PBS for 5 minutes and permeabilized/blocked in 1% normal donkey serum and 0.15% Triton X-100 (Fisher Scientific) in PBS for 1 hour. For detection of BrdU, sections were treated with 2 M hydrochloric acid for 30 minutes and then quenched with 0.1 M sodium borate for 5 minutes at room temperature prior to permeabilization and block.

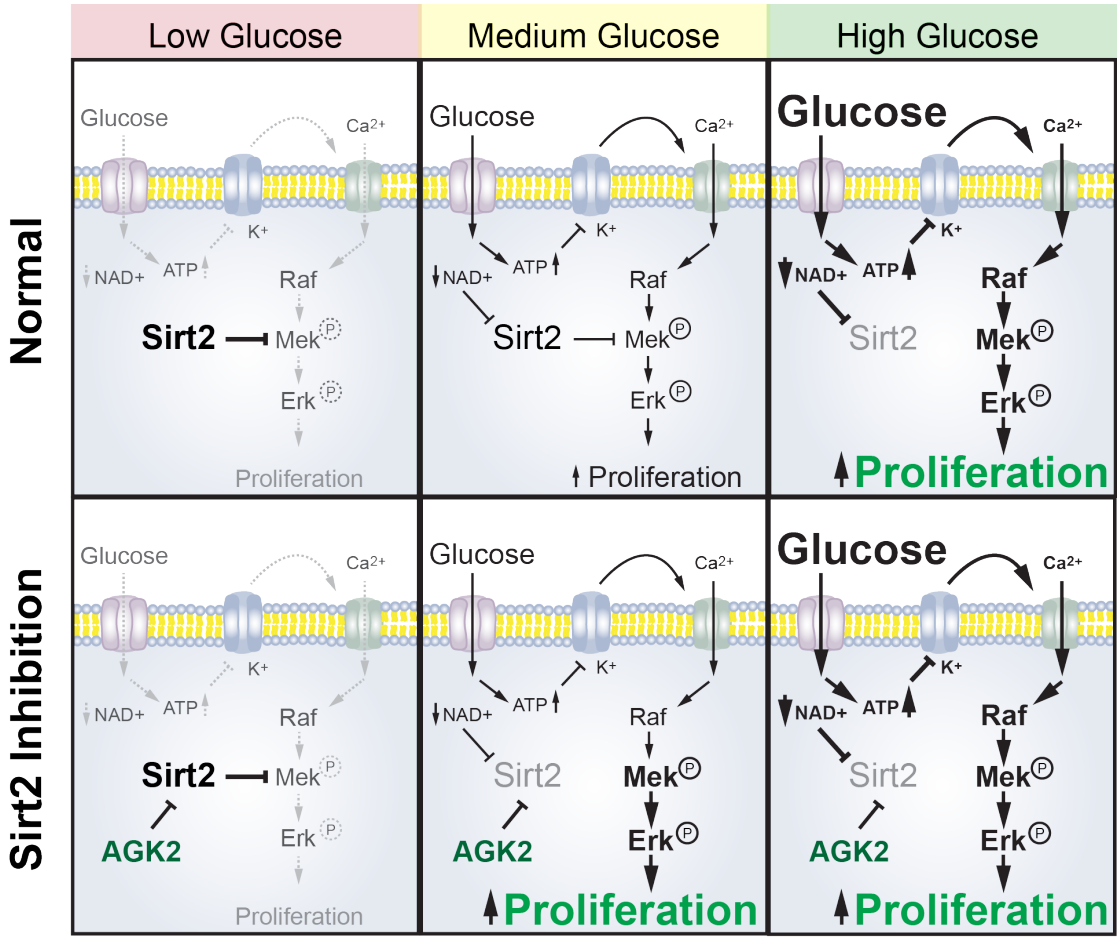

Figure 4.9 Model for Sirt2 regulation of the glucose-stimulated MAPK pathway. Upper panel represents how in normal, non-treated conditions varying concentrations of glucose affect MAPK-dependent stimulation of beta cell proliferation. Lower panel shows how pharmacological Sirt2 inhibition with AGK2 affects MAPK pathway activity and beta cell proliferation in varying glucose concentrations.

Primary and secondary antibodies are listed in Tables C1 and C2. Nuclei were counterstained with DAPI (Sigma) at 0.1μg/mL. For examination of apoptosis, TUNEL analysis was performed as specified by the manufacturer (Millipore). For area measurements, insulin+ area was imaged on three pancreas sections per animal. Area was quantified using Image J.

4.4.3 Microscopy and image analysis

All immunofluorescent images were acquired using a Zeiss AxioObserver.Z1 microscope (Carl Zeiss) with the Zeiss ApoTome module and processed in Zeiss Zen 2 and Adobe Photoshop and Illustrator CS5.1. Only brightness, contrast, and cropping have been applied to images. No specific feature in the images have been enhanced, moved, obscured, removed, or inserted.

4.4.4 Islet isolation and culture

Islet isolations were performed as previously described (136). Briefly, Liberase TL (Roche) was perfused into pancreata at a working concentration of 0.655 units/mL through the common hepatic bile duct. Pancreata were then removed and dissociated at 37^{o}C for roughly 15 minutes (dissociation time depends on age and size of pancreas). Islets were separated onto a gradient composed of HBSS (Cellgro) and Histopaque (Sigma) layers. Purified islets were then hand picked under a dissection microscope to minimize acinar contamination. Mouse islets were cultured in petri dishes with RPMI 1640 (Cellgro) supplemented with 10% FBS, 8 mM glucose, 2 mM L-Glut (Corning), 100 u/mL Penicillin/Streptomycin (Gibco), 1 mM Sodium Pyruvate (Cellgro), 10 mM Hepes (Gibco), 0.25 μg/mL Amphoterecin B.

Human islets were received through the Integrated Islet distribution program (IIDP). Islets were stained with 0.02 μg/mL dithizone, 0.1 mM ammonium hydroxide solution for 10 minutes at 37^{o}C to determine purity. Islets were hand picked and cultured in CMRL1066 (Cellgro) supplemented with 10% FBS, 1.22 μg/mL Nicotinamide, 1:1000 Insulin-Selenium-Transferrin (Gibco), 16.7 μM Zinc Sulfate, 5 mM Sodium Pyruvate (Cellgro), 2 mM GlutaMAX

(Gibco), 25 mM Hepes (Gibco), 100 u/mL Penicillin/Streptomycin (Gibco). For information on human islet samples see Table B1.

4.4.5 Glucose tolerance tests (GTT) and glucose stimulated insulin secretion (GSIS) assay.

GTT and GSIS assays were performed as previously described (14). For glucose tolerance tests, mice were fasted for 16 hours overnight and blood glucose levels were recorded (Bayer Contour glucometer; Bayer) before an intraperitoneal injection of a 1.5 mg/g body weight D-glucose solution in sterile water. Blood glucose levels were recorded at 20, 40, 60, 90, 120, and 150 minutes post injection.

For GSIS assays, mouse islets were isolated or human islets were obtained, and incubated in culture media as described above. Islets were then washed and pre-incubated for 1 hour in 2.8 mM glucose Krebs Ringer Buffer (KRB). Groups of 10 islets were then transferred to a 96-well dish into solutions of 2.8 mM or 16.8 mM glucose KRB. After incubation for 1 hour, supernatant was collected and islets were lysed overnight at 4^{o}C in a 20% acid:80% ethanol solution. Insulin was measured in supernatants and lysates using the mouse insulin ELISA (ALPCO). Insulin secreted was calculated as percentage of total insulin content per hour.

4.4.6 Incubation of islets with chemical compounds

Human islets were obtained and cultured as described above. One day after receiving human islets, islet media was changed and supplemented with 0.1% DMSO vehicle or 10 μM AGK2 (Tocris), 25 μM AK-1 (EMD-Millipore), 10 μM SirReal2 (Tocris), 10 μM U0126 (Cell Signaling), 100 μM Diazoxide (Sigma), 100 μM NMN (Sigma), 10 nM FK866 (Axon MedChem), or 16.8 mM high glucose, or combinations as indicated. To detect proliferating cells, media was also supplemented with 10 μM EdU. After 24 hours, media was refreshed

with new compound and 10 μM EdU. After 24 hours, islets were fixed and stained as described above.

4.4.7 Western blot analysis

To determine Sirt2 and phospho-Erk protein levels, islets were lysed in RIPA buffer and 30 μg of protein was loaded onto a 10% Tris-HCl SDSpolyacrylamide gel. Protein was then transferred to a PVDF membrane and membranes were blocked in 5% non-fat milk in PBS/0.1% tween followed by incubation with primary antibodies in blocking solution overnight at 4ºC and secondary antibodies for 1 hour at room temperature the following day. The membrane was incubated in SuperSignal West Pico Chemiluminescent Substrate (Thermo Fischer) and exposed to Blue Bio Film (Denville Scientific). Primary and secondary antibodies are listed in Table C1 and C2, respectively. Densitometry analysis was performed using Image J.

