EFFECTS OF INSULIN GENE DOSAGE
ON MURINE OBESITY AND LIFESPAN

by

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Abstract

There are numerous parallels between aging and obesity, and insulin may play a crucial role in modulating both conditions. For instance, elevated insulin levels are closely associated with obesity, although the causal role of insulin hypersecretion in the development of obesity remains controversial. Interestingly, genetically reducing components of insulin/insulin-like growth factor (IGF)-1 signaling can increase lifespan in invertebrates and mammals. However, impaired insulin-stimulated glucose disposal is a form of decreased insulin signaling that is paradoxically a detrimental feature of mammalian aging, whereas long-living mammals often show enhanced responsiveness to insulin stimulation. Therefore, the role of insulin/IGF-1 signaling for mammalian longevity, and the relative functions of the insulin and IGF-1 ligands, are still unclear. The lifelong effects of moderately decreasing insulin production in mammals had not been directly tested. In this dissertation, the goal was to further delineate effects of lowering insulin levels on obesity and metabolic health across the lifespan of a mammalian model organism, culminating in an evaluation of longevity. We used a model in which the rodent-specific insulin gene was fully inactivated (Ins1-null), and compared mice with full or partial expression of the ancestral insulin gene (Ins2). Male and female Ins1⁻/⁻:Ins2⁻/⁻ and Ins1⁻/⁻:Ins2⁺/⁻ littermates were fed a chow diet or high fat diet, and were evaluated across their lifetime to determine long-term effects of reducing insulin gene dosage on obesity, glucose homeostasis, and other physiological parameters. The studies herein show that murine insulin levels and metabolic homeostasis might be regulated in a sex-specific, environmentally-dependent manner, since inactivating one Ins2 allele unexpectedly did not cause a consistent reduction of circulating insulin in Ins1-null male mice, and we observed cross-cohort hyper-variability in circulating insulin of male mice. However, limiting insulin hypersecretion in young, growing female mice can confer long-term protection against obesity. Furthermore, we found that lowering circulating insulin has the potential to improve glucose homeostasis and insulin sensitivity in advanced age, as well as lead to lifespan extension in mammals. To our knowledge, these studies are the first to demonstrate that a targeted, moderate reduction of insulin may be sufficient to promote healthier aging and extend lifespan in mammals.
Preface

The studies presented in this dissertation were principally designed by the author, Nicole Templeman. The majority of the experiments and data analysis were also performed by Nicole Templeman, with assistance on various aspects as outlined below.

Xiaoke Hu assisted with *in vivo* animal work, as well as with the tissue harvests and islet isolations, and she also performed the islet perifusion experiments. Farnaz Taghizadeh also assisted with tissue harvests and islet isolations. Arya Mehran provided technical training and assistance with early *in vivo* animal work, and both Derek Dionne and Melissa Page were available to assist with animal monitoring when needed. Shannon O’Dwyer (from the laboratory of Timothy Kieffer) performed the surgical work for subcutaneously implanting mini osmotic pumps into a group of mice. Subashini Karunakaran (from the laboratory of Susanne Clee) provided extensive assistance for the metabolic cage experiments. Both Timothy Kieffer and Susanne Clee made laboratory equipment available, and provided expertise. The animal technician staff at in the Centre for Disease Modeling facility, with assistance from the veterinarian team, helped with animal health monitoring and care. In addition, Jana Hodasova and the Animal Care Services Diagnostic Laboratory assisted with necropsies and tissue preparation for histopathology. Nick Nation was the primary veterinary pathologist, and he provided very comprehensive information.

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Versions of Chapter 1, Chapter 4, and Chapter 5 will be submitted to peer-reviewed journals in the upcoming year.
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<th>Description</th>
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<tbody>
<tr>
<td>AMPK</td>
<td>AMP-activated protein kinase</td>
</tr>
<tr>
<td>ANCOVA</td>
<td>Analysis of covariance</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>AOC</td>
<td>Area over the curve</td>
</tr>
<tr>
<td>AUC</td>
<td>Area under the curve</td>
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<tr>
<td>AS160</td>
<td>AKT substrate of 160 kDa</td>
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<tr>
<td>BAD</td>
<td>Bcl-2-associated death promoter</td>
</tr>
<tr>
<td>BAT</td>
<td>Brown adipose tissue</td>
</tr>
<tr>
<td>CD</td>
<td>Chow diet</td>
</tr>
<tr>
<td>C/EBP</td>
<td>CCAAT/enhancer binding protein</td>
</tr>
<tr>
<td>DEXA</td>
<td>Dual-energy X-ray absorptiometry</td>
</tr>
<tr>
<td>ERK</td>
<td>Extracellular signal-regulated kinase</td>
</tr>
<tr>
<td>FOXO</td>
<td>Class O forkhead box</td>
</tr>
<tr>
<td>GH</td>
<td>Growth hormone</td>
</tr>
<tr>
<td>GIP</td>
<td>Glucose-dependent insulinotropic polypeptide</td>
</tr>
<tr>
<td>Glu</td>
<td>Glucose</td>
</tr>
<tr>
<td>GLUT</td>
<td>Glucose transporter</td>
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<tr>
<td>HFD</td>
<td>High fat diet</td>
</tr>
<tr>
<td>HOMA-IR</td>
<td>Homeostatic model assessment of insulin resistance</td>
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<tr>
<td>IGF</td>
<td>Insulin-like growth factor</td>
</tr>
<tr>
<td>IGF-1R</td>
<td>Insulin-like growth factor (IGF) 1 receptor</td>
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<tr>
<td>Term</td>
<td>Description</td>
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<td>--------------</td>
<td>---------------------------------------------------------</td>
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<tr>
<td>INSULIN2</td>
<td>Synthesized murine insulin 2 peptide</td>
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<tr>
<td>IR</td>
<td>Insulin receptor</td>
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<tr>
<td>IRS</td>
<td>Insulin receptor substrate</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen-activated protein kinase</td>
</tr>
<tr>
<td>MEK</td>
<td>Mitogen-activated protein kinase (MAPK) kinase</td>
</tr>
<tr>
<td>mTOR</td>
<td>Mechanistic target of rapamycin</td>
</tr>
<tr>
<td>mTORC1</td>
<td>Mechanistic target of rapamycin (mTOR) complex 1</td>
</tr>
<tr>
<td>NEFA</td>
<td>Non-esterified fatty acid</td>
</tr>
<tr>
<td>PI3K</td>
<td>Phosphatidylinositol 3-kinase</td>
</tr>
<tr>
<td>PI(3,4,5)P₃</td>
<td>Phosphotidylinositol 3,4,5-triphosphate</td>
</tr>
<tr>
<td>PI(4,5)P₂</td>
<td>Phosphotidylinositol 4,5-biphosphate</td>
</tr>
<tr>
<td>PPAR</td>
<td>Peroxisome proliferator-activated receptor</td>
</tr>
<tr>
<td>PTEN</td>
<td>Phosphatase and tensin homolog</td>
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<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
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<tr>
<td>SOS</td>
<td>Son-of-sevenless</td>
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<tr>
<td>SPF</td>
<td>Specific pathogen-free</td>
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<tr>
<td>TSC2</td>
<td>Tuberous sclerosis complex-2</td>
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<tr>
<td>WAT</td>
<td>White adipose tissue</td>
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Chapter 1: Introduction

1.1 Biological aging

Human demographics are shifting. In 2011, there were approximately 800 million people aged 60 or older; by 2050 the “aged” proportion of the population is expected to have doubled, with 22% of the world’s population – over 2 billion people – projected to be 60 years or older.\textsuperscript{1} Given these circumstances, it is becoming more important to identify means to extend the period of life spent generally healthy (referred to as the healthspan), and delay the onset of age-associated disease.\textsuperscript{2}

In this body of work, the term ‘aging’ is used in the context of growing old, or senescence. It is thereby defined as a progressive physiological decline after reproductive maturation, which is characterized by accrual of molecular and cellular damage that leads to tissue detriment, increased susceptibility to disease, and eventually death.\textsuperscript{2-6} This physiological deterioration is highlighted in the classic physical characteristics of advanced age, including decreased bone mass density and stature, reduced skeletal muscle strength and mass that corresponds with proportionally increased adiposity, thinner and less-elastic skin, impaired immune function that is linked to chronic inflammation, and diminished hearing, eyesight, and cognitive function.\textsuperscript{2,3,7} Many diseases associated with aging, such as type 2 diabetes, osteoporosis, arthritis, Alzheimer’s disease, cancers, and cardiovascular disease, are reflective of changed body composition, reduced immune function, or other progressive degeneration.\textsuperscript{3,8,9}

Thus, to facilitate healthy aging as a whole it is necessary to uncover root causes and mechanisms of the physiological decline of senescence, rather than solely respond to its associated conditions on an independent basis.\textsuperscript{8} To this end, the characterization of generalized
aging often highlights such hallmarks as mitochondrial dysfunction, genomic instability, epigenetic changes, cellular senescence, loss of protein homeostasis, and impairment of metabolic homeostasis.\textsuperscript{10-13}

1.1.1 Oxidative damage and mitochondrial dysfunction

Harman proposed the free radical/oxidative stress theory in 1956 as a potential explanation of the root source of aging. This well-known theory suggests that biological decline could result from accumulated oxidative damage to cellular macromolecules, caused by reactive oxygen species (ROS) which are endogenously produced through aerobic metabolism.\textsuperscript{14} Indeed, aging is widely associated with increasing ROS production and oxidative damage, concurrent with declining mitochondrial function.\textsuperscript{15} Many long-lived organisms exhibit oxidative stress resistance or diminished oxidative damage, although this is not universal.\textsuperscript{15} There is evidence that increasing expression of antioxidant defenses to alleviate oxidative damage can mitigate some age-associated pathological deterioration; however, this is not always sufficient to extend mammalian lifespan.\textsuperscript{16-18} Moreover, decreasing antioxidant defenses can impart increased sensitivity to oxidative stress without reducing lifespan in mice.\textsuperscript{17,18} It is possible that restricting an excess of ROS production would be more effective at imparting lifespan extension than elevating antioxidant defenses,\textsuperscript{19} or that ROS production has varying age- and tissue-specific effects.\textsuperscript{18} Indeed, acutely produced physiological levels of ROS have regulatory roles in normal cellular function, including roles in apoptosis, cell cycle progression, and as messengers or modulators of some signaling pathways.\textsuperscript{15,20} Therefore, while oxidative damage may contribute to aging, the original free radical/oxidative stress theory does not account for all of the associated complexities of ROS.
Mitochondrial dysfunction could also have detrimental effects independent of large-scale ROS production. For instance, mice that accrue mitochondrial DNA mutations due to defective mitochondrial polymerase activity exhibit accelerated aging without systemically elevated ROS production or oxidative damage. Mitochondria play a crucial role in energy homeostasis and pathways facilitating nutrient utilization, as well as in such processes as apoptosis. Accordingly, there are numerous means by which mitochondrial dysfunction could contribute to the physiological decline of aging.

1.1.2 Genomic or epigenomic instability, and cellular senescence

The accumulation of DNA damage is another hallmark of aging that may facilitate systemic deterioration. Transgenic mice with enhanced preservation of genomic integrity due to an overexpressed mitotic regulator are protected against tumourigenesis and age-related tissue decline, and have a longer lifespan. Nuclear DNA damage can be a downstream outcome of oxidative stress, but it can also result from exogenous physical, chemical, and biological insults, or replication errors and spontaneous mutations. In addition, epigenetic modifications shift with the advancement of age, with changes to DNA methylation patterns and chromatin architecture that can reveal previously closed regions of DNA for mutation or transcription, among other potential outcomes. Another form of damage that is associated with aging is the progressive shortening of telomeres, structures at the terminal ends of chromosomes that are integral for maintaining genomic stability. Telomere erosion can play a causal role in driving aging-related pathologies and mortality.

DNA damage or telomere attrition can cause replication-competent cells to enter a state of cellular senescence, or permanent growth arrest. While cellular senescence is likely a protective response to a variety of stresses, as it leads to the removal of damaged or potentially
oncogenic cells, the accumulation of senescent cells also perpetuates biological aging.\textsuperscript{12,27} For instance, somatic stem cells tend to undergo a decline in function with aging, and the accumulation of senescent stem cells represents a diminished regenerative capacity for certain tissues or cell types.\textsuperscript{12,28} In addition, senescent cells secrete an array of chemokines, proteases, and proinflammatory cytokines, which likely contribute to the detrimental chronic inflammation of advanced age.\textsuperscript{27} Interestingly, the inducible removal of senescent cells in a mouse model of progeria (rapid biological aging) delays the onset of age-related deterioration in a number of different tissues.\textsuperscript{29} Thus, cellular senescence represents a protective response to prevent such pathologies as cancers, but comes at the cost of potentially facilitating other forms of aging-related physiological deterioration.

1.1.3 Decreased protein homeostasis

Deregulated protein homeostasis can also have far-reaching consequences in the progressive decline of aging. Protein misfolding or protein aggregation can arise from numerous causes, from genetic mutations or translational errors to oxidation or other post-translational modifications.\textsuperscript{30} Since build-up of damaged proteins is cytotoxic, there is an extensive protective network in place to prevent protein damage, as well as repair or remove damaged proteins.\textsuperscript{30} This protective system depends on contributions by molecular chaperones, the endoplasmic reticulum unfolded protein response, the ubiquitin-proteasome system, and autophagy.\textsuperscript{12,30} If these mechanisms collectively fail at maintaining protein homeostasis, the result is the accumulation of damaged proteins, which is characteristic of aging.\textsuperscript{12,30} Mice with systemic deletion of a co-chaperone/ubiquitin ligase show accelerated aging that is characterized by increased cellular senescence and oxidative damage, as well as a significantly reduced lifespan.\textsuperscript{31} Indeed, protein
aggregation is a key feature of several neurodegenerative diseases that are predominantly associated with advanced age.\textsuperscript{30,32}

Protein damage can both result from and further perpetuate genomic instability or mitochondrial dysfunction.\textsuperscript{12} This highlights the large degree of integration that can be detected between many of the features believed to contribute to the aging process.\textsuperscript{12} The interrelated connections between the hallmarks of aging add ambiguity as to which features might play more causative upstream roles in the progressive physiological decline.