4.4.8 RNA-sequencing

For the analysis of RNA changes after DMSO or AGK2 treatment of human islets, islets were lysed in RLT and total RNA was extracted using the RNeasy Micro Kit (Qiagen) per manufacturer's instructions. TruSeq stranded mRNA libraries were prepared by UCSD Institute for Genomic Medicine Genomics Center and sequenced using HiSeq2500 Highoutput Run V4 (Illumina). The single-end 50 base pair reads were mapped to the UCSC human transcriptome (hg19) by STAR, allowing for up to 10 mismatches. Only the reads aligned uniquely to one genomic location were retained for subsequent analysis. Expression levels of all genes were estimated by Cufflink using only the reads with exact matches.

4.4.9 qRT-PCR

To detect *Sirt2* transcript, qRT-PCR was performed as previously described (134) on total RNA isolated from mouse islets. Results were normalized to GAPDH. Primers used are

as follows: Sirt2 (f- GCCTGGGTTCCCAAAAGGAG; r- GCCTGGGTTCCCAAAAGGAG); Gapdh (f-CATGTTCCAGTATGACTCCACTC; r-GGCCTCACCCCATTTGATGT).

4.4.10 Statistics

Unless otherwise stated, all values are shown as mean ± SEM; P-values were calculated using Student's T-Test for data with one variable in Microsoft Excel. P-values for 3 groups were calculated using One-Way ANOVA with StatPlus. $P<0.05$ was considered significant.

4.5 ACKNOWLEDGEMENTS

Chapter 4 includes material that is currently being prepared for submission as a manuscript. Benthuysen, Jacqueline R; Sander, M. "Sirtuin 2 is a novel regulator of human beta cell proliferation." The dissertation author was the primary investigator and author of this study. The authors would like to thank J. Auwerx for the *Sirt2*^{flox} mice, and acknowledge A. Carrano for critical reading and feedback, Y. Sui for processing RNA-seq data, F. Liu and T. Guan for supportive technical roles, and N. Rosenblatt for assistance in the mouse facility. J.B. designed and performed the experiments, analyzed the data, and prepared figures. J.B. and M.S. wrote the manuscript. The authors would also like to acknowledge the Integrative Islet Distribution Program for providing human islet samples for this study.

CHAPTER 5

Conclusions and Future Directions

A major goal in the diabetes therapeutics field is to identify successful strategies to replenish beta cell mass. One approach is to target factors that can stimulate beta cell replication; however, only a small number of factors have been identified that can promote proliferation in human beta cells. Beta cell proliferation is dynamically controlled during aging. Throughout early postnatal growth and maturation beta cell proliferation is elevated, but rapidly declines as juvenile beta cells gain refined insulin secretory function (4; 39; 138). And from juvenile to advanced age, beta cells undergo a slow decline in proliferation (3; 4). Likely, different layers of regulation occur to modulate beta cell proliferation during these different time periods of development, maturation, and aging.

The objective of this dissertation was to better understand the factors that contribute to and control beta cell proliferation. To this end, I have (1) characterized the transcription factor Nkx6.1 in regulating postnatal beta cell proliferation, (2) gained a more comprehensive understanding of pancreatic islet aging using quantitative proteomics to identify novel beta cell proliferation regulators, and (3) characterized an age-dependently regulated protein, Sirt2 in controlling human beta cell proliferation. Overall, it is clear from these three studies that glucose and nutrient sensing play important roles in not just beta cell function, but beta cell proliferation as well. Furthermore, as diabetes is a metabolic disease, it will be important to consider how therapeutics aimed at expanding beta cell mass will interplay with the ability of the beta cell to function properly.

5.1 IS NKX6.1 A THERAPEUTIC TARGET?

Nkx6.1 has been previously shown to be required for the maintenance of proper beta cell function in adult rodents (14). Here, we have found that Nkx6.1 is required for postnatal beta cell proliferation as beta cells deficient for Nkx6.1 proliferate less due to reduced expression of key nutrient sensing receptors Glut2 and Glp1r. While work by others have demonstrated that overexpressing Nkx6.1 *in vitro* can increase rodent and human beta cell

proliferation (135), in our previous studies we have not observed stimulation of beta cell proliferation *in vivo* by forced expression of Nkx6.1 in adult rodent beta cells (136). Because Nkx6.1 is critical for beta cell function and preventing de-differentiation, it could be beneficial to overexpress Nkx6.1 in diabetic beta cells that may be losing Nkx6.1 expression (185). Most successfully, Nkx6.1 has been used as a diagnostic marker for proper *in vitro* differentiation of beta cells from pluripotent stem cells (186-188). Further evaluation testing human beta cell mass expansion or human beta cell de-differentiation with Nkx6.1 overexpression will be necessary to determine whether Nkx6.1 could be an effective therapeutic target.

5.2 BETA CELL AGING

From our studies and from others, there is a growing body of work suggesting that early in life beta cells undergo a switch from highly proliferative with a broad window of glucose concentrations necessary to stimulate insulin secretion to a lowly proliferative state with tightly controlled insulin release (138). This mitogenic and functional switch appears to be regulated metabolically after weaning (39), and our quantitative proteomics analysis has demonstrated that this continues well into late adulthood with an upregulation of beta cell function proteins and downregulation of proteins important for replication. It is important that the inverse relationship of beta cell proliferation and function is considered when evaluating therapeutic targets for beta cell mass expansion because it is likely that stimulating beta cells to proliferate can diminish their functional state. Furthermore as beta cells age, the promoters for important cell cycle genes become methylated (13), which suggests that it becomes increasingly difficult to expand beta cell mass. Therefore, it will be critical to identify pathways that can re-open the accessibility of cell cycle genes to effectively stimulate aged beta cells to replicate. To this end, our age-dependent proteomics analysis identified a group of epigenetic regulators that could be interesting targets to further test.

5.3 IS SIRT2 A VIABLE THERAPEUTIC TARGET?

The ability for Sirt2 to stimulate beta cell proliferation in diabetic rodents as well as human beta cell proliferation *ex vivo* is promising for therapies targeting beta cell mass expansion in diabetic patients. As a protein with enzymatic activity, it is a druggable target and multiple compounds have been, and are currently being, developed to specifically inhibit its activity (166; 168; 189). Effective *in vivo* compounds have precluded efforts to test whether human beta cell proliferation can be stimulated after transplantation into diabetic mice, and this work is still ongoing. However, our findings demonstrating that stimulatory effects of these inhibitors can be modulated depending on glucose levels could alleviate concerns for oncogenic potential. Theoretically, once blood glucose levels are normalized, the Sirt2 inhibitors would no longer have a proliferative effect; and after increasing beta cell mass, the drug treatments would no longer be necessary. Long-term studies will be critical for evaluating the efficacy and oncogenic potential of Sirt2 inhibition over extended periods of time.

Given that beta cell proliferation and function are reciprocally related, it is also encouraging that Sirt2 inhibition in human islets does not detrimentally affect beta cell function or identity. Sirt2 co-factor, NAD+, modulation *in vivo* has been shown to have effects on beta cell function. NAMPT heterozygous mice, with depleted NAD+ levels, have impaired glucose tolerance, which can be rescued by NMN supplementation and increased NAD+ levels (181). But this effect is likely due to Sirt1 inhibition, as *in vivo* Sirt1 deletion in beta cells results in impaired glucose tolerance (182) and Sirt1 overexpression in beta cells *in vivo* leads to improved glucose tolerance (190). Sirt2-specific inhibition or deletion does not appear to have this effect, which is beneficial for the development of targeting Sirt2 for therapeutic usage.

5.4 THERAPEUTIC IMPLICATIONS FOR TARGETING BETA CELL MASS EXPANSION

Recent studies have found various promising candidates for targeting beta cell mass expansion (72; 87; 88). While each alone appear to have modest effects on beta cell

proliferation, it will be important to begin testing synergistic effects of targeting multiple candidates. Because there are numerous mitogenic pathways that can effect beta cell proliferation, it may be more effective to target several pathways simultaneously. Furthermore it would be of use to develop drugs that could target multiple pathways simultaneously, similar to how aminopyrazine compounds regulate both Dyrk1a and Gsk3β to promote human beta cell proliferation (Figure 1.1)(88).

A major challenge for utilizing compounds that target proliferation pathways is the potential to stimulate uncontrolled growth resulting in cancer. For example harmine upregulates the expression of C-myc (87), which is one of the Myc family proteins that potentiate cell growth in many tissues (191; 192). Harmine was shown to upregulate beta cell proliferation, however could also stimulate alpha and ductal cell proliferation *ex vivo* and was not evaluated for proliferation of other tissues types *in vivo* (87). Therefore, for many of the current drug targets that can stimulate human beta cell proliferation, it will be critical to evaluate the oncogenic status of beta cells and other cell types after long-term treatments. This will be a major hurdle for implementing this strategy as an effective therapy.