\textbf{1.1.4 Evolutionary theories of aging}

At a demographic level, aging is characterized by an accelerating risk of mortality after reproductive maturation, accompanied by reduced fecundity.\textsuperscript{4,33} The disposable soma theory suggests that evolutionary selection favouring reproductive fitness may ultimately contribute to the detriment of aging, due to an energetic trade-off shuttling resources towards reproduction rather than somatic maintenance.\textsuperscript{34} This is an extension of antagonistic pleiotropy, a concept whereby genes that have deleterious late-life effects might remain in a population if they also play beneficial roles early in life.\textsuperscript{35} Alternatively, the mutation accumulation theory posits that multiple harmful mutations with detrimental effects that are only evident late in life could be transferred passively to offspring, since natural selection is expected to exert most of its influences in the early ages when an organism has a greater chance of reproduction.\textsuperscript{36}

Based on evolutionary theories such as these, it was once widely believed that genetic mechanisms to modulate aging on a systemic level and prolong post-reproductive life could not have evolved.\textsuperscript{4,37,38} This was challenged by the discovery that single gene mutations could dramatically extend lifespan in the nematode \textit{Caenorhabditis elegans},\textsuperscript{39,40} and moreover, do so without always requiring a reproductive trade-off.\textsuperscript{4,33,38,40} The earliest lifespan-extension genes
that were uncovered\cite{39,40} were found to mediate insulin/insulin-like growth factor (IGF)-1–like signaling,\cite{41,42} and in fact many of the genes later revealed to impact longevity operate in the regulation of metabolism and energy homeostasis.\cite{5,43,45} Therefore, nutrient sensing networks such as the insulin/IGF-1–like signaling pathway have been identified as integral components in the physiological deterioration of aging.\cite{10,12,13}

### 1.2 Insulin/IGF-1–like signaling in invertebrates

The eventual cloning and molecular identification of these detected ‘lifespan-extension genes’ in *C. elegans* contributed to revealing the remarkable extent of the evolutionary conservation of the insulin/IGF-1 signaling pathway (Fig. 1.1). This pathway plays a critical role in coordinating nutrient availability with growth, metabolism, and energy balance in diverse species.\cite{5,43,44} Invertebrates generate numerous insulin-like peptides: there are at least 38 genes encoding putative insulin-like peptides in *C. elegans*,\cite{46,47} and eight known genes in *Drosophila melanogaster*.\cite{48,49} In both *C. elegans* and *D. melanogaster*, insulin-like peptides are released in response to sensory stimulation, and bind to a transmembrane tyrosine kinase receptor (encoded by *daf-2* in *C. elegans*,\cite{42} *dInR* in *D. melanogaster*;\cite{50} Fig. 1.1).\cite{44,51,52} The activated receptor recruits and activates effector phosphatidylinositol 3-kinase (PI3K, catalytic subunit encoded by *age-1* in *C. elegans*\cite{41}), either directly or utilizing a receptor substrate intermediate (encoded by *chico* in *D. melanogaster*;\cite{53} Fig. 1.1).\cite{44,51,52} Activated PI3K then converts phosphatidylinositol 4,5-biphosphate into second messenger phosphatidylinositol 3,4,5-triphosphate; this key step is also subject to negative regulation, by a phospholipid phosphatase analogous to the mammalian phosphatase and tensin homolog (PTEN, encoded by *daf-18* in *C. elegans*,\cite{54} *dPTEN* in *D. melanogaster*;\cite{55} Fig. 1.1).\cite{44,51,52} The second messenger stimulates recruitment and activation of
3-phosphoinositide dependent protein kinase-1 and its substrate, serine/threonine kinase AKT (Fig. 1.1).\textsuperscript{44,51,52} Once activated through phosphorylation, AKT phosphorylates many substrates important for cell survival, growth, and metabolic processes (Fig. 1.1).\textsuperscript{44,51,52} One key target is a homologue of the mammalian class O forkhead box (FOXO) family\textsuperscript{44,51,52} (encoded by \textit{daf-16} in \textit{C. elegans}, \textsuperscript{56,57} \textit{dFOXO} in \textit{D. melanogaster}\textsuperscript{58}), a transcription factor which is phosphorylated and excluded from the nucleus in response to insulin/IGF-1–like signaling (Fig. 1.1).\textsuperscript{59-61}

Reduction-of-function mutations of several intermediates in this signaling pathway can result in as much as a doubling of lifespan in \textit{C. elegans},\textsuperscript{39,40} and longevity can be extended by 40-85\% in \textit{D. melanogaster}, primarily in the female flies.\textsuperscript{62,63} Manipulations to achieve lifespan extension include reduction-of-function mutation of genes encoding the tyrosine kinase receptor \textit{daf-2}\textsuperscript{40} or \textit{dInR},\textsuperscript{62} heterozygous mutation of the receptor substrate \textit{chico},\textsuperscript{63,64} and reduction-of-function mutation of the catalytic subunit of PI3K \textit{age-1}.\textsuperscript{39} Concurrent reduction-of-function mutation of this pathway’s negative regulator \textit{daf-18} counteracts the lifespan expansion in \textit{C. elegans},\textsuperscript{65,66} whereas overexpression of the homologous phospholipid phosphatase \textit{dPTEN} in the fat body of \textit{D. melanogaster} can lead to extended lifespan.\textsuperscript{67} Ablation of cells that produce insulin-like peptide ligands in \textit{D. melanogaster} can also extend lifespan.\textsuperscript{68}

In both \textit{C. elegans}\textsuperscript{57,65,66} and \textit{D. melanogaster},\textsuperscript{69} the functional presence of a homologue of the mammalian FOXO family seems to be key for extending lifespan through these manipulations, and increasing \textit{dFOXO} expression in fat body alone leads to increased lifespan in \textit{D. melanogaster}.\textsuperscript{67,70} Nuclear activity of the FOXO transcription factors and their invertebrate homologues has been associated with: 1) altering metabolic processes, such as gluconeogenesis, and providing feedback control of insulin/IGF-1–like signaling; 2) imparting resistance to cell stress through DNA repair, oxidative stress protection, and promotion of other stress-resistant
programs; 3) promoting cell cycle arrest or cell death; and 4) reducing cellular protein flux by repressing translation initiation.\textsuperscript{52,71-75} Therefore, the lifespan-extending effects of reduced invertebrate insulin/IGF-1–like signaling could occur through modulation of multiple facets of aging, including metabolic processes, oxidative stress, DNA damage, cellular senescence, and protein homeostasis.

1.3 Insulin/IGF-1 signaling in mammals

1.3.1 Insulin

Insulin/IGF-1 signaling in mammals has a fundamental role in maintaining fuel homeostasis and energy balance, similar to the functions in invertebrates.\textsuperscript{5,43,44} The discovery of the hormone insulin in 1921-1922 was a major biological and medical advancement. In the late 1800’s it was determined by Minkowski and von Mering that the pancreas plays a pivotal function in the regulation of carbohydrate metabolism, since removal of the pancreas in dogs led to symptoms of severe diabetes mellitus – specifically, elevated blood glucose, glycosuria, and ketonuria, as well as weight loss and polyuria despite substantial food and water intake.\textsuperscript{76} The islets of Langerhans were eventually identified as the specific anatomical regions of the pancreas likely to influence carbohydrate metabolism, as it was observed by several groups that pancreatic duct ligation and acinar exocrine tissue degradation was not associated with diabetes as long as the islets were intact.\textsuperscript{77} Therefore, it seemed that the islet tissue might secrete a factor internally to control blood glucose levels.\textsuperscript{77} The collaborative work of Banting, Best, Macleod, and Collip was successful in extracting and purifying a pancreatic substance that was shown to treat the clinical symptoms of diabetic patients in 1922.\textsuperscript{78} The effective component of the pancreatic extraction was insulin.
In mammals, insulin is primarily secreted by pancreatic β cells in the islets of Langerhans, generally in response to elevated blood glucose in the postabsorptive state.\textsuperscript{79-81} Circulating insulin promotes nutrient uptake and storage in its major peripheral targets, primarily by: 1) stimulating glucose uptake in adipose tissue and muscle; 2) stimulating glycolysis and glycogen synthesis in muscle and liver, lipogenesis in adipose tissue and liver, and protein synthesis in muscle and liver, to store the influx of nutrients; and lastly 3) repressing glycogenolysis, lipolysis, and protein breakdown, as well as suppressing gluconeogenesis and ketogenesis in the liver, to prevent further release of nutrients into circulation.\textsuperscript{79-81} Insulin also influences metabolism and energy balance through more indirect means, such as modulating the central regulation of metabolic processes and nutrient intake,\textsuperscript{82,83} and exerting local effects to promote β cell growth and survival, as well as influencing its own production and secretion.\textsuperscript{84-87} Thus, insulin is a critical metabolic hormone.

The secreted insulin peptide that exerts these effects consists of a 21-amino acid A-chain bound by disulfide bonds to a 30-amino acid B-chain.\textsuperscript{88} The preproinsulin precursor encoded by the insulin gene is translocated into the lumen of the rough endoplasmic reticulum, facilitated by a signal peptide that is cleaved in the process to yield proinsulin.\textsuperscript{88} Proinsulin is folded into its three-dimensional conformation and transported into immature secretory vesicles via the Golgi apparatus, and is subsequently cleaved to yield the final 51-amino acid insulin and the excised C-peptide.\textsuperscript{88} Insulin is stored in the secretory granules packed as insoluble crystalline hexamers, which dissociate into the active monomeric insulin after secretion, due to electrostatic repulsion and a reduced insulin concentration gradient in circulation.\textsuperscript{88} The priming and fusion of insulin granules that leads to stimulated insulin secretion is triggered by a rise in intracellular calcium, which takes place downstream of glucose sensing.\textsuperscript{88} In brief, glucose enters the β cell via an
insulin-independent glucose transporter (GLUT) with low substrate affinity, and is phosphorylated by glucokinase, a type of hexokinase with a low substrate affinity that exhibits continued activity in the face of a high rate of glycolytic activity.\textsuperscript{88} Glycolysis and oxidative phosphorylation result in an elevated ATP:ADP ratio, which leads to closure of ATP-sensitive potassium channels and the ensuing depolarization of the plasma membrane, thus resulting in voltage-dependent calcium channels opening to allow an influx of calcium.\textsuperscript{88} In response to processes such as these, insulin levels in circulation oscillate, and the baseline pulsatile pattern as well as the biphasic stimulated secretory pattern are thought to facilitate more effective insulin signaling.\textsuperscript{89}

Although elevated glucose is the primary stimulant of insulin secretion, circulating insulin levels are also affected by other nutrients and numerous circulating factors, such as certain amino acids, fatty acids, sex hormones, melatonin, leptin, growth hormone (GH), glucose-dependent insulinotropic polypeptide, and glucagon-like peptide-1.\textsuperscript{88,90} Insulin levels can be tempered through stimulation or repression of its secretion, and insulin production is also subject to modulation, at the level of transcription, mRNA stability, translation, and processing.\textsuperscript{88,90} Thus, insulin is well suited to its role as a key metabolic hormone, since its circulating levels are adjusted through rapid fluctuations in response to such extrinsic stimuli as a postabsorptive rise in blood glucose, and through sustained basal alterations in response to chronic demand.\textsuperscript{88,91,92}

1.3.2 Murine insulin 1 and insulin 2

Mice and rats have two distinct insulin peptides that are produced from non-allelic insulin genes, unlike other mammals and humans.\textsuperscript{93} In mice, these genes reside on distinct chromosomes, with \textit{Ins1} on chromosome 19 and \textit{Ins2} on chromosome 7.\textsuperscript{94,95} \textit{Ins2} is the ancestral
insulin gene that is analogous to human INS, while rodent-specific \textit{Ins1} likely originated from the transposition of a reverse-transcribed, partially processed \textit{Ins2} mRNA.\textsuperscript{93} As a result, \textit{Ins1} lacks one of the two introns found in \textit{Ins2}.\textsuperscript{96} However, the fully processed insulin 1 and insulin 2 peptides only differ at the level of two B-chain amino acids, with additional slight changes also evident in the amino acid sequences of the respective C-peptides.\textsuperscript{96}

Despite sharing a high degree of sequence homology that extends up to 500 base pairs preceding the transcription initiation site, thus including many control elements,\textsuperscript{90,96} \textit{Ins1} and \textit{Ins2} do have notable differences with respect to certain promoter elements, general expression patterns, and imprinting status.\textsuperscript{97-102} For instance, murine \textit{Ins2} but not \textit{Ins1} is susceptible to temporal- and tissue-specific imprinting.\textsuperscript{98,103,104} \textit{Ins2} can be detected earlier in development, in the prepancreatic mouse embryo,\textsuperscript{97} and has a broad tissue distribution in adult mice that includes expression and/or promoter activity in regions of the brain\textsuperscript{102,105,106} and in the thymus.\textsuperscript{107-109} Conversely, murine \textit{Ins1} expression appears largely restricted to the pancreas of adults,\textsuperscript{102} and it does not contribute as much to pancreatic insulin production as \textit{Ins2},\textsuperscript{97,110-112} except in the mouse embryo during the early stages of pancreatic development.\textsuperscript{97} This may be partially explained by preferential translation or processing rates of the murine preproinsulin 2 compared to preproinsulin 1.\textsuperscript{110,111}

Complete inactivation of both \textit{Ins} genes in mice (\textit{i.e.} \textit{Ins1}\textsuperscript{−/−}:\textit{Ins2}\textsuperscript{−/−}) leads to severe diabetes and death within several days of birth,\textsuperscript{113} but genetic inactivation of either \textit{Ins} locus alone is not associated with metabolic deficiencies.\textsuperscript{114} Instead, there is a compensatory elevation of mRNA and protein of the corresponding non-deleted \textit{Ins} gene, and increased \(\beta\) cell mass, which suggests a degree of redundancy between \textit{Ins1} and \textit{Ins2}.\textsuperscript{114} However, there is evidence to suggest that \textit{Ins1} may have been subjected to positive selective mechanisms to maintain the
two-\textit{Ins}-gene system in mice, and it is unclear whether \textit{Ins1} and \textit{Ins2} or their peptide products have distinct functional roles.