Successful induction of beta cell proliferation will likely require cell type-specific delivery systems for application in humans. Local delivery of regeneration factors could be achieved by ultrasound destruction of microbubbles carrying plasmid DNA administered into the pancreatic microcirculation (193). An alternative approach for delivering molecules directly to the beta cell could be by tethering the molecule to the ligand of a beta cell-specific receptor. Efficacy of this approach has recently been demonstrated for GLP-1-estrogen conjugates in beta cells (194).

Perhaps the most challenging issue is protecting the newly generated beta cells from autoimmune destruction in T1D. Recent studies suggest that specific gene editing strategies can be used to generate hPSCs that are invisible to the immune system and could escape at least allogenic rejection (195). Similar strategies could perhaps also allow for evasion of the cells from autoimmune destruction. Overall, significant progress has been made in the past

decade and the coming decade will show whether targeting beta cell mass expansion holds promise for translation into clinical therapies.

5.5 ACKNOWLEDGEMENTS

Chapter 5 includes material, in part, currently being prepared for submission as a review article. Benthuysen, Jacqueline R; Carrano, A; Sander, M. “Advances in Beta Cell Regeneration-Strategies for the Treatment of Diabetes Mellitus.” The dissertation author was the primary investigator and author of this manuscript. J.B., A.C., and M.S. wrote the manuscript. J.B. prepared figures.

APPENDIX A

Table of proteins differentially expressed during pancreatic islet aging

Table A1 Proteins changed during pancreatic islet cell aging. Blue=down with age, red=up with age.

Protein Name	Fold Change down with age	ANOVA p-value	Total # of Quantitated 14N/15N Pairs	Protein Name	Fold Change up with age	ANOVA p-value	Total # of Quantitated 14N/15N Pairs
Inpp5f	7.66	1.41E-02	4	Aldh1l2	9.11	0.00E+00	16
Hist1h1b	5.63	0.00E+00	14	Nol3	7.89	0.00E+00	10
Msln	4.03	1.73E-02	9	Ftl1	5.92	0.00E+00	16
Oat	3.98	1.63E-02	8	Fth1	5.03	0.00E+00	14
Dmbt1	3.63	1.05E-02	4	Cpa1	4.17	4.49E-02	13
Serpinh1	2.96	0.00E+00	16	Fam129a	4.02	1.00E-05	12
Gpx2	2.75	0.00E+00	10	Cel	3.92	1.99E-03	19
Sult1d1	2.63	0.00E+00	14	Cbr3	3.68	9.80E-04	12
Insm1	2.60	6.97E-03	7	Rtn1	3.66	5.61E-03	13
LOC100046823	2.59	1.57E-02	4	Erp27	3.56	3.31E-03	5
Pcna	2.59	1.57E-02	4	Ppt1	3.44	7.00E-05	11
LOC100045442	2.32	1.81E-02	6	Ctrl	3.35	4.63E-02	16
Gamt	2.31	4.67E-02	6	Ckb	3.29	0.00E+00	25
Bak1	2.27	4.00E-04	6	Fabp4	3.28	1.79E-02	6
Tbl2	2.16	3.00E-05	11	Amy2	3.24	2.50E-04	29
Msh6	2.14	3.20E-02	5	Phgdh	3.24	2.40E-04	13
Hmgcl	2.13	6.00E-05	4	Vat1	2.96	0.00E+00	21
Foxk1	2.12	1.51E-02	4	Crmp1	2.95	0.00E+00	14
Serping1	2.11	6.60E-04	14	Hapln4	2.95	0.00E+00	15
Nt5dc2	2.09	0.00E+00	19	Apoe	2.95	4.50E-04	8
Arg1	2.07	0.00E+00	19	Gstm2	2.93	1.00E-05	20
Cc2d1a	2.00	1.45E-02	4	Pnlip	2.82	1.55E-02	16
Ocrl	1.98	5.70E-04	10	Tpp1	2.72	0.00E+00	16
Emd	1.98	4.04E-03	10	Rnase4	2.64	0.00E+00	13
Ckmt1	1.91	5.00E-05	16	Atp8a1	2.63	0.00E+00	14
Nasp	1.91	4.00E-04	11	Ela3	2.52	2.89E-02	18
Rpa3	1.91	0.00E+00	13	Abcb9	2.50	2.46E-02	4
Dis3	1.90	2.77E-02	6	Dpp7	2.49	2.00E-05	13
Vps29	1.88	1.57E-02	11	Fam169a	2.47	0.00E+00	13
Hist1h3h	1.88	6.50E-04	24	Gpld1	2.46	1.66E-03	9
LOC100048313	1.85	8.90E-04	6	Gdpd1	2.46	4.33E-03	8
Mov10	1.85	8.09E-03	10	Eno2	2.43	0.00E+00	15
Igfbp7	1.83	0.00E+00	18	Asah1	2.40	0.00E+00	14
Gcg	1.81	0.00E+00	30	Lcp1	2.30	0.00E+00	16
Klc3	1.78	4.25E-03	11	Gsn	2.29	0.00E+00	23
Tbce	1.75	4.32E-02	8	Igfals	2.29	1.00E-05	17
Rgl3	1.74	3.48E-02	8	Enpp2	2.28	2.20E-04	13
Dpysl3	1.74	7.10E-04	8	Coro1a	2.28	0.00E+00	14
Adss	1.73	3.40E-02	14	Slc27a1	2.28	4.53E-02	6
Nudcd2	1.72	2.94E-02	7	Gstm1	2.27	0.00E+00	18
Aacs	1.72	3.20E-04	10	Comtd1	2.27	7.29E-03	6
D430028G21Rik	1.72	1.22E-02	8	Gstm3	2.22	1.70E-04	12
Irgq	1.71	1.24E-02	6	Spock1	2.22	0.00E+00	22
Csda	1.70	2.40E-04	12	Dhtkd1	2.21	0.00E+00	20
Pls1	1.70	2.00E-05	12	Pnpo	2.20	0.00E+00	20
Rad21	1.70	5.80E-04	16	Gstm7	2.19	2.30E-04	10
Pyy	1.68	5.20E-04	14	Pde5a	2.19	0.00E+00	14
Ints4	1.67	4.54E-02	13	Cntnap2	2.16	4.63E-02	4
Pgam5	1.66	3.35E-03	5	Tmem22	2.15	8.72E-03	7
Prdx4	1.64	8.90E-04	12	Sepp1	2.14	4.73E-02	4
Papss1	1.64	4.20E-04	14	Got1	2.10	0.00E+00	23
Mttp	1.62	2.35E-02	19	Fmn2	2.09	7.17E-03	7
Tspan8	1.62	1.13E-02	11	Hint3	2.07	1.08E-03	9
Fasn	1.62	0.00E+00	18	Chga	2.05	0.00E+00	23

Table A1, continued. Proteins changed during pancreatic islet cell aging. Blue=down with age, red=up with age.

Protein Name	Fold Change down with age	ANOVA p-value	Total # of Quantitated 14N/15N Pairs	Protein Name	Fold Change up with age	ANOVA p-value	Total # of Quantitated 14N/15N Pairs
Tfrc	1.61	1.30E-04	15	G6pdx	2.04	0.00E+00	20
Aoc2	1.61	7.35E-03	8	Bace2	2.03	2.94E-03	12
Aoc3	1.61	7.35E-03	8	Scamp3	2.01	2.62E-03	14
Chd5	1.61	1.41E-02	8	Cntfr	2.00	2.24E-02	6
Lage3	1.59	2.28E-02	7	Hibadh	2.00	0.00E+00	15
Unc45a	1.59	2.44E-02	6	Maoa	2.00	0.00E+00	16
Anxa4	1.58	0.00E+00	26	LOC100047762	1.97	0.00E+00	9
Pdia5	1.58	3.00E-05	21	Acot9	1.93	2.00E-05	11
Syne2	1.58	8.73E-03	13	Psap	1.91	0.00E+00	21
EG665509	1.58	1.40E-03	9	Adh1	1.90	0.00E+00	22
S100a11	1.58	1.40E-03	9	Idua	1.89	3.79E-02	6
Itpr1	1.58	7.50E-04	9	Tceal3	1.89	6.81E-03	8
Itpr3	1.57	1.16E-02	18	Scg2	1.88	0.00E+00	25
Wipi1	1.56	4.46E-03	8	Copz2	1.87	5.33E-03	4
Madd	1.56	4.80E-04	6	Mecp2	1.87	0.00E+00	16
Lmnb1	1.55	0.00E+00	26	Them2	1.87	1.20E-04	8
Ppie	1.55	5.35E-03	10	Ldha	1.86	1.52E-02	6
Gfpt1	1.55	0.00E+00	21	Emilin1	1.86	0.00E+00	25
Itga6	1.54	1.51E-02	10	Nfxl1	1.86	1.89E-03	5
Gck	1.54	3.20E-02	8	Cdc42bpa	1.85	2.75E-02	4
Acsl5	1.54	2.70E-03	16	Pcca	1.83	0.00E+00	20
Iqgap2	1.54	2.41E-03	10	Tmed5	1.82	1.00E-05	14
Marcks	1.54	1.19E-02	7	Slc9a7	1.82	9.01E-03	5
Itfg3	1.54	8.53E-03	11	Mettl7a1	1.82	4.00E-05	9
Tmpo	1.54	0.00E+00	19	Rab3a	1.82	0.00E+00	13
H2afv	1.53	0.00E+00	19	Pccb	1.81	0.00E+00	27
H2afz	1.53	0.00E+00	19	Creg1	1.81	5.00E-05	17
Dido1	1.52	3.73E-03	8	Tfb1m	1.80	2.21E-02	9
Nipbl	1.52	6.72E-03	8	Dnajb9	1.80	0.00E+00	16
2010309E21Rik	1.51	2.31E-02	9	Myo5a	1.79	0.00E+00	24
Nedd4l	1.51	1.45E-02	13	Mthfd2	1.79	1.41E-03	12
Cdk9	1.51	2.56E-02	10	Gaa	1.79	0.00E+00	16
Dek	1.50	6.40E-04	16	Vgf	1.79	6.70E-03	10
Dsp	1.50	6.35E-03	22	Ehd4	1.78	0.00E+00	14
ENSMUSG00000069083	1.50	1.33E-03	8	C030006K11Rik	1.78	1.20E-04	8
Slc25a24	1.50	1.12E-02	7	Aldh18a1	1.77	0.00E+00	23
Srp54c	1.50	2.85E-03	4	Nnt	1.77	0.00E+00	14
Etfdh	1.49	2.92E-02	6	Hint2	1.76	1.00E-05	14
Tpm4	1.49	2.00E-05	13	Rph3al	1.76	0.00E+00	17
Pus7	1.48	1.25E-03	6	Sqrdl	1.76	0.00E+00	25
Vps36	1.48	3.36E-02	9	Pacsin1	1.75	2.59E-02	5
Mpp1	1.48	7.07E-03	5	Flad1	1.75	1.81E-03	7
Ctse	1.47	1.29E-02	16	Mccc1	1.75	0.00E+00	22
EG665955	1.47	1.29E-02	16	Tha1	1.75	2.00E-05	12
Pold2	1.47	2.32E-03	10	Aldh5a1	1.74	0.00E+00	19
Ank	1.47	2.90E-04	12	Prnd	1.74	1.70E-04	15
Fdps	1.46	3.00E-05	13	Prnp	1.74	1.00E-05	17
Larp4	1.46	3.00E-05	13	E330026B02Rik	1.72	2.76E-03	12
Dnajc8	1.45	2.87E-02	10	LOC677317	1.72	1.20E-04	14
Mms19	1.45	9.03E-03	9	Me1	1.72	1.20E-04	14
Abcb10	1.44	2.10E-04	16	Spock2	1.71	0.00E+00	21
Spata5	1.44	2.19E-02	8	Nars2	1.71	2.80E-04	13
Hist1h3e	1.44	4.89E-03	22	Aadacl1	1.71	0.00E+00	19
Tmx4	1.44	3.01E-03	9	Slc25a42	1.70	0.00E+00	14

Table A1, continued. Proteins changed during pancreatic islet cell aging. Blue=down with age, red=up with age.

Protein Name	Fold Change down with age	ANOVA p-value	Total # of Quantitated 14N/15N Pairs	Protein Name	Fold Change up with age	ANOVA p-value	Total # of Quantitated 14N/15N Pairs
Vim	1.44	7.05E-03	17	9030617O03Rik	1.70	1.02E-02	11
Hist1h1c	1.44	0.00E+00	25	Impact	1.70	1.26E-02	11
Kpna2	1.44	2.60E-04	11	E430002G05Rik	1.69	1.00E-05	21
Stambp	1.44	1.43E-03	10	Hsd17b11	1.69	2.00E-05	17
Ctps	1.44	9.00E-05	13	Macrod1	1.69	3.80E-04	9
Rbm3	1.44	0.00E+00	22	C2cd2l	1.69	8.37E-03	12
LOC100047016	1.43	3.30E-04	20	Hsdl2	1.69	8.61E-03	6
Polr2a	1.43	1.49E-02	13	Stxbp5	1.69	0.00E+00	16
Stag2	1.43	1.70E-04	19	Stard5	1.68	1.16E-02	9
Ipo9	1.43	2.00E-05	14	Ndrg3	1.65	1.78E-02	7
Slc35b2	1.43	2.00E-02	4	Bmp1	1.65	5.00E-05	15
Skiv2l2	1.42	3.21E-03	11	Suclg2	1.64	0.00E+00	14
Smarcc1	1.42	1.00E-05	16	Mccc2	1.64	0.00E+00	23
Ddx56	1.42	5.76E-03	9	Ivd	1.64	0.00E+00	25
Nmt2	1.42	1.82E-02	12	Scg3	1.63	0.00E+00	34
5730453I16Rik	1.41	1.44E-02	8	Chgb	1.63	0.00E+00	20
Lgtn	1.41	3.28E-02	7	Arsa	1.63	0.00E+00	16
Prcc	1.41	1.02E-02	6	Pdxk	1.63	1.00E-05	14
Ube2v2	1.41	2.21E-02	5	Stx3	1.63	0.00E+00	14
Usp4	1.40	3.91E-03	12	Acaa2	1.62	0.00E+00	21
Arid1a	1.40	6.00E-05	16	BC039632	1.62	5.00E-05	21
Cbx3	1.40	4.60E-04	17	Gnai2	1.62	0.00E+00	27
Ak2	1.40	0.00E+00	17	Hdhd3	1.62	4.03E-02	9
Acat2	1.40	5.70E-04	14	Lima1	1.62	1.83E-02	12
Snrpd2	1.40	1.10E-04	12	Mapk14	1.61	3.00E-02	8
Glt8d3	1.39	4.98E-02	6	1700052N19Rik	1.61	2.58E-02	6
Ppat	1.39	5.00E-05	16	Isoc2b	1.61	1.60E-04	11
Drg1	1.39	4.00E-04	12	Endod1	1.61	0.00E+00	17
Usp15	1.39	4.90E-04	17	2510003E04Rik	1.60	4.47E-02	5
LOC100045132	1.39	6.80E-03	6	Dnajc5	1.60	5.00E-05	13
Lpgat1	1.39	5.92E-03	13	Atp6v0a1	1.60	0.00E+00	31
Tagln2	1.39	0.00E+00	23	Echdc2	1.59	9.10E-04	16
Gcn1l1	1.39	4.00E-05	24	A230050P20Rik	1.59	3.70E-02	11
Trf	1.39	1.22E-03	16	Cbs	1.58	3.02E-02	14
Amt	1.39	1.60E-04	16	Prpsap1	1.58	1.80E-04	15
Smc1a	1.38	3.61E-03	16	Sv2a	1.58	0.00E+00	19
Creld2	1.38	3.85E-03	13	Cryz	1.58	0.00E+00	19
Htra2	1.38	8.90E-04	4	Rhpn2	1.58	7.55E-03	12
Fxyd6	1.38	6.35E-03	10	Stk32a	1.58	1.00E-05	12
Pbrm1	1.38	3.49E-02	7	Ucn3	1.58	8.00E-05	10
Banf1	1.37	1.50E-04	12	Vps13a	1.57	3.00E-04	13
Rock2	1.37	2.75E-03	16	Abcc1	1.57	4.50E-02	8
BC057079	1.37	1.71E-02	8	Dnajc13	1.57	2.54E-02	12
Bub3	1.37	0.00E+00	16	Ilvbl	1.57	2.43E-03	13
Bxdc2	1.37	2.80E-04	5	Dhdh	1.56	3.00E-05	16
Rprd2	1.37	1.13E-03	12	Mapt	1.56	2.12E-02	16
Anp32b	1.37	0.00E+00	14	Fmo5	1.56	2.72E-02	6
Ckap4	1.37	5.33E-03	14	LOC100046051	1.56	2.72E-02	6
Ctcf	1.37	5.80E-04	16	Rab27a	1.56	0.00E+00	18
Myo1b	1.37	4.97E-03	16	Gcat	1.56	2.00E-05	20
Hdac2	1.37	3.00E-05	16	Spnb3	1.55	0.00E+00	24
Akr1c14	1.36	1.30E-04	17	Lgals3bp	1.55	0.00E+00	24
Rbbp4	1.36	1.41E-02	19	Slc9a3r2	1.55	2.29E-02	16
2610301G19Rik	1.36	0.00E+00	14	Casr	1.55	4.58E-02	9

Table A1, continued. Proteins changed during pancreatic islet cell aging. Blue=down with age, red=up with age.