1.3.3 Diabetes

Diabetes mellitus is a type of metabolic disease involving extensively disrupted fuel homeostasis, widely characterized by the chronically elevated blood glucose that results from insufficient insulin production. The immediate clinical benefit of insulin’s discovery in 1922 was the ability to treat type 1 diabetes, which is distinguished by deficient endogenous insulin levels due to the autoimmune destruction of insulin-producing \( \beta \) cells. Without exogenous insulin treatment, uncontrolled type 1 diabetes has widespread detrimental consequences that include glycosuria, ketoacidosis, coma, and ultimately death.

Type 2 diabetes, which is by far the most prevalent form of diabetes, is closely associated with obesity, and excessive nutrient intake and/or diminished energy output; underlying factors that contribute to both obesity and susceptibility to type 2 diabetes include genetic and epigenetic components, lifestyle, and environmental conditions. Chronic fuel surplus can lead to type 2 diabetes when there is a failure to compensate by adequately elevating insulin levels to meet demand. Thus, a decline in \( \beta \) cell function and mass is thought to play a key role in the onset of full-blown type 2 diabetes, and progression towards this state appears to be perpetuated by the metabolic disruptions associated with impaired tissue responsiveness to circulating insulin. The term ‘insulin resistance’ is widely used to specify an impairment of glucose disposal in response to insulin stimulation; however, this term is misleading, because not all insulin-regulated processes are equally affected in the insulin-resistant state that characterizes glucose intolerance and an increased risk of type 2 diabetes. Since peripheral insulin resistance is closely related to elevated circulating insulin levels, both of these features are
linked to glucose intolerance. Other factors that contribute to the type 2 diabetes pathology include chronic inflammation, elevated secretion of insulin’s counter-regulatory hormone glucagon, and an insufficient partitioning of excess nutrients to white adipose tissue (especially the subcutaneous depots), which would prevent accumulation in other tissues where surfeit fuel storage can cause damage. There are numerous etiologies that can lead to insufficient insulin production or diminished response to insulin, and thus cause diabetes.

1.3.4 IGF-1

The insulin-like growth factors are more closely associated with mitogenic rather than metabolic effects, and thereby represent a degree of divergence between the functions of the insulin and IGF ligands in vertebrates. The mammalian insulin and IGF-1 peptides are encoded by distinct genetic loci, but share a high degree of homology and appear to have evolved from a common ancestral gene. Unlike insulin, levels of bioactive IGFs are maintained at a fairly constant level in circulation throughout the day, due to IGF binding proteins. The IGF-1 and -2 ligands in circulation are primarily produced and secreted by the liver in response to GH stimulation, but IGFs are also locally secreted by other tissues, thus operating in an autocrine and paracrine manner. IGF-2 has important growth stimulatory effects during gestation, but IGF-1 is the primary insulin-like growth factor produced in adult rodents, and little is known about the postnatal role of IGF-2 in other mammals. IGF-1 plays a major role in the promotion of cell proliferation, growth, and survival. The simplified view holds that the IGFs are the ligands responsible for most of the mitogenic component of insulin/IGF-1 signaling. Indeed, null mutants of the IGF-1 or -2 genes can be ~40% smaller in neonatal body mass compared to their littermates, in contrast to the ~20% reduction in body mass of neonatal $Ins1^{-/-}:Ins2^{-/-}$ pups. However, although there is likely to be some degree of divergence
in function between insulin and IGF-1, supported by the fact that skeletal muscle is the only major insulin-target tissue with substantial content of IGF-1 receptors, there is not a clear division of labour between these ligands.\textsuperscript{91,92,131} Divergence between the roles of insulin and IGF-1 is tempered by signaling cross-talk and incomplete functional specificity, likely influenced by their common origin and the close similarities between their respective receptors.\textsuperscript{92,124}

### 1.3.5 Mammalian insulin/IGF-1 signaling network

The core mammalian insulin/IGF-1 system is analogous to that of invertebrates in many respects (Fig. 1.1), and in fact the PI3K/AKT signaling cascade is thought to be the primary pathway for insulin’s metabolic effects in mammals.\textsuperscript{44,80} However, there are important distinctions between the invertebrate model organisms and mammals (Fig. 1.1). For instance, there is only one known receptor and signaling pathway in invertebrates, and differential control of this pathway likely depends on variation between insulin-like peptide ligands.\textsuperscript{132} Like invertebrates, mammals also have several insulin-like products, but mammalian insulin/IGF-1 signaling additionally involves multiple receptors, and a network of signaling pathways can be triggered through activation of any single receptor, with the added complexity of interactions and crosstalk between the downstream pathways.\textsuperscript{44,80,121,133}

The classical tyrosine kinase receptor ligands in mammals are insulin, IGF-1 and IGF-2; other members of the mammalian insulin-like family include relaxins and four additional insulin-like peptides.\textsuperscript{83,126} The classical ligands can bind with variable affinity to the primary mammalian insulin/IGF tyrosine kinase receptors, consisting of the insulin receptor (IR), which has two functionally distinct splice isoforms (IRa, IRb), and the IGF-1 receptor (IGF-1R).\textsuperscript{44,80,126,133} Moreover, since these receptors are tetrameric proteins, functional hybrid
heterodimers can form between insulin and IGF-1 receptor subunits (e.g. IRa:IGF-1R, IRb:IGF-1R; Fig. 1.1). Receptor activation and subsequent phosphorylation of cellular receptor substrates, such as the scaffolding proteins insulin receptor substrate (IRS) 1 or IRS2, can initiate signal transduction via two major branches of insulin/IGF-1 signaling: PI3K/AKT (Fig. 1.1), and the mitogen-activated protein kinase (MAPK) pathway. Downstream of PI3K, AKT phosphorylates additional mammalian targets besides the FOXO family of transcription factors, including the Rab-GTPase-activating protein AS160 (AKT substrate of 160 kDa), which is involved in membrane trafficking, glycogen synthase kinase-3, which represses glycogen synthase activity, TSC2 (tuberous sclerosis complex-2), a component of the negative regulatory complex of mechanistic target of rapamycin (mTOR) complex 1 (mTORC1), and the pro-apoptosis protein BAD (Bcl-2-associated death promoter). Therefore, the downstream effects of mammalian AKT kinase activity can range from triggering translocation of the glucose transporter GLUT4 and promoting glycogen synthesis to stimulating protein synthesis and promoting cell survival. This is in addition to outcomes of excluding FOXO transcription factors from the nucleus, such as altering expression of gluconeogenic and lipogenic enzymes.

The MAPK branch of insulin/IGF-1 signaling is also largely comprised of a kinase cascade. The binding of the adapter protein GRB2 and exchange protein SOS (Son-of-sevenless) to receptor substrates such as IRS facilitates activation of the small GTPase Ras. This leads to the stepwise activation of the serine/threonine kinase Raf, the MAPK kinase (MEK) 1 and MEK2, and subsequently the MAPKs: ERK (extracellular signal-regulated kinase) 1 and ERK2. Downstream targets of phosphorylation by the activated ERKs, including the p90
ribosomal protein S6 kinase and the transcription factor ELK1, cumulatively contribute to cell growth, proliferation, differentiation, and survival.\textsuperscript{79,80} Thus, the Ras/MAPK pathway primarily contributes to the mitogenic side of insulin/IGF-1 signaling in mammals.\textsuperscript{79,80}

The precise mechanisms differentiating the downstream signaling of insulin receptors from IGF-1 receptors are not fully delineated.\textsuperscript{126} Notably, there are multiple forms and isoforms of key components of the insulin/IGF-1 signaling network, with differences in tissue distribution, cellular localization, activation kinetics, and binding partner interactions.\textsuperscript{44,80,121} Moreover, other receptors, such as the GH receptor, can modulate insulin/IGF-1 signaling by such actions as the recruitment or phosphorylation of IRS1 and IRS2.\textsuperscript{131,133} Therefore, the capacity exists for a fine level of systemic control of ligand effects.\textsuperscript{44,80} For example, there is evidence that the receptor substrate IRS1 plays an important role as an effector of IGF-1 signaling, since \textit{Irs1} knockout mice exhibit a prenatal and postnatal generalized growth reduction, similar to \textit{Igf1} knockout mice.\textsuperscript{80,126} This is accompanied by deficient insulin-stimulated glucose uptake in the muscle, suggesting that IRS1 also contributes to metabolic actions in certain tissues.\textsuperscript{80,126} In contrast, IRS2 may be more tightly coupled to the insulin receptor, since \textit{Irs2} removal is associated with normal growth in most tissues, but pronounced defects in insulin signaling in the liver and adipose tissue, as well as hyperglycemia or full diabetes that is linked to impaired β cell growth.\textsuperscript{80,126} However, the degree of overlap in function between insulin and IGF-1 remains unclear.\textsuperscript{126}

Collectively, insulin/IGF-1 signaling has metabolic effects, via the phosphorylation cascade of PI3K/AKT, as well as mitogenic effects, principally regulated by the MAPK pathway in mammals.\textsuperscript{79,80} This signaling network is also associated with such pleiotropic outcomes as suppressing stress resistance, and enhancing the proinflammatory response.\textsuperscript{44,134} Moreover, there
is evidence to suggest that insulin/IGF-1 signaling influences longevity in mammals, largely based on experiments performed using mouse models.

Mice, which are widely used as mammalian model organisms, are subject to considerable variability with respect to many physiological characteristics. This is reflected in the wide range of lifespans that can be detected across 31 inbred mouse strains held in a single specific pathogen-free (SPF) facility.\textsuperscript{135,136} However, there are some basic life history patterns that are fairly typical. Following a 19-21 day gestation period, the postnatal nesting period is characterized by linear growth rates, and ends with weaning at around 2-3 weeks of age.\textsuperscript{137} Puberty can occur as early as at four weeks of age, and female mice tend to be fertile until approximately one year.\textsuperscript{137} In the general sense, mice cannot be considered to be ‘old’ until they reach the age when the survival curve defining their population starts to show a rapid decline, or the point of 50\% population survival.\textsuperscript{138} Although mouse lifespan can depend on numerous factors, including strain, environmental conditions, and sex, the median lifespan for many standard inbred mouse strains tends to range between 600-900 days, or ~1.5-2.5 years.\textsuperscript{135-138}

1.4 Influences of nutrient-sensing pathways on mammalian aging and lifespan

1.4.1 Somatotropic and insulin/IGF-1 signaling pathways

Numerous lines of evidence have demonstrated that, as observed in invertebrates, a moderate reduction in insulin/IGF-1 signaling can lead to mammalian lifespan extension. However, it is clear that extreme downregulation tends to be detrimental in mammals.\textsuperscript{139-141} This is consistent with invertebrate models, where it is usually the reduction-of-function mutations rather than the loss-of-function mutations that positively affect lifespan.\textsuperscript{142} Deletion of insulin receptors in the adipose tissue has been found to lead to an 18\% extension of lifespan, in both
male and female mice. In contrast, the increase in longevity that results from a whole-body partial inactivation of IGF-1 receptors is only prominent in female mice, which experience an extension of 11-33% (dependent on strain). Interestingly, a comparative study across 16 diverse rodent species showed a negative correlation between maximum lifespan and IGF-1 receptor levels in the brain. Further downstream, lack of the receptor substrate IRS1 in the whole body leads to as much as a 16-32% (dependent on sex) extension of lifespan, and reducing IRS2 levels, either through a whole-body haploinsufficiency or deletion specifically in the brain, can also extend lifespan in in some mouse models, by 14-18% (depending on the manipulation). Reducing PI3K/AKT signaling by transgenically overexpressing Pten can extend mouse lifespan by 12%. In addition, a lifelong heterozygous inactivation of the p110α catalytic subunit of PI3K can improve late-life metabolic health and impart a modest 6% extension of lifespan.

Other long-lived mutant mice exhibit reduced insulin/IGF-1 signaling through more indirect means. For example, mice transgenically overexpressing Klotho experience a 19-31% (dependent on sex) extension of lifespan. KLOTHO is a transmembrane protein that appears to repress insulin/IGF-1 receptor autophosphorylation and receptor substrate phosphorylation, but these mice also exhibit a number of other physiological changes that could affect longevity. Transgenic overexpression of Fgf21 imparts a 30-40% (dependent on sex) increase in mouse lifespan, which may be in part because fibroblast growth factor 21 is associated with inhibited GH/IGF-1 signaling, amongst other metabolic changes. In addition, deletion of the p66shc splice variant of SHC transforming protein 1, a signal transducer that mediates signaling downstream of growth factor receptors such as the IGF-1 receptor, also extends lifespan by
28%.\textsuperscript{153} All in all, there is a great deal of evidence demonstrating the connection between reduced insulin/IGF-1 signaling and mammalian lifespan extension.

Studies performed to examine aging in human populations have revealed that the insulin/IGF-1 signaling network may also impact human longevity. In genome-wide association studies or targeted investigations looking at components of insulin/IGF-1 signaling, genetic variation in \textit{AKT},\textsuperscript{154} \textit{FOXO1},\textsuperscript{155,156} and \textit{FOXO3A}\textsuperscript{154,156-159} can be linked with a long human lifespan. Of these, \textit{FOXO3A} shows strongest associations, as it has been repeatedly detected in studies involving distinct long-living populations.\textsuperscript{154,156-159} Furthermore, variation in the insulin receptor gene\textsuperscript{160} or reduction-of-function mutations of the IGF-1 receptor\textsuperscript{161} can also be associated with increased human longevity, and lower composite insulin/IGF-1 signaling scores were associated with reduced mortality in women.\textsuperscript{162} Genetic variation in the IGF-1 receptor gene that are associated with lowering circulating IGF-1 levels can also be detected with increased frequency in long-lived individuals.\textsuperscript{163} Therefore, it seems that moderately lowering insulin/IGF-1 signaling could have evolutionarily conserved lifespan-extension effects from invertebrates to humans.