Protein Name	Fold Change down with age	ANOVA p-value	Total # of Quantitated 14N/15N Pairs	Protein Name	Fold Change up with age	ANOVA p-value	Total # of Quantitated 14N/15N Pairs
Hbs1l	1.36	2.52E-02	14	Vps13c	1.54	0.00E+00	21
Mospd2	1.36	1.00E-02	14	Fam114a2	1.54	8.76E-03	8
Ncor2	1.36	4.25E-02	11	Hapln1	1.54	1.64E-02	13
Ap1m1	1.36	4.76E-02	13	D330012F22Rik	1.54	2.02E-03	11
Ybx1	1.35	5.50E-04	15	Gstp1	1.54	3.00E-05	18
Pank4	1.35	1.79E-03	10	Tom1	1.54	0.00E+00	16
Xpnpep1	1.35	0.00E+00	12	Phospho2	1.53	3.76E-02	8
EG225228	1.35	4.81E-02	4	Bsdc1	1.53	1.30E-02	12
Enoph1	1.35	6.31E-03	11	Tex10	1.53	2.69E-02	8
Rbm25	1.35	3.05E-02	11	LOC640369	1.53	5.50E-04	17
Uba2	1.35	0.00E+00	16	Rab5b	1.53	5.50E-04	17
Ppp1r12a	1.34	2.44E-02	16	Gnpda1	1.52	3.00E-05	14
Prep	1.34	1.02E-03	17	BC026585	1.52	1.00E-05	16
Ilkap	1.34	1.27E-02	8	BC003331	1.52	2.31E-03	9
Klraq1	1.34	2.71E-03	13	Pcsk2	1.51	0.00E+00	20
Rbm39	1.34	8.60E-04	22	Hexb	1.51	4.00E-05	18
Snrnp70	1.34	2.30E-02	14	Fahd2a	1.51	4.00E-05	11
Cpox	1.34	5.47E-03	14	Iapp	1.51	0.00E+00	14
Papss2	1.34	0.00E+00	26	H2-Ke6	1.51	3.91E-03	9
Clic1	1.33	3.04E-03	14	Tacc2	1.51	4.50E-02	13
Tpm1	1.33	1.42E-02	12	Blvra	1.51	4.11E-02	16
Bccip	1.33	1.68E-02	18	Extl2	1.51	5.00E-05	14
Rbm14	1.33	1.33E-03	13	Ctnnd2	1.50	2.50E-04	6
Creld1	1.33	2.37E-02	8	Lamp2	1.50	1.00E-05	12
Impdh2	1.33	0.00E+00	17	Mtap	1.50	0.00E+00	16
OTTMUSG00000019498	1.33	0.00E+00	17	Sdr39u1	1.50	0.00E+00	14
Flna	1.33	0.00E+00	24	Atp6v0d1	1.49	0.00E+00	21
Bat2d	1.33	8.15E-03	18	Akt1	1.49	2.00E-02	13
Bclaf1	1.33	2.20E-04	11	LOC100047666	1.49	2.00E-02	13
Cnbp	1.33	8.72E-03	12	Atl1	1.49	4.56E-02	4
Snrnp40	1.33	1.90E-03	14	Tcn2	1.49	3.44E-03	12
Zfml	1.33	1.44E-02	11	Cpe	1.49	0.00E+00	31
Puf60	1.32	0.00E+00	19	Serpina10	1.48	1.50E-03	14
C330023M02Rik	1.32	4.90E-04	12	Gsr	1.47	0.00E+00	18
Nmral1	1.32	1.03E-02	17	Hadh	1.47	0.00E+00	21
Gprin1	1.32	3.00E-05	18	Gusb	1.46	0.00E+00	18
Gsto1	1.32	3.92E-02	13	Ehd3	1.46	0.00E+00	16
Mbd3	1.32	9.19E-03	7	Pdap1	1.46	4.50E-04	13
Srrm2	1.31	3.50E-04	6	Man1a	1.45	2.20E-04	14
Sf3a2	1.31	1.18E-02	8	Ctsa	1.45	0.00E+00	18
Plrg1	1.31	1.65E-03	12	Sult1c2	1.45	3.54E-02	11
Birc6	1.31	1.00E-03	16	Echdc2	1.45	4.27E-02	11
Eif6	1.31	2.00E-03	18	Pitpna	1.45	2.03E-02	16
Mtap1s	1.31	1.00E-04	12	Rbbp9	1.45	2.00E-05	13
Tmem27	1.31	3.63E-02	13	Ccdc44	1.45	7.80E-04	13
Aof2	1.31	5.00E-05	14	Mrpl46	1.45	2.94E-02	10
LOC100046934	1.31	5.00E-05	14	Nipsnap1	1.44	0.00E+00	20
Dync1li2	1.31	3.00E-05	11	Ephx1	1.44	1.00E-05	14
Pxn	1.31	3.74E-02	11	Gbas	1.44	0.00E+00	16
Tbc1d1	1.30	1.25E-02	4	Gstm5	1.44	3.09E-03	15
Ctnnbl1	1.30	1.71E-03	14	Alb	1.44	4.00E-05	16
Dock11	1.30	4.92E-02	12	Cbr4	1.44	3.00E-05	13
LOC100047971	1.30	1.04E-02	11	Decr1	1.44	0.00E+00	18
Ptma	1.30	1.04E-02	11	Aldh9a1	1.44	0.00E+00	22

Table A1, continued. Proteins changed during pancreatic islet cell aging. Blue=down with age, red=up with age.

Protein Name	Fold Change down with age	ANOVA p-value	Total # of Quantitated 14N/15N Pairs	Protein Name	Fold Change up with age	ANOVA p-value	Total # of Quantitated 14N/15N Pairs
Nudt21	1.30	1.10E-04	12	Aldh6a1	1.43	1.75E-03	16
Atox1	1.30	1.50E-02	12	Asns	1.43	2.84E-02	18
Srrt	1.30	4.00E-05	15	Prkd1	1.43	9.46E-03	9
Myh9	1.30	0.00E+00	18	Qsox1	1.43	0.00E+00	19
Sdf2l1	1.30	9.30E-04	16	Aldh4a1	1.43	3.00E-05	18
Api5	1.30	2.00E-04	14	Bcs1l	1.43	3.87E-03	15
Tceb1	1.30	2.55E-03	5	Slc25a25	1.43	2.73E-03	12
Ogt	1.30	1.10E-03	10	Thnsl1	1.43	7.90E-04	17
Uchl5	1.30	6.62E-03	12	Gar1	1.43	1.48E-02	4
Sae1	1.29	0.00E+00	19	Rars2	1.42	6.00E-05	20
Ak3	1.29	2.71E-02	14	C1qbp	1.42	0.00E+00	16
Ddx39	1.29	2.86E-03	8	Ahcy	1.42	0.00E+00	22
Rbm12	1.29	9.87E-03	10	Cyb5r1	1.42	1.17E-02	16
Syncrip	1.29	0.00E+00	18	Preb	1.42	3.00E-05	16
Pabpc1	1.29	2.00E-05	14	D10Jhu81e	1.42	6.09E-03	18
Krt8	1.29	1.05E-02	18	LOC100046684	1.42	1.77E-02	16
Nbea	1.29	3.80E-04	18	Pts	1.42	6.66E-03	10
Cul4a	1.29	0.00E+00	29	Isoc2a	1.41	0.00E+00	19
Ddx21	1.29	1.08E-02	18	Sgpl1	1.41	4.90E-04	12
Hnrnpab	1.29	1.70E-04	23	Akr1c19	1.41	8.50E-04	18
Myh10	1.29	0.00E+00	10	A230046K03Rik	1.41	6.47E-03	11
Pcyt2	1.29	5.34E-03	10	Ctsl	1.41	1.49E-03	13
Wdr81	1.29	6.34E-03	5	Pgcp	1.41	0.00E+00	18
Calb1	1.29	1.61E-03	13	Pcsk1	1.41	0.00E+00	27
Cherp	1.29	1.40E-04	16	Itgav	1.40	1.16E-03	15
Mtmr7	1.29	3.00E-04	23	Pck2	1.40	0.00E+00	19
Tsg101	1.29	6.34E-03	14	Nolc1	1.40	3.82E-03	12
Pea15a	1.28	3.35E-02	12	Cadps2	1.40	6.00E-05	15
Nup98	1.28	1.64E-02	8	Clic4	1.40	2.64E-03	14
Tmx1	1.28	4.31E-02	13	Bcat2	1.40	3.22E-02	17
6430527G18Rik	1.28	5.40E-04	10	Naga	1.40	0.00E+00	27
Limd1	1.28	7.98E-03	14	Pla2g15	1.40	1.58E-02	13
Sf1	1.28	3.50E-04	14	B4galt3	1.39	1.35E-02	11
Ppp1r8	1.28	2.27E-02	5	Acss2	1.39	2.93E-03	12
Glud1	1.27	0.00E+00	20	Ins2	1.39	7.10E-04	23
Ubap2l	1.27	3.90E-02	17	Abhd12	1.39	2.66E-02	14
Prpf40a	1.27	2.07E-02	11	Cryl1	1.39	0.00E+00	23
Blmh	1.27	3.40E-03	16	Csad	1.39	9.00E-05	13
Ipo5	1.27	1.07E-02	22	Coro7	1.39	0.00E+00	20
Jtv1	1.27	9.00E-04	10	2400001E08Rik	1.39	1.00E-05	14
Khdrbs1	1.27	5.00E-05	15	Ctsd	1.39	0.00E+00	21
Sfrs11	1.27	1.40E-04	14	Vamp2	1.39	0.00E+00	17
Osgep	1.27	8.93E-03	8	Stx16	1.39	9.00E-05	12
Dnajb11	1.27	1.29E-03	17	Mcfd2	1.39	3.10E-04	16
Cse1l	1.27	1.00E-05	18	Sfxn1	1.39	0.00E+00	20
Isoc1	1.27	0.00E+00	15	Ddc	1.38	0.00E+00	19
Eif4h	1.27	2.10E-02	15	A2ld1	1.38	2.08E-03	9
Fus	1.27	0.00E+00	14	Mpi	1.38	0.00E+00	17
Elp2	1.27	6.35E-03	16	2310047M10Rik	1.38	3.52E-02	4
Gtf2i	1.27	4.57E-02	18	Hexa	1.38	0.00E+00	22
Oxct1	1.27	0.00E+00	21	Ins1	1.38	1.13E-03	19
Gspt1	1.27	9.00E-05	22	Bckdhb	1.38	3.88E-03	16
Ranbp3	1.27	3.10E-04	11	Aldoart1	1.38	1.00E-05	13
Sltm	1.27	7.89E-03	14	9130011E15Rik	1.38	3.12E-02	11

Table A1, continued. Proteins changed during pancreatic islet cell aging. Blue=down with age, red=up with age.

Protein Name	Fold Change down with age	ANOVA p-value	Total # of Quantitated 14N/15N Pairs	Protein Name	Fold Change up with age	ANOVA p-value	Total # of Quantitated 14N/15N Pairs
EG627352	1.26	3.69E-02	7	Pgm3	1.38	0.00E+00	32
Gls	1.26	1.64E-03	18	Rab2a	1.38	0.00E+00	17
Morf4l1	1.26	3.69E-02	7	Mgll	1.37	4.99E-02	12
Myh11	1.26	2.60E-03	14	Cutc	1.37	5.79E-03	13
Smc3	1.26	4.00E-04	23	Lta4h	1.37	0.00E+00	18
Nrd1	1.26	9.00E-05	13	Hdgfrp3	1.37	1.33E-02	16
Ssrp1	1.26	4.60E-04	15	Scrn2	1.37	5.18E-03	13
Bat1a	1.26	0.00E+00	20	Rogdi	1.37	2.95E-02	8
Sf3b3	1.26	5.00E-05	17	Abhd14b	1.37	0.00E+00	14
Upf1	1.26	0.00E+00	14	Pkp4	1.37	0.00E+00	21
Cotl1	1.26	1.48E-03	14	LOC100047632	1.37	2.70E-02	7
Snrpe	1.26	6.34E-03	9	LOC100047636	1.37	2.70E-02	7
Ppib	1.26	2.09E-03	19	Cops2	1.37	5.60E-04	4
Cugbp1	1.26	1.20E-04	18	Nsf	1.37	2.00E-05	16
Cdc73	1.26	1.36E-02	13	Insrr	1.36	7.00E-05	14
LOC100047915	1.26	5.24E-03	12	Asah2	1.36	1.72E-02	8
Spnb4	1.26	8.30E-03	9	Glt8d1	1.36	4.60E-04	12
LOC100047573	1.26	1.19E-02	17	Eppk1	1.36	1.86E-03	4
Rbp4	1.26	1.19E-02	17	Scarb2	1.36	3.00E-05	13
Snrpd3	1.26	2.00E-02	9	Lmnb2	1.36	3.70E-04	27
Hdgf	1.26	6.78E-03	17	Rab3d	1.36	5.10E-04	16
Ace2	1.26	1.03E-03	17	Tom1l2	1.36	4.00E-05	17
Akr1e1	1.26	2.46E-03	4	Pdcd4	1.36	1.12E-02	10
Ankrd44	1.26	7.98E-03	13	Hspe1	1.35	0.00E+00	14
Atp1a3	1.25	3.22E-02	8	Smpd1	1.35	6.59E-03	10
Sart1	1.25	5.10E-03	12	LOC100048117	1.35	0.00E+00	19
Grb2	1.25	1.31E-02	10	Fech	1.35	0.00E+00	13
Ube4b	1.25	3.35E-03	15	Gcsh	1.35	1.72E-02	7
Spg20	1.25	2.88E-03	10	Sfrp5	1.35	2.22E-03	11
Xpo5	1.25	9.28E-03	21	Snap25	1.35	2.36E-02	9
Thoc5	1.25	9.00E-05	13	Uap1l1	1.35	2.00E-05	13
Actl6a	1.25	8.90E-04	14	Src	1.34	9.50E-04	12
Ap1g1	1.25	0.00E+00	16	Ccdc51	1.34	1.10E-03	7
Ppm1g	1.25	1.87E-02	14	Mif	1.34	4.15E-03	14
Ywhah	1.25	4.00E-05	19	Mrpl37	1.34	8.13E-03	9
Gyg	1.25	1.00E-04	13	Mut	1.34	5.10E-04	14
Mapre1	1.24	8.20E-04	14	Rraga	1.34	2.20E-04	14
Lmf2	1.24	6.88E-03	17	Aldoc	1.34	1.50E-04	14
Zfp516	1.24	5.20E-04	7	Ppm1l	1.34	0.00E+00	13
Dpy30	1.24	6.85E-03	18	Sil1	1.34	3.15E-03	17
Npm1	1.24	0.00E+00	17	SG00000069070	1.34	4.41E-02	32
Isyna1	1.24	1.11E-02	13	Acadsb	1.34	1.60E-03	15
Sf3b1	1.24	3.00E-05	16	Acp2	1.33	0.00E+00	14
100042959	1.24	8.00E-05	19	Fam49b	1.33	1.33E-02	11
Ube3a	1.24	1.38E-02	7	Ghdc	1.33	3.64E-02	9
Rps6ka3	1.24	1.00E-05	14	Lsm4	1.33	2.43E-02	13
Ranbp1	1.24	3.03E-03	12	Osbpl1a	1.33	2.65E-03	9
Saps3	1.24	1.25E-03	18	Plod3	1.33	1.10E-04	18
Hdac1	1.24	6.00E-05	15	Sars	1.33	0.00E+00	28
Cstf2	1.24	2.00E-05	15	Sfxn3	1.33	6.20E-04	12
Nsun2	1.24	2.80E-02	15	Sirt5	1.33	1.28E-02	10
Ddx42	1.24	9.80E-04	14	Tollip	1.33	0.00E+00	13
Son	1.23	4.79E-02	8	Cog8	1.33	6.23E-03	12
Idh1	1.23	3.30E-04	17	Ptprn	1.33	1.40E-04	13

Table A1, continued. Proteins changed during pancreatic islet cell aging. Blue=down with age, red=up with age.