However, it is difficult to discern the precise roles of insulin versus IGF-1 when components of this signaling network are disrupted in mammals.\textsuperscript{44,164} At the ligand level, reduced cumulative IGF-1 has been repeatedly associated with lifespan extension. For example, dwarf mice with mutations in \textit{Prop1} or \textit{Pit1}, two transcription factors controlling pituitary development, have dramatically reduced IGF-1 levels, and can live between 26 and 68\% longer than wild-type controls.\textsuperscript{165-167} This is also the case for mice with loss of GH-releasing hormone\textsuperscript{168} or a mutated GH releasing hormone receptor gene,\textsuperscript{166} as well as for mice with disrupted GH receptors\textsuperscript{169} or GH receptor/binding proteins.\textsuperscript{170} Diminishing IGF-1 receptor levels in the mouse
brain was also found to reduce somatotropic (i.e. related to the GH-signaling axis) function, thus leading to general growth retardation and metabolic alterations, as well as extending mean lifespan by 8-13% (dependent on sex).\textsuperscript{171}

Reduced exposure to IGF-1 is thought to be a primary mediator of lifespan extension in these models, but mutant mice with disruptions to the somatotropic axis do have extensive endocrine changes that include all downstream effects of decreased GH signaling. Indeed, the binding of GH to its receptor can exert downstream effects on the insulin/IGF-1 signaling pathway independently of IGF-1.\textsuperscript{14,127} In support of the IGF-1 ligand itself as a mediator of longevity, female mice with reduced circulating IGF-1 due to liver-specific inactivation of the \textit{Igf1} gene show a 16% increase in mean lifespan,\textsuperscript{172} whereas a distinct line of mice with lowered levels of IGF-1 show a 10% increase in maximum lifespan, but no significant effects on mean lifespan.\textsuperscript{173} In general, based on the greater magnitude of lifespan extension that tends to result from disrupting GH signaling instead of targeting IGF-1 specifically, it seems likely that other mediators play a role in extending lifespan of mice with reduced somatotropic signaling.\textsuperscript{127} However, mice lacking a protein which degrades IGF binding proteins (pregnancy-associated plasma protein A) have reduced availability of bioactive IGF-1, and these animals exhibit a 33-41% increase in lifespan.\textsuperscript{174} In addition, consistent with the important mitogenic role of IGF-1 in mammals, it has been demonstrated that increased body size corresponds with elevated IGF-1 in mice,\textsuperscript{136,175} dogs,\textsuperscript{176,177} and humans,\textsuperscript{178} and moreover greater body size or higher IGF-1 correlates with reduced lifespan within each of these species.\textsuperscript{135,136,175,179-181}

The role of insulin in modulating mammalian aging and lifespan has remained unclear. Although it has been demonstrated that genetically reducing components of insulin/IGF-1 signaling can increase mammalian longevity, a paradox exists whereby long-lived mammals tend
to show enhanced responsiveness to insulin stimulation; peripheral insulin resistance is instead a
detrimental characteristic of mammalian aging that is a risk factor for a number of aging-related
pathologies, such as type 2 diabetes.\textsuperscript{43,182} The ambiguity is further compounded by the
convention of defining ‘insulin resistance’ as impaired insulin-stimulated glucose uptake on a
broad, whole-body level.\textsuperscript{79,121} In fact, not all insulin-regulated processes in all of the target
tissues are equally affected in this state.\textsuperscript{79,121} For example, in obese humans, muscle and adipose
tissue can exhibit a reduction in insulin receptor tyrosine kinase activity, IRS1-associated PI3K
activity, and glucose uptake, while AKT and MAPK activity is maintained at normal levels in
muscle, and IRS2-associated PI3K activity appears unaffected in adipose tissue.\textsuperscript{79,121} Since
circulating insulin levels rise in correspondence with impaired insulin-stimulated glucose uptake
in peripheral tissues,\textsuperscript{119,122} it is likely that conventionally-defined ‘insulin resistance’ – which is
sometimes imprecisely designated as simply a high ratio of circulating insulin:glucose\textsuperscript{122} –
actually represents a condition wherein some downstream effects of insulin/IGF-1 signaling are
reduced while other downstream effects may be simultaneously elevated.\textsuperscript{121}

Therefore, it is controversial whether it is reduced overall insulin signaling or enhanced
whole-animal insulin sensitivity (\textit{i.e.} presumably some degree of increased insulin signaling, at
least within specific branches of the signaling network) that would be expected to benefit
mammalian healthspan and longevity. While low circulating insulin levels have been detected in
many long-lived mammals, including humans,\textsuperscript{148,149,152,168,169,183-185} it is not clear if reduced
insulin in these models has a causal role in extending lifespan, or if it is simply a secondary
feature of enhanced insulin sensitivity. It had not yet been tested whether moderately reducing
the insulin ligand itself could promote healthier aging and extend lifespan in mammals.
1.4.2 Other nutrient-sensing systems

Changes in other nutrient-sensing systems besides insulin/IGF-1 signaling have also been found to influence aging and lifespan, in both invertebrates and mammals. For instance, serine/threonine kinase mTOR regulates such cellular processes as protein flux and growth, and mTORC1 promotes mRNA translation and protein synthesis in addition to repressing autophagy.\textsuperscript{186} mTORC1 can be activated through the PI3K/AKT pathway of insulin/IGF-1 signaling, as well as stimulated by nutrients such as amino acids.\textsuperscript{186} Treatment with rapamycin, a negative regulator of mTORC1, has been shown to extend murine lifespan by 9-26\% (with sex-dependent and dose-dependent variation), even when treatment is not initiated until mice are nearly two years old.\textsuperscript{187-189} In addition, deletion of ribosomal protein S6 kinase 1, one of the positive regulators of protein synthesis that is activated by mTORC1, leads to a 19\% increase in lifespan in female mice.\textsuperscript{190} Interestingly, S6 kinase 1 deletion is associated with increased activity of AMP-activated protein kinase (AMPK),\textsuperscript{190,191} a sensor of cellular energy status that induces catabolic pathways and represses anabolic pathways, and in fact negatively regulates mTORC1.\textsuperscript{186} A low, chronic dose of metformin, a drug commonly used to treat type 2 diabetics that acts at least in part through AMPK activation, can extend murine lifespan by 6\%.\textsuperscript{192} The sirtuins, a family of NAD\textsuperscript{+}-dependent protein and histone deacetylases, represent another energy-sensing system that has the potential to affect mammalian aging and longevity.\textsuperscript{43,193,194} Transgenic overexpression of Sirt6 led to a 12\% increase in median lifespan for male mice without having significant effects for females,\textsuperscript{195} and transgenic mice overexpressing Sirt1 in regions of the brain show a 9-16\% (dependent on sex) lifespan extension.\textsuperscript{194} In addition, some studies have shown that male mice treated with an activator of sirtuin 1 have extended healthspan and lifespan.\textsuperscript{196,197} Importantly, in their capacity as nutrient-sensing systems, it seems
that insulin/IGF-1 signaling, the mTOR pathway, AMPK activation, and the sirtuins may also contribute to the mechanisms underlying the healthspan and lifespan extensions imparted by dietary restriction.43,198

1.4.3 Dietary restriction

In 1935 it was found that a reduction of total calorie intake without malnutrition could significantly prolong the lifespan of rodents.199 Today, dietary restriction interventions remain one of the most effective environmental manipulations for extending healthy lifespan in a range of diverse organisms that includes yeast, invertebrates, fish, rodents, and non-human primates.198 Importantly, dietary restriction can be associated with not merely extending lifespan, but also delaying the onset and slowing the rate of aging.198,200 This is supported in part by a reduced incidence of age-associated cardiovascular diseases, metabolic dysfunction, and cancers in non-human primates under dietary restriction,201,202 as well as some beneficial health outcomes in diet-restricted humans.198,200

The specific mechanisms underlying the beneficial effects of dietary restriction are still unclear. Moreover, these mechanisms and their effectiveness likely depend on numerous factors, including the specific dietary restriction that is imposed and its severity, the background genetics of the experimental subjects or strains, and the life history phase.43,198,203,204 Interestingly, mounting evidence suggests that a restriction of calories per se may not be the key factor for imparting the health benefits of dietary restriction.198 For instance, using dietary dilution to reduce total caloric intake without limiting feeding time or volume of food intake was not observed to extend lifespan in mice, whereas progressively decreasing the protein:carbohydrate ratio in an isocaloric diet had positive effects on lifespan.205 Intermittent energy restriction or time-restricted feeding without reducing cumulative energy intake has beneficial effects on
health and longevity that likely contribute to some ‘caloric restriction’ experimental
designs, and restriction of certain nutrients or macronutrients, or even specific amino
acids such as methionine, may also be a key contributor to healthspan and lifespan extension.

Notably, long-lived mutant mice tend to share some of the distinguishing traits of
organisms under dietary restriction, including reduced body size or
adiposity, reduced ROS production or signs of superior
oxidative stress resistance, improved insulin sensitivity and/or
glucose homeostasis, and reductions in circulating levels of IGF-I
and/or insulin that are supplementary to the experimental genetic
manipulation. These similarities point to some of the key conserved
mechanisms for prolonging lifespan.

1.5 Elevated insulin levels and obesity

At the opposite end of the spectrum from dietary restriction, excessive nutrient intake
and/or diminished energy output can produce an obese phenotype that features many of the
unhealthy hallmarks of aging. Indeed, central adiposity tends to increase with aging, and many of
the pathologic conditions or diseases associated with obesity, including glucose intolerance and
type 2 diabetes, insulin resistance, a proinflammatory state, coronary heart disease, hypertension,
atherosclerosis, and some cancers, are also associated with biological aging. In 2013, the
World Health Organization estimated that the worldwide incidence of obesity had nearly doubled
since 1980, and over 40 million children under the age of five were classified as overweight in
2011. The escalation of obesity in youth is leading to an increasing prevalence of traditionally
‘aging-associated’ disorders in younger populations.
Although obesity is broadly considered as a state of increased adiposity, there are multiple types of adipose tissue with distinct functions, spatial distribution, and contributions to obesity. Brown adipose tissue (BAT), as well as the brown-like “brite”/“beige” adipocytes that can be found in some white adipose tissue depots, are distinguished by a marked capacity to uncouple the electron transport chain-generated proton gradient from ATP synthesis, and thus expend energy through thermogenesis.\textsuperscript{218} In contrast, white adipose tissue (WAT) serves as an energy reserve, as well as secreting an array of factors with autocrine, paracrine, and endocrine functions.\textsuperscript{219,220} Subcutaneous and visceral depots of white adipose tissue have distinct characteristics, differing in cellular composition, innervation, metabolic characteristics, and secretory profile; in general, visceral WAT is more implicated in perpetuating metabolic dysfunction, compared to subcutaneous WAT.\textsuperscript{220-222} The mechanisms regulating the specific expansion of distinct WAT depots are not well understood, although physiological factors such as age and sex have an influence on adipose tissue distribution.\textsuperscript{222,223}

It seems that certain life stages, such as the perinatal period and adolescence, are particularly influential for determining metabolic and adipocyte characteristics and thus shaping future susceptibility to obesity.\textsuperscript{220,224,225} The expansion of WAT that defines obesity can occur through both adipocyte hypertrophy (lipid filling) and hyperplasia (adipogenesis, or increased differentiation of adipocyte precursor cells into adipocytes), but the mechanisms controlling these processes have not been fully delineated.\textsuperscript{220} Notably, insulin plays a key role in regulating white adipocyte fat accumulation, which depends on the degree to which lipogenesis offsets lipolysis and fatty acid oxidation.\textsuperscript{79,226} Likewise, exposure to insulin can enhance adipogenesis, an orchestrated progression of events involving transient expression of the transcription factors CCAAT/enhancer binding protein (C/EBP) β and C/EBPδ leading to induction of the master
adipogenic regulators C/EBPα and peroxisome proliferator-activated receptor (PPAR) γ.\textsuperscript{219,220} Although the mechanisms guiding WAT growth are not fully understood, insulin appears to be an integral regulator at several prominent steps.

Obesity is often associated with hyperinsulinemia (\textit{i.e.} elevated insulin levels, for the purpose of this body of work; similarly, ‘insulin hypersecretion’ is used to broadly refer to elevated basal and/or stimulated insulin secretion) and also peripheral insulin resistance (\textit{i.e.} decreased general, whole-animal insulin-stimulated glucose disposal).\textsuperscript{119,217} Insulin resistance within tissues can arise from numerous etiologies, including a decrease in the levels of signaling pathway components from reduced production or elevated degradation, an impairment of component activity through post-translational modifications such as serine phosphorylation, an increase in the levels or activities of endogenous negative regulators of insulin signaling, and exposure to inhibitors, such as the suppressors of cytokine signaling proteins that are induced by proinflammatory cytokines.\textsuperscript{79,120,121} The commonly accepted paradigm proposes that the hyperbolic relationship between impairment of insulin-stimulated glucose uptake and insulin secretion signifies that hyperinsulinemia is a compensatory response to prevent hyperglycemia when peripheral tissues are failing to take in excess glucose due to conditions resulting from obesity.\textsuperscript{122,217,227}

However, the chronology of obesity, hyperinsulinemia, and insulin resistance is not always clear. Characteristics of the obese state, such as toxic accumulation of lipids in non-adipose tissue and increased levels of proinflammatory cytokines, can induce or exacerbate insulin resistance, and the resultant elevated blood glucose has the potential to stimulate increased insulin secretion.\textsuperscript{79,120,121} On the other hand, alleviating or preventing hyperinsulinemia has been shown to reduce obesity and improve whole-body insulin-stimulated glucose uptake.
Clinical and experimental evidence indicates that hyperinsulinemia can precede and promote both obesity\textsuperscript{102,228-231} and insulin resistance or dysglycemia.\textsuperscript{232-234} Moreover, drugs that reduce insulin secretion lead to weight loss\textsuperscript{235-237} and/or enhancement of insulin-stimulated glucose uptake.\textsuperscript{235,238,239} Diet intervention studies have indicated that obese individuals with the highest insulin secretion respond best to diets that reduce postprandial glycaemia and insulinemia, whereas obese subjects with relatively lower insulin levels experience equivalent weight loss on low-fat diets.\textsuperscript{214,215} One of the major metabolic functions of insulin is to promote fat accumulation in white adipose tissue,\textsuperscript{79} and hyperinsulinemia can be associated with elevated insulin-induced lipid storage even if there is an ‘insulin resistant’ state with respect to glucose uptake.\textsuperscript{121,217} Interestingly, mouse models with adipose tissue-level inactivation of either insulin receptors alone or insulin and IGF-1 receptors concurrently are protected from obesity.\textsuperscript{240,241} Collectively, these studies suggest that insulin has more of a causal role in perpetuating obesity and peripheral insulin resistance than is widely supposed.

\subsection*{1.6 Specific research aims}

Insulin is an essential hormone, and variation in circulating insulin levels can have far-reaching consequences that may be highly relevant in the context of both aging and obesity. Measured baseline insulin levels within healthy human and mouse populations are subject to wide variance,\textsuperscript{242-244} suggesting that endogenous insulin levels could be a potential predisposing factor underlying a multitude of downstream effects. In addition, despite the insulin secretory defects that can develop with age, biological aging in humans is often associated with maintained or elevated circulating insulin levels due to such physiological changes as elevated adiposity, chronic inflammation, reduced insulin-stimulated glucose uptake in peripheral tissues, and
reduced insulin clearance.\textsuperscript{245-247} Collectively, these conditions are strikingly similar to the obese state, and can likewise lead to an increased risk of glucose intolerance and type 2 diabetes.\textsuperscript{245-248} It is clear that there are numerous parallels between biological aging and obesity, and insulin may be a crucial factor in modulating both of these phenomena.

Recent work in our lab showed that $\text{Ins2}^{+/-}$ male mice with reduced $\text{Ins1}$ gene dosage experienced a pronounced lifelong suppression of basal insulin levels with high fat feeding and were protected from high fat diet-induced obesity, unlike their hyperinsulinemic $\text{Ins1}^{+/+}:\text{Ins2}^{-/-}$ littermates.\textsuperscript{102} In contrast, female $\text{Ins1}^{+/-}:\text{Ins2}^{-/-}$ mice did not show significant differences in circulating insulin, compared to $\text{Ins1}^{+/+}:\text{Ins2}^{-/-}$ females.\textsuperscript{102} Since murine $\text{Ins1}$ and $\text{Ins2}$ genes may have distinct functional roles, in our current studies we focused on the effects of altering dosage of the ancestral $\text{Ins2}$ gene. We used a mouse model in which the $\text{Ins1}$ gene was fully inactivated ($\text{Ins1}^{-/-}$) to provide the capacity to substantially lower endogenous insulin production while focusing on the effects of reducing $\text{Ins2}$ expression, without potential compensation by rodent-specific $\text{Ins1}$.

The first research aim was to examine the effects of reducing dosage of the ancestral $\text{Ins2}$ gene on high fat diet-induced hyperinsulinemia and obesity, in both male and female $\text{Ins1}$-null mice. We predicted that reducing $\text{Ins2}$ dosage would repress circulating insulin levels and thereby impart protection from high fat diet-induced obesity. Interestingly, we discovered model- and sex-specific outcomes in these experiments, allowing us to: 1) investigate whether protection from obesity could be sustained beyond the clear repression of insulin hypersecretion evident in young, growing female $\text{Ins1}^{+/-}:\text{Ins2}^{-/-}$ mice (Chapter 3); and 2) explore the phenotypic hyper-variability in circulating insulin and response to high fat feeding of the male littermates (Chapter 4).
The second major research aim was to determine how reducing *Ins2* gene dosage and moderately lowering circulating insulin levels would affect mammalian aging and lifespan. We predicted that a modest cumulative reduction in circulating insulin levels would have beneficial effects in the context of aging, and lead to an extension of lifespan. These predictions were tested in both male and female *Ins1*-null mice (Chapter 5).

The overall goal of this dissertation was to further delineate the effects of limiting or moderately lowering insulin levels on obesity and metabolic health across a mammal’s lifespan, culminating in an assessment of impact on longevity.
Figure

<table>
<thead>
<tr>
<th>C. elegans:</th>
<th>D. melanogaster:</th>
<th>Mammals:</th>
</tr>
</thead>
<tbody>
<tr>
<td>1) Ligand</td>
<td>38 Insulin-like peptides</td>
<td>8 Insulin-like peptides</td>
</tr>
<tr>
<td>2) Tyrosine kinase receptor</td>
<td>DAF-2</td>
<td>InR</td>
</tr>
<tr>
<td>3) Receptor substrate</td>
<td>Chico</td>
<td>IRS1, IRS2 (plus others)</td>
</tr>
<tr>
<td>4) Phosphatidylinositol 3-kinase</td>
<td>AGE-1 (catalytic subunit)</td>
<td>PI3K</td>
</tr>
<tr>
<td>5) 2nd messenger PI(3,4,5)P\textsubscript{3}</td>
<td>DAF-18</td>
<td>Pten</td>
</tr>
<tr>
<td>6) 3-phosphoinositide dependent protein kinase-1</td>
<td></td>
<td>PTEN</td>
</tr>
<tr>
<td>7) Serine/threonine kinase AKT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>8) Transcription factor homologous to class O forkhead box (FOXO) family</td>
<td>DAF-16</td>
<td>Foxo</td>
</tr>
<tr>
<td></td>
<td></td>
<td>FOXO family</td>
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</tbody>
</table>
Figure 1.1 Schematic of the PI3K/AKT signaling pathway in invertebrates and mammals

A simplified depiction of insulin/IGF-1–like signaling in invertebrates and the analogous PI3K/AKT branch of insulin/IGF-1 signaling in mammals, highlighting some of the differences between the organisms, as well as several of the primary signaling component proteins in *C. elegans*, *D. melanogaster*, and mammals. In brief, insulin-like peptide ligands are released in response to sensory stimulation, and bind to a transmembrane tyrosine kinase receptor, which auto-phosphorylates and then recruits and activates the effector phosphatidylinositol 3-kinase, either directly or using a receptor substrate intermediate. Activated phosphatidylinositol 3-kinase converts PI(4,5)P₂ (phosphotidylinositol 4,5-biphosphate) into second messenger PI(3,4,5)P₃ (phosphotidylinositol 3,4,5-triphosphate); this step is subject to negative regulation by a phospholipid phosphatase. PI(3,4,5)P₃ stimulates recruitment and activation of 3-phosphoinositide dependent protein kinase-1 and its substrate, serine/threonine kinase AKT. Once activated through phosphorylation, AKT phosphorylates many substrates, including a transcription factor that is homologous to the mammalian class O forkhead box family, which is phosphorylated and excluded from the nucleus in response to phosphorylation by AKT. Blue arrows represent kinase activity; red arrows represent phosphatase activity.
Chapter 2: Materials and methods

2.1 Experimental animals

All animal procedures were approved by the University of British Columbia Animal Care Committee, following Canadian Council for Animal Care guidelines. The Ins1-null and Ins2-null alleles were generated by J. Jami and colleagues as previously described. Experimental mice had a mixed genetic background, comprised of approximately equal parts C57BL/6 and 129 strains (according to single-nucleotide polymorphism genome scanning analysis performed by the Jackson Laboratory, Bar Harbor, ME, USA). Data presented are from male and female littermates, and were therefore collected in the same time-frames and under nearly identical conditions. All animals were predominantly handled by the same female researcher, which is relevant since stress levels and physiological responses of mice is influenced by the sex of the human to which they are exposed. The mice tracked across time were in two major cohorts: cohort A, born October 2011 – December 2011, and cohort B, born approximately one year later (October 2012 – February 2013). The dams and sires of cohort B experimental mice were themselves born of parents from cohort A stock, thus minimizing the chance of significant genetic drift, and the same room was used for breeding across both cohorts. For female mice, phenotypes were consistent between cohorts, and therefore both cohorts were pooled in the presented data.

Shortly after weaning (i.e. at 3-4 weeks of age), Ins1−/−:Ins2+/+ and Ins1−/−:Ins2+/− male and female mice were assigned to one of two diet groups: a moderate-energy chow diet (CD: 4.68 kcal/g, 25% fat, 55% carbohydrate, 20% protein content; LabDiet 5LJ5; PMI Nutrition International, St. Louis, MO, USA) or a high-energy high fat diet (HFD: 5.56 kcal/g, 58% fat,
26% carbohydrate, 16% protein content; Research Diets D12330; Research Diets, New Brunswick, NJ, USA). These specific diets were consistent with those used in previous experiments assessing the effect of reduced \textit{Ins1} gene dosage on obesity.\textsuperscript{102} Each litter was distributed between the two diet assignments, based on matching the initial average body mass between diet groups for each combination of sex and genotype. Food was provided \textit{ad libitum}, except during fasting periods. Mice were housed under specific pathogen-free (SPF) conditions at 21°C, on a 12:12 h light:dark cycle, in the same room for both cohorts. Nearly all females were housed with cage-mates for the large part of their lifespan, but the vast majority of male mice from both cohorts were singly housed, due to fighting between young male cage-mates. Mice were assessed based on whether their dam or sire had the disrupted \textit{Ins2} allele, to control for possible parental imprinting,\textsuperscript{98,103} but we combined ‘parental groups' as no clear differences were observed when groups were separated. In addition, when the factor of “parental effect” was incorporated into analyses of body mass or fasting insulin levels, there were not consistent, statistically significant effects. Experimenters were blind to mouse genotype, diet (where possible), and parental group while performing and analyzing each experiment.

\section*{2.2 Lifespan evaluation}

We attempted to achieve a balance between performing the necessary experiments for fulfilling our research aims while also minimizing manipulations and stresses that could undermine an assessment of longevity. Most \textit{in vivo} experiments and phenotypic characterizations were performed within the first \~1.5 years of murine life (Fig. 2.1). After this, the aging animals were left largely undisturbed, with the exception of daily visual assessment, regular health monitoring, and any necessitated treatments (\textit{e.g.} lancing of preputial gland
abscesses, treatment for retraction of penile prolapse, analgesics and/or a brief course of antibiotics to alleviate pain or prevent infection in any cases of open wounds, temporary treatment with eye drops and/or eye lubrication to prevent problems associated with the few cases of corneal swelling). For each sex, the number of mice receiving treatments appeared to be approximately evenly distributed between groups. Mice noted to be ill were subject to more frequent health monitoring, and were euthanized if they reached a humane endpoint, which was determined based on a set of veterinarian-approved criteria. This involved scoring the severity of clinical signs in several categories, including but not restricted to: body weight changes (cumulative and/or rapid weight loss), activity levels and responsiveness, tumour size and/or ulceration, and physical signs of well-being such as posture, piloerection, and any changes to body temperature that were detectable by touch. Researchers were blinded to genotype while scoring mouse health and assessing humane endpoint. For the purposes of these studies, euthanization at humane endpoint was considered to be an estimate of natural lifespan.

Necropsies were performed on nearly all mice, after death due to euthanization or natural causes. Following fixation in 10% neutral buffered formalin, major vital organs (i.e. liver, heart, kidney, spleen, lung, pancreas, stomach, uterus) as well as any gross tissue abnormalities such as tumours were sectioned, stained with hematoxylin and eosin, and assessed by a board-certified veterinary pathologist. The pathologist, who was blind to genotype, scored the severity of lesions and also provided an evaluation of which lesions likely contributed to natural death or the clinical signs leading to euthanization.
2.3 Glucose homeostasis and plasma analytes

We performed longitudinal monitoring of insulin levels and glucose homeostasis to track the effects of reduced $Ins2$ gene dosage. Mice were fasted for 4 h during the light period to ensure a post-absorptive state for all blood sampling. Glucose levels were measured in fresh blood using OneTouch Ultra2 glucose meters (LifeScan Canada Ltd, Burnaby, BC, Canada). Additional blood samples were stored prior to analyses, at -20°C (samples collected in heparin-coated capillary tubes at 8, 15, 27, 52, and 80 weeks of age to measure insulin, as well as C-peptide and corticosterone) or -80°C (samples collected in ethylene diamine tetracetate-coated capillary tubes at 30, 40, and 90 weeks of age to measure other analytes). Fasting blood glucose was assessed weekly for cohort A animals up to one year, after which it was measured every five weeks up to ~1.5 years of age. For cohort B mice, fasting blood glucose was measured every five weeks from 5 weeks to 1.5 years of age. Fasting and intraperitoneal glucose-stimulated (2 g/kg) insulin secretion was assessed periodically, as well as blood glucose response to intraperitoneal delivery of glucose (2 g/kg) or an insulin analog (0.75 U/kg of Humalog; Eli Lilly, Indianapolis, IN, USA). Plasma insulin was measured with a mouse insulin ELISA (Alpco Diagnostics, Salem, NH, USA), according the manufacturers’ instructions. Notably, this insulin ELISA also has a high cross-reactivity to Humalog without significant cross-reactivity to rodent C-peptides, IGF-1 peptides, or IGF-2 peptides. Our assessments with murine insulin peptides synthesized by Novo Nordisk showed that the Alpco mouse insulin ELISA could detect both murine insulin peptides, although there appeared to be a greater degree of cross-reactivity for the synthesized insulin 2 solution, compared to the solution containing synthesized insulin 1. Area over or under the curve was calculated to evaluate statistical differences in glucose- or insulin-stimulated tests. In aged mice, we also used the human homeostatic model assessment to perform an alternative
quantification of the parameter of ‘insulin resistance’ (HOMA-IR) based on fasting blood glucose and insulin levels (i.e. fasting glucose • fasting insulin / 22.5).\textsuperscript{250}

We also performed further characterization of other plasma analytes. C-peptide was measured in a subset of 27 week-old female mice, using a mouse C-peptide ELISA (Alpco Diagnostics) in accordance with manufacturers’ instructions. Although the ELISA could detect both murine C-peptides, murine C-peptide 2 has 150% cross-reactivity, compared to 100% cross-reactivity for C-peptide 1. Corticosterone was measured in plasma collected from 27 week-old male mice in the early afternoons (collected across multiple weeks for each cohort), using a mouse/rat corticosterone ELISA (Alpco Diagnostics) according the manufacturers’ instructions.