Protein Name	Fold Change down with age	ANOVA p-value	Total # of Quantitated 14N/15N Pairs	Protein Name	Fold Change up with age	ANOVA p-value	Total # of Quantitated 14N/15N Pairs
Ppp6c	1.23	2.95E-02	14	Ano10	1.33	1.67E-03	12
Dak	1.23	8.97E-03	16	Aldoa	1.33	1.04E-03	4
EG665839	1.23	8.30E-04	20	Cox4i1	1.33	0.00E+00	16
Apex1	1.23	8.00E-05	12	Clpp	1.33	1.90E-03	14
Parp1	1.23	1.70E-04	20	Fam62b	1.33	0.00E+00	18
Smarcc2	1.23	1.00E-05	16	Hadhb	1.33	0.00E+00	18
Trnt1	1.23	4.16E-02	12	Nt5c	1.32	3.67E-02	17
Hmgb1	1.23	0.00E+00	21	Yars	1.32	1.30E-04	24
LOC637733	1.23	5.00E-05	17	Ggt7	1.32	5.25E-03	11
Drg2	1.23	1.61E-03	14	Myh7b	1.32	5.25E-03	11
Srm	1.23	4.92E-03	19	Nt5dc3	1.32	3.06E-02	10
Iqgap1	1.23	0.00E+00	16	Hadha	1.32	0.00E+00	23
Tsfm	1.23	4.70E-04	17	Ddt	1.32	0.00E+00	22
Twsg1	1.23	1.63E-03	14	Erp44	1.32	0.00E+00	30
Ddx5	1.23	5.00E-05	14	Atp6v0c	1.32	2.60E-04	12
Ppp2r5c	1.23	1.12E-02	14	Spr	1.32	8.40E-04	15
Chd3	1.22	1.53E-02	12	Tmem55a	1.32	3.33E-02	6
Eif4a3	1.22	2.80E-04	15	Cadps	1.31	0.00E+00	18
Nptn	1.22	4.82E-02	8	Ptpn9	1.31	3.50E-04	12
Rbmx	1.22	5.37E-03	8	Gns	1.31	8.00E-04	17
Slc27a4	1.22	4.75E-02	18	Stx12	1.31	9.94E-03	14
Cpne1	1.22	2.24E-02	8	Psmb10	1.31	2.15E-02	8
Ddx47	1.22	1.00E-03	11	Grhpr	1.31	3.10E-04	17
Pfdn5	1.22	2.87E-02	14	Hsdl1	1.31	2.51E-02	8
Pofut2	1.22	2.28E-02	14	Mtap4	1.31	3.85E-02	17
Eif4g1	1.22	1.13E-03	12	Tor1aip1	1.31	2.87E-03	13
Rpia	1.22	1.11E-03	14	Hist2h2aa1	1.31	1.00E-05	25
100041985	1.22	1.12E-03	17	Atp6v1e1	1.31	2.00E-05	15
Capza1	1.22	1.12E-03	17	Cars	1.31	4.90E-04	16
Spcs3	1.22	3.40E-02	10	Vamp3	1.31	6.00E-05	13
LOC100039215	1.22	1.22E-02	14	Fn3krp	1.31	7.01E-03	4
Tbcb	1.22	7.52E-03	6	Mdh1	1.31	0.00E+00	27
Tsnax	1.22	1.22E-02	14	Acad10	1.31	0.00E+00	22
Trip12	1.22	4.18E-02	18	Grn	1.31	6.10E-04	14
Mybbp1a	1.22	2.00E-05	26	Lace1	1.31	2.52E-03	12
Srrm1	1.21	1.32E-03	12	Pcx	1.31	0.00E+00	21
Ap1m2	1.21	4.50E-02	18	Fdxr	1.30	1.40E-03	17
Dcps	1.21	1.00E-05	14	Mgat2	1.30	1.85E-03	14
Hnrnpr	1.21	7.50E-04	19	Bphl	1.30	0.00E+00	18
Dbr1	1.21	1.56E-02	12	Mrps30	1.30	1.54E-03	14
Npepl1	1.21	6.48E-03	17	Slc25a1	1.30	0.00E+00	16
Pex19	1.21	2.20E-04	11	1300010F03Rik	1.30	0.00E+00	20
LOC637796	1.21	4.29E-02	11	Fabp3	1.30	2.22E-03	10
Mff	1.21	4.29E-02	11	LOC100047867	1.30	2.22E-03	10
Ak1	1.21	0.00E+00	22	Rab5a	1.30	3.80E-04	16
Hnrpdl	1.21	1.10E-04	14	Gatm	1.29	1.28E-02	23
Me2	1.21	3.26E-02	14	Gpx1	1.29	0.00E+00	17
Hnrnpk	1.21	1.60E-04	8	Abat	1.29	0.00E+00	22
Myl12b	1.21	1.23E-02	13	Itga1	1.29	4.76E-02	7
Prpf19	1.21	0.00E+00	21	0610011F06Rik	1.29	2.70E-04	14
Cdc5l	1.21	1.30E-04	17	Ndufb7	1.29	2.53E-02	14
2900073G15Rik	1.21	1.32E-02	10	Trmu	1.29	1.80E-02	9
Anp32a	1.21	2.94E-03	18	Rtn4	1.29	1.65E-03	19
Gimap4	1.21	2.00E-02	13	Atp9a	1.29	1.01E-03	14

Table A1, continued. Proteins changed during pancreatic islet cell aging. Blue=down with age, red=up with age.

Protein Name	Fold Change down with age	ANOVA p-value	Total # of Quantitated 14N/15N Pairs	Protein Name	Fold Change up with age	ANOVA p-value	Total # of Quantitated 14N/15N Pairs
Ddx17	1.21	0.00E+00	17	Adk	1.27	1.38E-02	16
Exosc4	1.21	4.72E-02	11	Sst	1.27	9.93E-03	18
Impa1	1.21	1.03E-03	4	Ctage5	1.26	1.90E-03	22
Dnaja1	1.21	1.12E-03	22	Casc4	1.26	9.86E-03	16
Hnrnph1	1.21	0.00E+00	17	Nit1	1.26	5.62E-03	4
Rpl10a	1.21	2.00E-05	7	Skp1a	1.26	2.10E-02	16
Pds5a	1.21	2.38E-02	14	Anxa7	1.26	2.00E-05	17
Cnot1	1.20	1.59E-02	10	Dnm3	1.26	2.02E-02	14
Hccs	1.20	2.33E-02	8	Lonp1	1.26	2.00E-05	19
Wdr42a	1.20	1.58E-02	12	Dgke	1.26	9.84E-03	11
Shc1	1.20	4.44E-02	8	Epb4.1l3	1.26	1.64E-02	13
Snrnp200	1.20	0.00E+00	24	Mvp	1.26	5.00E-05	14
Eef2	1.20	0.00E+00	21	9330129D05Rik	1.26	1.31E-03	14
Smu1	1.20	3.33E-03	11	Mrpl12	1.26	1.60E-04	14
Tjp1	1.20	3.15E-03	14	Wdr7	1.26	8.50E-04	16
Protein Name	**Fold Change up with age**	**ANOVA p-value**	**Total # of Quantitated 14N/15N Pairs**	Nars	1.26	0.00E+00	31
				Ndufb10	1.26	1.00E-05	16
				Ehd1	1.25	2.10E-02	4
				Bpnt1	1.25	1.00E-05	24
				Auh	1.25	0.00E+00	14
Gorasp2	1.29	4.00E-03	15	Lman2	1.25	1.00E-05	19
Cox5a	1.28	0.00E+00	18	Lypla1	1.25	1.28E-02	13
Acly	1.28	0.00E+00	23	Ndufv3	1.25	5.90E-04	16
Cmbl	1.28	5.43E-03	14	Glg1	1.25	4.00E-05	18
Mboat7	1.28	3.82E-02	6	Nhp2	1.25	2.79E-03	11
Ndufb4	1.28	3.00E-05	16	Pip4k2c	1.25	2.32E-02	15
Chid1	1.28	3.00E-05	14	Tprkb	1.25	1.21E-02	8
BC017158	1.28	3.95E-02	17	Tspo	1.25	4.02E-02	4
Acadl	1.28	6.00E-05	15	Hist2h2ab	1.25	0.00E+00	32
Capns1	1.28	4.69E-02	4	Dpm1	1.25	5.00E-04	18
Ddhd2	1.28	2.86E-02	12	Slc25a12	1.25	0.00E+00	21
Gosr2	1.28	2.00E-05	16	Prdx6	1.25	0.00E+00	18
Itpkb	1.28	4.27E-03	10	Prps2	1.25	3.22E-03	14
Sdha	1.28	2.00E-05	16	Sacm1l	1.25	1.20E-04	22
6330409N04Rik	1.28	2.25E-02	12	Fam151a	1.25	3.00E-05	16
Acat1	1.28	0.00E+00	23	Sdf4	1.25	1.66E-02	18
Ddi2	1.28	1.39E-02	13	2810407C02Rik	1.25	2.49E-02	16
Pgm1	1.28	1.50E-04	14	Ahcyl2	1.24	4.00E-04	14
Rab7	1.28	3.10E-04	19	Man2b1	1.24	1.00E-05	28
Pdk3	1.27	5.00E-05	14	Pdha1	1.24	0.00E+00	23
Samhd1	1.27	3.40E-04	21	Rab3gap1	1.24	1.00E-05	20
St13	1.27	2.00E-05	14	Stx18	1.24	4.41E-02	11
Tor1b	1.27	9.00E-05	16	Gfm1	1.24	2.18E-02	14
Arl8b	1.27	6.50E-03	14	Hspa9	1.24	0.00E+00	18
Dmxl2	1.27	7.66E-03	17	Tufm	1.24	0.00E+00	24
Aldh2	1.27	0.00E+00	21	Ganab	1.24	0.00E+00	26
Dennd4c	1.27	4.70E-04	14	Lactb2	1.24	3.29E-03	13
Gmpr	1.27	2.60E-04	14	Ptplad1	1.24	1.00E-05	20
Stxbp1	1.27	0.00E+00	23	Agpat1	1.24	2.49E-03	13
Clu	1.27	2.40E-04	16	Gnal1	1.24	5.29E-03	15
Atp6ap1	1.27	1.00E-05	18	Ahcyl1	1.24	2.00E-05	17
Avl9	1.27	5.50E-03	14	Ndufs4	1.24	2.00E-05	16
Hax1	1.27	4.14E-03	11	Coasy	1.24	7.00E-04	23
Gcdh	1.27	0.00E+00	19	Slc2a2	1.24	1.70E-02	16

Table A1, continued. Proteins changed during pancreatic islet cell aging. Blue=down with age, red=up with age.