In plasma collected from cohort B male mice at 40 weeks and from female mice at 40 and 90 weeks, we used colorimetric kits to measure total cholesterol (Cholesterol E kit; Wako Chemicals, Richmond, VA, USA), triglycerides (Serum Triglyceride kit; Sigma-Aldrich, St Louis, MO, USA), and non-esterified fatty acid levels (NEFA-HR[2] kit; Wako Chemicals), in addition to using a mouse magnetic bead panel assay that utilized Luminex technology (Millipore, St. Charles, MO, USA) to evaluate leptin, resistin, interleukin 6, glucose-dependent insulinotropic polypeptide (GIP), peptide YY, and glucagon levels, in accordance with manufacturers’ instructions. Plasma IGF-1 levels were measured at 30 and 90 weeks in a subset of female mice, using a mouse/rat IGF-1 ELISA (Alpco Diagnostics) according the manufacturers’ instructions. Lastly, aliquots of the plasma collected from fasting 30- and 90-week-old female mice were stored at -80°C in the presence of 0.005% butylated hydroxytoluene, and an 8-isoprostane EIA kit (Cayman Chemical, Ann Arbor, MI, USA) was used to measure 8-isoprostane, a marker of lipid peroxidation. This was performed according to
manufacturers’ instructions, including an alkaline hydrolysis step to facilitate determination of total levels of plasma 8-isoprostane.

2.4 Body composition, grip strength tests, and metabolic cage experiments

A subset of pups from cohort B was weighed prior to weaning. After weaning, all mice were weighed weekly. *In vivo* body composition, including total fat and fat-free mass as well as bone mineral density, was assessed periodically in cohort B mice using dual-energy X-ray absorptiometry (DEXA; Lunar PIXImus densitometer; GE Medical Systems LUNAR, Madison, WI, USA). For these analyses, mice were anaesthetized with isoflurane for approximately five minutes, while the DEXA assessment was performed as described in manufacturers’ instructions.

At similar time points throughout their lifetime, a grip strength meter (Columbus Instruments, Columbis, OH) was used to evaluate forelimb grip strength in a subset of male and female mice from cohort B. For each test, the mouse, gripping a horizontal bar with its forelimbs, was held near the base of its tail and gently but steadily pulled away from the bar until the negative force necessitated letting go, with peak force registered by the apparatus. Each mouse was tested three times in succession with a minimum of one resting minute between tests, and the mean of the best two values was considered to be the grip strength for each individual, thus controlling for any instances of reduced effort.

To assess whole-body energy expenditure based on oxygen consumption and respiratory exchange ratio, as well as gauge activity levels and food intake, a subset of HFD-fed 17 week-old mice from cohort B was evaluated in PhenoMaster metabolic cages (TSE Systems, Chesterfield, MO, USA) as described. Mice were first individually housed for at least a week, and acclimated to the metabolic cage environment for at least four days prior to data collection.
During data collection, the environmental temperature was maintained at 21ºC, with animals continuing to be exposed to a 12:12 h light:dark cycle. Data were averaged from across 48-84 h of continual acquisition, after excluding the initial light and dark periods when animals were first transferred into the metabolic cages. We controlled for differences in fat and fat-free mass between genotypes by analyzing energy expenditure with the covariates of lean and fat mass. Food intake was assessed as average consumption per mouse, but we did not observe differences in statistical outcomes when including covariates of lean and fat mass.

2.5 INSULIN2 treatment

We performed a small experiment to independently test the long-term effects of transient exposure to elevated insulin levels. A separate group of female mice were put on HFD at 15 weeks and subcutaneously implanted at 17 weeks with mini osmotic pumps (Alzet 2004; DURECT, Cupertino, CA, USA) designed to continually release a synthesized murine insulin 2 peptide (“INSULIN2”; 0.1 U/day; generously provided by Novo Nordisk, Bagsvaerd, Denmark) or vehicle control for 28 days. INSULIN2-treated $\text{Ins1}^{+/-}\text{:Ins2}^{+/-}$ mice and vehicle-treated $\text{Ins1}^{+/-}\text{:Ins2}^{+/-}$ and $\text{Ins1}^{+/-}\text{:Ins2}^{+/+}$ mice were tracked to 50 weeks of age.

2.6 Tissue analyses

Tissue-level analyses, including islet $\text{Ins2}$ mRNA, insulin content, and function, were assessed in groups of mice that were discrete from the longitudinally tracked mice from cohorts A and B. These groups included 25 week-old CD- and HFD-fed male and female mice (born April 2014), female mice from the 50 week-old HFD-fed mini osmotic pump group (born November 2013), and 70 week-old HFD-fed male mice (born April 2012). Tissues were
dissected from mice euthanized after 4 h of fasting, with the exception of 70 week-old male mice, which were euthanized after a minimum of 1 h of fasting.

Islets were isolated with collagenase and filtration, using a previously described protocol\textsuperscript{252} with minor modifications. Islets were handpicked using brightfield microscopy, and cultured at 37 °C and 5% CO\textsubscript{2} for at least 16 h prior to any analyses, in RPMI-1640 medium (Invitrogen, Burlington, ON, Canada) supplemented with 11 mM glucose, 100 U/mL penicillin, 100 µg/mL streptomycin and 10% (volume/volume) fetal bovine serum. Islet perifusion experiments were carried out as described,\textsuperscript{253} using groups of 150 size-matched islets and evaluating 3 mM glucose basal insulin secretion as well as stimulatory conditions of 15 mM glucose or 30 mM KCl. Islet insulin content was averaged from 10 size-matched islets per mouse, lysed at -20°C in 0.02 M HCl in 70% ethanol, and sonicated a minimum of 30 s before dilution for measurement with a mouse insulin ELISA kit (Alpco Diagnostics).

Other tissues were weighed and flash-frozen in liquid nitrogen before -80°C storage, except half of the gonadal white adipose tissue depot from female mice, which was fixed in 4% paraformaldehyde, embedded in paraffin, and cut into 5 µm-thick sections that were stained for perilipin (antibody 9349S; Cell Signaling Technology, Danvers, MA, USA) using an Alexa fluor 488-conjugated secondary antibody (Life Technologies, Burlington, ON, Canada), and DAPI. Images with identical exposure times were taken with a Zeiss 200M inverted microscope (Carl Zeiss, Oberkochen, Germany) and lipid droplet areas were determined using CellProfiler 2.1.0 (http://www.cellprofiler.org/).\textsuperscript{254}

For quantitative reverse transcription PCR, total RNA was extracted from islets using the Qiagen RNeasy Mini kit (Qiagen, Mississauga, ON, Canada) in accordance with manufacturers’ instructions. For adipose tissue of female mice, total RNA was isolated using acid guanidinium
thiocyanate-phenol-chloroform extraction with TRIzol (Invitrogen) followed by Qiagen RNeasy Mini columns.\textsuperscript{255} RNA was quantified at 260 nm with a NanoDrop 2000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA), and cDNA was generated using a qScript cDNA synthesis kit (Quanta Biosciences, Gaithersburg, MD, USA). Transcript levels were measured with TaqMan assays or SYBR Green primer sets (see Table 2.1 for primer details). Reactions were performed in duplicate on a StepOnePlus real-time PCR system (Applied Biosystems, Foster City, CA, USA), using TaqMan Fast Universal PCR Master Mix or Fast SYBR Green Master Mix (Applied Biosystems) and a fast-mode thermal program consisting of a 20-s activation at 95°C, then 40 cycles of 95°C melting for 1 s and 60°C annealing for 20 s. Values were normalized to β-actin, Tbp or Hprt expression levels using the $2^{-\Delta Ct}$ method. The specific internal control gene used for each tissue was determined based on the internal control gene having stable expression levels across the groups.

2.7 Statistical analyses

Statistical analyses were performed with SPSS 15.0 software, although Graphpad Prism 6.0 software was used to generate and assess any linear regressions. The critical α-level was set at $p \leq 0.05$. For most analyses, we used two-way analysis of variance (ANOVA) models (for male mice, this was performed within each cohort for most variables) to assess factors of genotype and diet, and a significant interaction led to one-way ANOVA comparing HFD-fed $Ins1^{+/+}:Ins2^{+/+}$, CD-fed $Ins1^{+/+}:Ins2^{+/+}$, HFD-fed $Ins1^{+/+}:Ins2^{+/+}$, and CD-fed $Ins1^{+/+}:Ins2^{+/+}$ groups, with Bonferroni corrections. Three-way ANOVA was used for incorporating additional factors, such as cohort or parental effect, and one-way ANOVA was used if only one factor was being tested, as with data from the 50 week-old HFD-fed mini osmotic pump group of female mice.
2-tailed independent \( t \)-tests were used to assess differences if only two groups were compared. Analysis of covariance (ANCOVA) was employed to test energy expenditure with covariates of lean and fat mass. In all of these cases, Levene’s test was used to evaluate the assumption of homogeneity of variance. Where the homogeneous variance assumption was violated, as with some of the insulin measures and body mass values of the male mice, for instance, logarithmic transformations were applied and generally stabilized data variance. Chi-square tests were used to statistically evaluate tumour incidence, and incidence of hepatic steatosis in HFD-fed female mice. Lastly, survival curves consisted of: 1) assessments of lifespan incorporating mortality due to all causes, with the exceptions of euthanization due to severe fight wounds, self-mutilation, or malocclusions (which led to eight animals being censored, evenly distributed between genotypes); and 2) assessments of mortality associated with the main categories of identified pathologies. Kaplan-Meir analyses were performed with log-rank comparisons stratified by diet, including censored mice up to the date of euthanization. In addition, survival curve results were also validated with Cox proportional hazards regression analyses incorporating covariates of diet and cohort.
Figure 2.1 Experimental timeline of mouse ages during data collection.

Timeline outlining mouse age (in weeks) for the majority of the in vivo experiments and plasma or tissue analyses performed in Ins1\(^{−/−}\):Ins2\(^{+/+}\) and Ins1\(^{−/−}\):Ins2\(^{−/−}\) male and female mice. Experiments were primarily performed across the first 90 weeks of mouse life.
### 2.9 Table

**Table 2.1 Primer sequences used for quantitative reverse transcription PCR.**

<table>
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<th>Gene</th>
<th>Forward primer (5’ to 3’)</th>
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<td><em>Ins2</em></td>
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<td>CAAATTTACAGGCGGATTCAGG</td>
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</tbody>
</table>

1Commercial primer assay (Catalog number 4352341E, Applied Biosystems)  
Other primers/PrimeTime qPCR assays are from Integrated DNA Technologies, Toronto, ON, Canada.
Chapter 3: Suppression of hyperinsulinemia in growing female mice provides long-term protection against obesity

3.1 Chapter preface and acknowledgements

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3.2 Introduction

Worldwide, more than 40 million children younger than the age of five were overweight in 2011, and being overweight or obese during childhood and adolescence is also a predictor of adult obesity. Since obesity is a risk factor for numerous pathologies, diseases that are traditionally associated with adulthood are thereby occurring with increasing prevalence in
youth. Hyperinsulinemia and insulin resistance are two characteristics of the obese state that likely contribute to its detrimental health effects. Although it is widely held that obesity leads to insulin resistance, causing a subsequent compensatory rise in insulin to prevent hyperglycemia, evidence suggests that hyperinsulinemia can precede and promote obesity. Insulin is known to suppress lipolysis and stimulate lipogenesis in white adipose tissue, and mouse models with reduced adipose tissue insulin signaling are protected from obesity.

We exploited the existence of two rodent insulin genes to genetically manipulate endogenous insulin production. Recent work in our laboratory demonstrated that continuous suppression of fasting hyperinsulinemia through reducing Ins1 dosage (in an Ins2-null background) prevented diet-induced obesity in male mice. However, Ins1 is a rodent-specific gene, and there are differences in promoter elements and expression patterns between Ins1 and Ins2. As it is unclear whether Ins1 and Ins2 have distinct roles, we felt it important to examine the effects of reduced Ins2 dosage in the development of high fat diet-induced obesity.

Certain life stages are important for adipocyte hyperplasia and hypertrophy, but the mechanisms controlling white adipose tissue expansion, and their timing, remain to be fully elucidated. White adipocyte cell number is thought to stabilize towards the end of adolescence in non-obese humans and rodents, which suggests that conditions during this programming period could influence future adiposity. Indeed, adolescence has been identified as one of the key life stages for the development of obesity in humans, since the presence or onset of obesity during adolescence is associated with an increased incidence of its associated morbidities in adults. In our previous study, the Ins1+/−:Ins2−/− genetic manipulation resulted in lifelong prevention of hyperinsulinemia, which precluded an assessment of whether anti-obesity
effects would persist without sustained repression of insulin. In the present study, we found that high-fat fed female \textit{Ins1}^{-/-}:\textit{Ins2}^{+/+} mice had reduced insulin secretion at a young age, but by one year they had reached an equivalent degree of hyperinsulinemia as high fat-fed \textit{Ins1}^{-/-}:\textit{Ins2}^{+/+} littermates. This provided a unique model to test the hypothesis that a reduction of insulin secretion in young, growing mice could provide long-term protection against diet-induced obesity. We found that this was the case, and that the attenuation of obesity persisted despite the late-onset emergence of hyperinsulinemia in HFD-fed \textit{Ins1}^{-/-}:\textit{Ins2}^{+/+} mice.

3.3 Results

3.3.1 Attenuated hyperinsulinemia in young HFD-fed \textit{Ins1}^{-/-}:\textit{Ins2}^{+/+} mice

To characterize this novel model, we first examined the effect of reduced \textit{Ins2} gene dosage on insulin mRNA, protein and secretion. Islets of 25 week-old \textit{Ins1}^{-/-}:\textit{Ins2}^{+/+} mice predictably had significantly less \textit{Ins2} expression than \textit{Ins1}^{-/-}:\textit{Ins2}^{+/+} islets, although this difference was less marked in islets from HFD-fed mice at 50 weeks (Figs. 3.1b,c). Insulin protein content also showed a more pronounced difference in islets from 25 week-old rather than 50 week-old mice, with approximately 50\% insulin content in \textit{Ins1}^{-/-}:\textit{Ins2}^{+/+} islets compared to \textit{Ins1}^{-/-}:\textit{Ins2}^{+/+} islets at 25 weeks (Figs. 3.1d,e). Insulin secretion was modestly, but consistently, lower in islets isolated from \textit{Ins1}^{-/-}:\textit{Ins2}^{+/+} mice, with a significant reduction in first-phase response to stimulatory glucose in chow-fed mice at 25 weeks of age (Figs. 3.1f-h). Collectively, these data demonstrate that reduced \textit{Ins2} dosage resulted in lower insulin production in female mice, although there is potential for age-dependent compensation with HFD feeding.

Reduced fasting insulin was detected in \textit{Ins1}^{-/-}:\textit{Ins2}^{+/+} mice at eight weeks, compared to \textit{Ins1}^{-/-}:\textit{Ins2}^{+/+} mice (Fig. 3.1i). Statistically significant HFD-induced fasting hyperinsulinemia
was observed in \( \text{Ins1}^{-/-} : \text{Ins2}^{+/+} \) mice by 27 weeks, and it remained elevated at one year (Fig. 3.1i). Importantly, \( \text{Ins1}^{-/-} : \text{Ins2}^{+/+} \) mice were protected from HFD-induced fasting hyperinsulinemia until at least 27 weeks (Fig. 3.1i). \( \text{Ins1}^{-/-} : \text{Ins2}^{+/+} \) mice were unable to increase fasting C-peptide in response to HFD at this age, confirming that reduced basal insulin secretion, rather than increased insulin clearance, accounted for these differences (Fig. 3.1j). At all measured time points, HFD led to higher glucose-stimulated insulin secretion than CD, but at eight and 15 weeks, glucose-stimulated insulin secretion was lower in \( \text{Ins1}^{-/-} : \text{Ins2}^{+/+} \) mice than \( \text{Ins1}^{-/-} : \text{Ins2}^{+/+} \) littermates (Fig. 3.2a). However, suppression of insulin secretion was not sustained in this mouse model, since by one year of age \( \text{Ins1}^{-/-} : \text{Ins2}^{+/+} \) females had equivalently high fasting and glucose-stimulated insulin levels as \( \text{Ins1}^{-/-} : \text{Ins2}^{+/+} \) mice on HFD (Figs. 3.1i, 3.2a).

### 3.3.2 Longitudinal analysis of glucose homeostasis

We found that reduced \( \text{Ins2} \) dosage had only minor effects on glucose homeostasis (Figs. 3.2b-d). At six weeks, \( \text{Ins1}^{-/-} : \text{Ins2}^{+/+} \) mice had slightly impaired glucose tolerance compared to \( \text{Ins1}^{-/-} : \text{Ins2}^{+/+} \) littermates (Fig. 3.2b). Since marginally elevated fasting glucose was also observed in 5-8 week-old \( \text{Ins1}^{-/-} : \text{Ins2}^{+/+} \) mice (Fig. 3.2d), insulin secretion may not be completely sufficient for normal glycemic control in very young \( \text{Ins1}^{-/-} : \text{Ins2}^{+/+} \) animals. However, these differences were only evident for a brief period (Figs. 3.2b,d), suggesting that there were no lasting negative repercussions in \( \text{Ins1}^{-/-} : \text{Ins2}^{+/+} \) mice. There were only modest long-term HFD effects on glucose homeostasis (Fig. 3.2b). Moderate glucose intolerance in the HFD group was associated with significant insulin resistance, with a notable decline in insulin-stimulated blood glucose response at 26 weeks and beyond in HFD-fed versus CD-fed mice (Fig. 3.2c). By 16 weeks, all HFD-fed animals had elevated fasting blood glucose compared to CD-fed mice (Fig. 3.2d). Therefore, any differences between HFD-fed \( \text{Ins1}^{-/-} : \text{Ins2}^{+/+} \) and \( \text{Ins1}^{-/-} : \text{Ins2}^{+/+} \) females would likely be driven by
attenuated hyperinsulinemia in young $Ins1^{-/-}:Ins2^{+/+}$ mice, rather than altered glucose homeostasis.

### 3.3.3 Lasting protection from obesity in HFD-fed $Ins1^{-/-}:Ins2^{+/+}$ mice

Next, we tested our primary hypothesis that reduced insulin early in life might attenuate diet-induced obesity. No significant mass differences were observed between pre-weaned $Ins1^{-/-}:Ins2^{+/+}$ and $Ins1^{-/-}:Ins2^{+/+}$ female pups (Fig. 3.3a). However, weight differences between high fat-fed $Ins1^{-/-}:Ins2^{+/+}$ and $Ins1^{-/-}:Ins2^{+/+}$ littermates were apparent almost immediately after introduction of HFD (Fig. 3.3a). While HFD-fed $Ins1^{-/-}:Ins2^{+/+}$ mice were significantly heavier than CD-fed $Ins1^{-/-}:Ins2^{+/+}$ littermates as early as 18 weeks, HFD had no detectable effect on $Ins1^{-/-}:Ins2^{+/+}$ mass until after 32 weeks (Fig. 3.3a). Furthermore, on HFD $Ins1^{-/-}:Ins2^{+/+}$ mice were still significantly lighter than $Ins1^{-/-}:Ins2^{+/+}$ littermates past one year of age (Fig. 3.3a), a time when fasting insulin levels were equivalent (Fig. 3.1i). At all time points studied, the HFD increased absolute fat mass (Fig. 3.3b) and proportional fat mass in $Ins1^{-/-}:Ins2^{+/+}$ mice. Moreover, HFD-fed $Ins1^{-/-}:Ins2^{+/+}$ mice showed a significant attenuation of adipose tissue expansion compared to $Ins1^{-/-}:Ins2^{+/+}$ mice (Fig. 3.3b). These data demonstrate that lower circulating insulin in young $Ins1^{-/-}:Ins2^{+/+}$ mice corresponded with a delayed and reduced degree of HFD-induced weight gain and obesity.

To look for evidence of adipose programming underlying differences in obesity, we examined inguinal (subcutaneous) and gonadal (visceral) WAT depots of 25 week-old mice. In gonadal WAT, perilipin staining revealed that while all HFD-fed mice had relatively fewer small lipid droplets and more large lipid droplets than CD-fed mice, $Ins1^{-/-}:Ins2^{+/+}$ mice tended to have a greater proportion of the largest lipid droplets (Figs. 3.3c,d). However, there were no
significant differences detected in transcript levels of measured WAT adipogenic, lipogenic, or lipolytic genes at this age (Figs. 3.3e,f).

3.3.4 Effects of HFD on circulating lipids and metabolic factors

To further characterize effects of lower insulin and attenuated obesity in HFD-fed $Ins1^{-/-}:Ins2^{+/+}$ mice, we measured lipids and metabolic factors in plasma from fasted 40 week-old mice. All HFD-fed mice had higher cholesterol and non-esterified fatty acids than CD-fed animals, but triglycerides were not significantly different (Figs. 3.4a-c). As expected based on body composition, only $Ins1^{-/-}:Ins2^{+/+}$ animals showed elevated leptin on HFD, compared to their chow-fed controls (Fig. 3.4d). However, all HFD-fed mice had increased resistin (Fig. 3.4e). We did not detect significant differences in inflammatory state based on interleukin 6 levels (Fig. 3.4f). Glucose-dependent insulino tropic polypeptide levels were similarly elevated in all HFD-fed mice (Fig. 3.4g). No significant differences were detected in concentrations of peptide YY (Fig. 3.4h) or glucagon (Fig. 3.4i). Therefore, while adult $Ins1^{-/-}:Ins2^{+/+}$ mice had attenuated HFD-induced weight gain and adiposity (Fig. 3.3), they showed many similarities to HFD-fed $Ins1^{-/-}:Ins2^{+/+}$ mice with respect to circulating metabolic factors, except leptin. These observations support the concept that attenuated obesity in $Ins1^{-/-}:Ins2^{+/+}$ mice was due to the transient reduction of insulin, rather than other factors.

3.3.5 Energy homeostasis in young mice

We examined energy balance of HFD-fed $Ins1^{-/-}:Ins2^{+/+}$ and $Ins1^{-/-}:Ins2^{+/+}$ littermates at 17 weeks, during their growth divergence. Trends suggest $Ins1^{-/-}:Ins2^{+/+}$ mice were more active than $Ins1^{-/-}:Ins2^{+/+}$ mice in early hours of the dark cycle (Fig. 3.5a). While there were no significant differences in food intake or respiratory exchange ratios (Figs. 3.5b,c), $Ins1^{-/-}:Ins2^{+/+}$ mice exhibited reduced energy expenditure compared to $Ins1^{-/-}:Ins2^{+/+}$ littermates
(Fig. 3.5d). *Ins1<sup>−/−</sup>:Ins2<sup>+/−</sup>* mice also had significantly reduced brown adipose tissue (BAT) mass compared to *Ins1<sup>−/−</sup>:Ins2<sup>++/−</sup>* littermates at 25 weeks (*p* < 0.05, Fig. 3.5e; *p* = 0.102 when normalized to body mass, Fig. 3.5f), and lower BAT *Cebpa* expression (Fig. 3.5g). Overall, attenuated adiposity in HFD-fed *Ins1<sup>−/−</sup>:Ins2<sup>+/−</sup>* mice was achieved despite decreased energy expenditure and BAT mass.

### 3.3.6 Long-term effects of short-term INSULIN2 treatment

In a small experiment designed to independently assess effects of elevating insulin during growth, HFD-fed 17 week-old mice were implanted with mini osmotic pumps secreting murine INSULIN2 or vehicle control for four weeks. INSULIN2-treated *Ins1<sup>−/−</sup>:Ins2<sup>+/−</sup>* mice tended to reach similar fasting insulin levels as *Ins1<sup>−/−</sup>:Ins2<sup>++/−</sup>* mice during the treatment, without immediate effects on body mass (Figs. 3.6a,b). However, by one year of age, *Ins1<sup>−/−</sup>:Ins2<sup>+/−</sup>* mice that had been previously INSULIN2-treated had body masses and WAT masses that appeared to be intermediate between vehicle-treated *Ins1<sup>−/−</sup>:Ins2<sup>+/−</sup>* and *Ins1<sup>−/−</sup>:Ins2<sup>++/−</sup>* mice (Figs. 3.6b,c). Regardless of INSULIN2 treatment, all HFD-fed *Ins1<sup>−/−</sup>:Ins2<sup>+/−</sup>* mice tended to exhibit relatively more of the small lipid droplets than *Ins1<sup>−/−</sup>:Ins2<sup>++/−</sup>* mice in gonadal WAT, with modest trends to suggest that INSULIN2 treatment may be associated with more of the largest droplets (Figs. 3.6d,e). Vehicle-treated *Ins1<sup>−/−</sup>:Ins2<sup>+/−</sup>* mice tended to have higher transcript levels of genes associated with fatty acid uptake and lipogenesis in gonadal WAT, compared to the other groups (*e.g. p* = 0.058 for *Lpl*; Fig. 3.6f), and had lower inguinal WAT *Cebpb* mRNA than other groups (Fig. 3.6g). Trends also suggested that INSULIN2 treatment was associated with increased BAT expression of *Prdm16*, a transcriptional coregulator that promotes development of brown adipocytes (Fig. 3.6h). While this experiment did not definitively show that short-term
INSULIN2 treatment was sufficient to promote obesity in \textit{Ins1}^{-/-}:\textit{Ins2}^{+/+} mice, it points to the potential for lasting effects, and should catalyze future studies.

### 3.4 Discussion

Our objective was to test the hypothesis that reducing insulin secretion by partial disruption of the \textit{Ins2} gene would prevent diet-induced obesity. A transient attenuation of insulin hypersecretion in young, growing \textit{Ins1}^{-/-}:\textit{Ins2}^{+/+} mice allowed us to test the secondary hypothesis that these anti-obesity effects would persist despite late-onset HFD-induced elevations in insulin. Our data indicate that, under these experimental conditions, reduced dosage of the ancestral \textit{Ins2} gene can provide a similar protection from obesity as reducing dosage of rodent-specific \textit{Ins1}. Importantly, the current study also identified the growth period of adolescence and young adulthood as a potentially critical time to suppress insulin escalation.

It is an intriguing concept that there could be key interventional periods for influencing obesity and associated health risks. In humans, the rate of body mass index rise during pubescence and the maximum body mass index during young adulthood (22-24 years) can both be stronger predictors of adiposity at mature adulthood (35-45 years) than adult lifestyle variables. It has previously been shown that early life manipulations such as short-term insulin treatment in neonatal rats leads to increased weight gain, as well as impairment of glucose tolerance and insulin responsiveness into adulthood. Our experiments with a short-term INSULIN2 treatment in \textit{Ins1}^{-/-}:\textit{Ins2}^{+/+} mice also pointed to possible long-term effects on weight gain, although the limited period of insulin treatment could have led to impaired insulin sensitivity and a lasting elevation of insulin secretion, as previously seen. Collectively, our
investigation indicates that repression of hyperinsulinemia in young, HFD-fed mice can attenuate obesity throughout adulthood.