Protein Name	Fold Change up with age	ANOVA p-value	Total # of Quantitated 14N/15N Pairs	Protein Name	Fold Change up with age	ANOVA p-value	Total # of Quantitated 14N/15N Pairs
Immt	1.24	2.60E-04	11	Glrx5	1.22	3.56E-02	11
1810034K20Rik	1.24	2.57E-03	12	Dlat	1.22	6.00E-05	17
Napg	1.24	1.87E-03	14	Fbl	1.22	2.71E-02	13
Gnpda2	1.24	2.99E-02	14	Tfam	1.22	4.14E-02	9
Ptprn2	1.24	0.00E+00	34	Dctn4	1.22	4.96E-02	14
Cox5b	1.23	6.02E-03	16	Uchl3	1.22	3.00E-02	14
Larp1	1.23	4.20E-04	16	Scg5	1.21	3.56E-02	23
Park7	1.23	1.00E-05	23	Qsox2	1.21	1.52E-02	14
Txndc17	1.23	1.66E-02	13	Arl6ip5	1.21	1.40E-04	13
Aldh3a2	1.23	1.00E-05	21	Slc25a5	1.21	0.00E+00	27
Alg2	1.23	0.00E+00	30	Ampd2	1.21	2.00E-05	14
Fh1	1.23	1.20E-04	16	Ndufa8	1.21	0.00E+00	14
Fhit	1.23	2.26E-03	4	Prkca	1.21	2.00E-05	17
Hibch	1.23	0.00E+00	20	Prkcb	1.21	1.00E-04	12
Myo1c	1.23	1.52E-03	20	Gba	1.21	1.04E-03	14
Ppa2	1.23	7.00E-05	14	Cds2	1.21	6.70E-04	12
Golph3	1.23	2.00E-04	17	Cln6	1.21	4.65E-03	12
Idh3g	1.23	6.30E-03	21	Pcbd1	1.21	2.58E-03	16
Tmed10	1.23	3.00E-05	19	Nop56	1.21	2.14E-03	13
Dnaja4	1.23	2.12E-03	23	AU021838	1.21	0.00E+00	28
mt-Co2	1.23	8.00E-05	18	Iah1	1.21	1.01E-02	16
Apoa1bp	1.23	0.00E+00	17	Mapksp1	1.21	8.60E-04	12
Gng12	1.23	3.23E-03	9	Nbas	1.21	1.00E-05	19
Fuk	1.23	6.20E-04	14	Prps1	1.21	0.00E+00	28
Ndufs1	1.23	0.00E+00	20	Ndufa12	1.21	5.90E-04	19
Scfd2	1.23	2.15E-03	14	Cmas	1.21	3.65E-02	13
Aars	1.22	1.00E-04	23	Mbc2	1.21	3.73E-03	16
Ndufa9	1.22	0.00E+00	20	Pebp1	1.21	1.00E-04	22
Cenpv	1.22	9.00E-05	17	Fkbp2	1.21	9.20E-04	18
E430025E21Rik	1.22	6.29E-03	14	Ndufv2	1.21	2.00E-04	16
H2afy	1.22	3.00E-05	22	Rint1	1.21	2.60E-03	13
Myo18a	1.22	3.85E-03	19	Samm50	1.21	1.84E-03	17
Nucb1	1.22	0.00E+00	21	Ndufb8	1.21	4.57E-03	14
Pfas	1.22	1.00E-05	18	LOC100047696	1.21	8.50E-04	6
Tmem63a	1.22	9.00E-05	16	Prosc	1.20	1.53E-02	16
Sirt2	1.22	1.83E-03	13	Lman1	1.20	1.71E-03	18
Arfgef2	1.22	7.50E-04	17	Sh3glb2	1.20	1.47E-02	11
Plod1	1.22	2.32E-02	16	Ndufb6	1.20	1.00E-05	11
2310005E10Rik	1.22	3.00E-04	19	Actr10	1.20	3.98E-03	14
Cplx2	1.22	5.80E-04	24	Atp6v1h	1.20	1.00E-05	16
Pcsk1n	1.22	1.30E-04	16	Dhx32	1.20	2.68E-02	16
Qpctl	1.22	4.26E-02	7	Pgrmc2	1.20	1.00E-05	16
Cd2ap	1.22	1.87E-02	14				

APPENDIX B

Table of human islet cadaver donor information

Table B1 Characteristics of human islet donors used for *in vitro* beta cell proliferation and insulin secretion experiments (related to Figure 4.2-4.8).

UNOS ID	Gender	Age	Body Mass Index	Race
AAI1303	M	53	27.2	Caucasian
AAJ1433	F	50	27.8	Caucasian
ABAW280	M	40	35.4	Caucasian
ABB2090	M	56	23.8	Hispanic/Latino
ABDE148	F	47	22	Asian
ABDV142	M	32	23	Caucasian
ABEI419A	M	57	29.8	Caucasian
ABE1388	M	59	21.5	Hispanic/Latino
ABGY290	F	49	36.9	Caucasian
ABHV347	F	61	31	African American
ABIT447	M	52	36.7	African American
ABJE155	F	46	20	African American
ABJU206A	F	52	31	Caucasian
ABKI103	M	29	32.6	Caucasian
ABK1181	M	50	31.7	African American
ACA1298	F	23	24.5	Caucasian
ACBZ224A	F	24	35.3	Caucasian
ACCQ267	M	35	32.6	Caucasian
ACCV204A	M	19	20	Caucasian
ACD1125A	M	68	26.7	Caucasian
ACFE127A	M	23	33.6	Hispanic/Latino
ACFV445B	M	21	23.9	Caucasian
ACHI421	F	30	18.4	Caucasian
ACIN402	M	49	40.1	Hispanic/Latino
ACJV169	M	26	44.8	African American
ACJY368A	F	42	23.1	Caucasian
ADAM295	F	56	33.4	African American
ADCE360A	M	20	24.4	Caucasian
ADDM439	M	36	28.5	Caucasian

APPENDIX C

Antibodies used for immunofluorescence and western blot analysis

Table C1 List of primary antibodies used in immunofluorescence staining and Western blot analysis.

Primary Antibodies				
Antigen	**Host**	**Dilution**	**Source**	**Catalogue #**
Insulin	Guinea Pig	1:1000	Dakocytomation	A0564
Glucagon	Goat	1:1000	Santa Cruz Biotechnology	SC-7780
Nkx6.1	Rabbit	1:250	Lifespan Biosciences	LS-C143534
GFP	Goat	1:500	Abcam	Ab6673
BrdU	Rat	1:200	Novus Biologicals	NB500-169
Ki67	Rabbit	1:200	Lab Vision	RM-9106-S0
γH2AX	Rabbit	1:100	Cell Signaling	2577
Sirt2	Rabbit	1:250	Santa Cruz Biotechnology	SC-20966
Beta-Tubulin	Mouse	1:1000	Sigma Aldrich	T5201
Erk	Mouse	1:5000	BD Transduction Lab	610123
Phospho-Erk1/2	Rabbit	1:500	Cell Signaling	4370

Table C2 List of secondary antibodies used in immunofluorescence staining and Western blot analysis.

Secondary Antibodies			
Antigen	**Conjugation**	**Dilution**	**Source**
Rabbit/Goat/Guinea Pig/Rat/Mouse	Alexa-488	1:2000	Jackson Immunoresearch
Rabbit/Goat/Guinea Pig/Rat/Mouse	Cy3	1:2000	Jackson Immunoresearch
Rabbit/Goat/Guinea Pig/Rat/Mouse	Cy5	1:500	Jackson Immunoresearch
Rabbit/Mouse	HRP	1:5000	GE Healthcare

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