Tissue analyses showed subtle dissimilarities in lipid droplet size distributions between $\text{Ins1}^{+/+}:\text{Ins2}^{+/+}$ and $\text{Ins1}^{-/-}:\text{Ins2}^{+/+}$ mice in the gonadal WAT at 25 weeks, implying possible divergence in adipocyte hypertrophy or hyperplasia, although no significant transcriptional changes were detected. By 50 weeks, HFD-fed $\text{Ins1}^{+/+}:\text{Ins2}^{+/+}$ mice showed a tendency for increased expression of genes associated with fatty acid uptake and lipogenesis in gonadal WAT. Although changes were not statistically significant in this small group of mice, these trends could indicate continued maintenance of adipocyte function and energy storage capacity, suggested to be metabolically beneficial in both humans and mice,\textsuperscript{262-265} and it may have been counteracted by exogenous INSULIN2 treatment. Interestingly, we did not detect elevated adipose $Ucp1$ expression in $\text{Ins1}^{+/+}:\text{Ins2}^{+/+}$ mice, unlike $\text{Ins1}^{+/+}:\text{Ins2}^{+/+}$ HFD-fed males.\textsuperscript{102} Rather, young $\text{Ins1}^{+/+}:\text{Ins2}^{+/+}$ females had smaller interscapular depots of BAT than $\text{Ins1}^{+/+}:\text{Ins2}^{+/+}$ littermates, similar to the effect of knocking the insulin receptor out of BAT,\textsuperscript{266} and this may have contributed to the lower energy expenditure. A caveat complicating the energy expenditure interpretation is that there were differences in body composition,\textsuperscript{267} and human studies have also shown that obese individuals may not show reduced total energy expenditure even if they are less active than lean subjects.\textsuperscript{268,269} Therefore, distinct adipose-level changes from those outlined in our previous study\textsuperscript{102} may have contributed to attenuated adiposity in HFD-fed $\text{Ins1}^{+/+}:\text{Ins2}^{+/+}$ females.

The HFD-fed $\text{Ins1}^{+/+}:\text{Ins2}^{+/+}$ female mice maintained protection from obesity into adulthood, despite the fact that their suppression of fasting insulin levels had reverted by one year of age. At 50 weeks, islet $\text{Ins2}$ mRNA and insulin content of HFD-fed $\text{Ins1}^{+/+}:\text{Ins2}^{+/+}$ mice
approached levels of their $Ins1^{-/-}:Ins2^{+/+}$ littermates. Although we could not show chronology of the relationship between this late-onset hyperinsulinemia and insulin resistance, it is clear from our results that reducing adipose tissue expansion and weight gain cannot always prevent the decline in glucose tolerance and insulin sensitivity that is associated with high fat feeding.

In conclusion, results from this investigation support the body of literature that places hyperinsulinemia mechanistically upstream of diet-induced obesity. The growth period of adolescence and young adulthood may be a critical time for shaping future adiposity, and our study demonstrates that in mice, repression of insulin hypersecretion during this life stage can provide long-term protection against obesity. Interestingly, pubescence in humans is associated with a transient period of reduced blood glucose responsiveness to insulin, and elevated insulin secretion.\textsuperscript{270,271} It could be worthwhile to explore whether limiting insulin hypersecretion during this phase could have lasting anti-obesity effects in humans.
3.5 Figures

Figures a and b show the relative Ins2 mRNA levels over time, comparing Chow Diet and High Fat Diet groups.

Figures c through j illustrate various physiological responses, including islet insulin, AUC, and fasting insulin levels, across different time points and dietary conditions.
Figure 3.1 Transiently attenuated HFD-induced hyperinsulinemia in \textit{Ins}1⁻⁻:\textit{Ins}2⁺⁺ mice.

(a) Experimental design of \textit{Ins}1⁻⁻:\textit{Ins}2⁺⁺ and \textit{Ins}1⁻⁻:\textit{Ins}2⁺⁺ littermates fed chow diet (CD) or high fat diet (HFD). Islet \textit{Ins}2 mRNA is corrected against β-actin and normalized to (b) CD-fed \textit{Ins}1⁻⁻:\textit{Ins}2⁺⁺ mice at 25 weeks (n = 3-5), or (c) HFD-fed \textit{Ins}1⁻⁻:\textit{Ins}2⁺⁺ mice at 50 weeks (n = 3). Islet insulin content is shown in (d) 25- and (e) 50-week mice, as is (f,g,h) insulin secretion by 150 islets perifused with 3 mM glucose (basal), 15 mM glucose (Glu), and 30 mM KCl, with area under the curve (y-axis units of ng/mL•min) depicted for each, including phases I/II of glucose stimulation (n = 3). 4-h fasted (i) insulin (n = 17-21) and (j) C-peptide (n = 5-6) is from \textit{in vivo} sampling. Data are means ± SEM. Dark blue and dark purple represent CD- and HFD-fed \textit{Ins}1⁻⁻:\textit{Ins}2⁺⁺ mice, respectively; pale blue and pale purple represent CD- and HFD-fed \textit{Ins}1⁻⁻:\textit{Ins}2⁺⁺ mice, respectively. \( p \leq 0.05 \) denoted by * for CD vs HFD, and # for \textit{Ins}1⁻⁻:\textit{Ins}2⁺⁺ vs \textit{Ins}1⁻⁻:\textit{Ins}2⁺⁺.
Figure 3.2 Effects of HFD and reduced *Ins2* dosage on longitudinal glucose homeostasis.

Periodic measurements of (a) glucose-stimulated insulin secretion (n = 17-21), blood glucose responses to intraperitoneal (b) glucose (n = 29-34) and (c) insulin analog (n = 29-34) is shown, with area under or over the curve (y-axis units of a, ng/mL•min, b mmol/L•min, c percent•min) shown in panel insets, and (d) 4-h fasted blood glucose (n = 15-18, most time points). Data are means ± SEM. Dark blue and dark purple represent CD- and HFD-fed *Ins1<sup>+/−:Ins2<sup>+/+</sup> mice*, respectively; pale blue and pale purple represent CD- and HFD-fed *Ins1<sup>+/−:Ins2<sup>+/−</sup> mice*, respectively. *p* ≤ 0.05 denoted by * for CD vs HFD, and # for *Ins1<sup>+/−:Ins2<sup>+/+</sup> vs *Ins1<sup>+/−:Ins2<sup>+/−</sup>.*
Figure 3.3 Attenuated obesity in HFD-fed $Ins1^{+/+}:Ins2^{+/+}$ mice.

(a) Body mass was tracked in pre-weaned pups (n = 19-29, inset) and weaned mice (n = 29-33, most time points). (b) DEXA-measured fat mass is shown (n = 8-11). (c) Staining (50X magnification) for perilipin (yellow) and DAPI (blue) in gonadal WAT of 25 week-old mice is quantified (n = 3) in (d) as frequency per size category. (e) Gonadal and (f) inguinal WAT mRNA is corrected against $Hprt$, and normalized to CD-fed $Ins1^{+/+}:Ins2^{+/+}$. Data are means ± SEM. Dark blue and dark purple represent CD- and HFD-fed $Ins1^{+/+}:Ins2^{+/+}$ mice, respectively; pale blue and pale purple represent CD- and HFD-fed $Ins1^{+/+}:Ins2^{+/+}$ mice, respectively. $p \leq 0.05$ denoted by * and $p = 0.051$ denoted by (*) for CD vs HFD, $p \leq 0.05$ denoted by # and $p = 0.054$ denoted by (#) for $Ins1^{+/+}:Ins2^{+/+}$ vs $Ins1^{+/+}:Ins2^{+/+}$, $p \leq 0.05$ denoted by *$^+$ for CD- vs HFD-fed $Ins1^{+/+}:Ins2^{+/+}$ mice, *$^+$ for CD- vs HFD-fed $Ins1^{+/+}:Ins2^{+/+}$ mice, and #$^{HFD}$ for HFD-fed $Ins1^{+/+}:Ins2^{+/+}$ vs $Ins1^{+/+}:Ins2^{+/+}$ mice.
Figure 3.4 Effects of HFD and reduced Ins2 dosage on plasma lipids and metabolic factors.

4-h fasted (a) cholesterol, (b) triglycerides, (c) non-esterified fatty acids (NEFAs), (d) leptin, (e) resistin, (f) interleukin 6, (g) glucose-dependent insulinotropic polypeptide (GIP), (h) peptide YY, and (i) glucagon is shown in 40 week-old mice. n = 8-12. Data are means ± SEM. Dark blue and dark purple represent CD- and HFD-fed \textit{Ins1}\textsuperscript{+/+}:\textit{Ins2}\textsuperscript{+/+} mice, respectively; pale blue and pale purple represent CD- and HFD-fed \textit{Ins1}\textsuperscript{−/−}:\textit{Ins2}\textsuperscript{+/+} mice, respectively. \(p \leq 0.05\) denoted by * for CD- vs HFD-fed, *\textsuperscript{+/+} for CD- vs HFD-fed \textit{Ins1}\textsuperscript{−/−}:\textit{Ins2}\textsuperscript{+/+} mice, and #\textsuperscript{HFD} for HFD-fed \textit{Ins1}\textsuperscript{−/−}:\textit{Ins2}\textsuperscript{+/+} vs \textit{Ins1}\textsuperscript{−/−}:\textit{Ins2}\textsuperscript{−/−} mice.
Figure 3.5 *In vivo* energy homeostasis and brown adipose tissue.

In HFD-fed 17 week-old mice (n = 6-8), (a) 24-h activity levels, (b) food intake, (c) respiratory exchange ratio, and (d) energy expenditure (adjusted for covariates of lean and fat mass) were averaged across 48-84 h, with grey indicating dark cycles. In 25 week-old mice (n = 5-7), BAT depot mass is shown as (e) absolute values and (f) proportional to body mass. (g) BAT mRNA is corrected against *Tbp* and normalized to CD-fed *Ins1<sup>−/−**:Ins2<sup>++</sup>*. Energy expenditure is shown as estimated marginal means ± SEM, adjusted for covariates of lean and fat mass; other data are simple means ± SEM. Dark blue and dark purple represent CD- and HFD-fed *Ins1<sup>−/−**:Ins2<sup>++</sup>* mice, respectively; pale blue and pale purple represent CD- and HFD-fed *Ins1<sup>−/−**:Ins2<sup>++</sup>* mice, respectively. *p* ≤ 0.05 denoted by * for CD vs HFD, and # for *Ins1<sup>−/−**:Ins2<sup>++</sup>* vs *Ins1<sup>−/−**:Ins2<sup>++</sup>*.
Figure 3.6 Long-term effects of HFD and transient INSULIN2 treatment.

(a) 4-h fasted insulin and (b) body mass is shown in mice implanted with mini osmotic pumps releasing INSULIN2 or vehicle for four weeks (indicated by hatching). n = 3. At 50 weeks, characterization included (c) WAT depot wet weight proportional to body mass, (d) gonadal WAT staining (50X magnification) for perilipin (yellow) and DAPI (blue), quantified (n = 3) in (e) as frequency per size category, and (f) gonadal and (g) inguinal WAT and (h) BAT mRNA corrected against *Tbp* or *Hprt*, and normalized to *Ins1-/-:Ins2+/-*. Data are means ± SEM. Dark purple represents vehicle-treated *Ins1-/-:Ins2+/-* mice, pale purple represents vehicle-treated *Ins1-/-:Ins2+/-* mice, and pale purple with dark hatching represents INSULIN2-treated *Ins1-/-:Ins2+/-* mice. ‡ denotes *p* ≤ 0.05, and (‡) denotes *p* ≤ 0.058.
Chapter 4: Hyper-variability in circulating insulin levels and response to high fat feeding in male $\text{Ins}^{+/--}:\text{Ins}^{+/+}$ mice

4.1 Chapter preface and acknowledgements

For the data presented in this chapter, Xiaoke Hu and Subashini Karunakaran assisted with animal studies. Xiaoke Hu and Farnaz Taghizadeh provided assistance with the tissue harvests and islet isolations, and performed the islet perifusion experiments. In addition, Timothy Kieffer provided use of the Luminex multiplexing instrument, and Susanne Clee provided use of the metabolic cages. Arya Mehran contributed to the study’s conceptualization and performed the preceding study, which was undertaken in a conventional animal facility.

4.2 Introduction

Variations in circulating insulin levels have far-reaching metabolic consequences. In addition to the expected fluctuations in insulin secretion that are associated with blood glucose, circulating insulin levels are affected by a number of hormones and circulating factors. In mice, the mean 5-h fasted insulin levels in non-obese, 12 week-old males can range from 0.5 to 1.2 ng/mL, across four commonly used strains. In humans, fasting insulin levels can range from 0.04 to 3.43 ng/mL in a nondiabetic adult population, and evidence suggests that less than half of the variance in fasting insulin can be accounted for by genetic variability.

Murine $\text{Ins}1$ and $\text{Ins}2$ have distinct promoter elements, tissue- and temporal-specific expression patterns, and imprinting status. In addition, differential translation or processing rates of the two murine preproinsulins have been reported. It is therefore
possible that levels of the fully processed murine insulin 1 and insulin 2 peptides are divergently susceptible to modulation under various conditions, which could underlie the evolutionary retention of both genes. When one insulin gene is inactivated, elevated transcript and protein level of the non-deleted insulin gene can at least partially compensate for the loss, but the exact nature of this reciprocal relationship remains understudied.

We have performed a series of investigations to examine how murine Ins1 and Ins2 gene dosage impacts the onset of high fat diet-induced hyperinsulinemia and the development of obesity. Previous work in our laboratory showed that reducing Ins1 gene dosage (on an Ins2-null background) results in continuous suppression of fasting hyperinsulinemia in male mice, thereby preventing diet-induced obesity. Interestingly, circulating insulin levels were not similarly modulated in the female littermates from this study, suggesting the possibility of sex-specific differences in the relationship between insulin gene dosage and circulating insulin levels. In the converse genetic manipulation, reduced Ins2 dosage (on an Ins1-null background) led to high-fat fed female Ins1−/−:Ins2+/− mice having lower insulin secretion than their Ins1−/−:Ins2+/+ controls at a young age, which again corresponded with attenuated obesity. The phenotype of the female Ins1−/−:Ins2+/− mice was highly consistent between the two large cohorts of animals studied under specific pathogen-free conditions, and was congruent to preliminary evaluations in a conventional facility.

We report herein on circulating insulin levels and the metabolic phenotype of the male Ins1−/−:Ins2+/− and Ins1−/−:Ins2+/+ littermates of the female mice that were the subject of our recent investigation (Chapter 3). Contrary to expectations, inactivating one Ins2 allele did not cause a consistent reduction of circulating insulin in Ins1-null mice, which precluded us from properly testing the hypothesis that reduced Ins2 dosage and lower insulin levels would lead to protection
from obesity in males. Specifically, we report that across cohorts, the effects of high fat feeding on glucose homeostasis, insulin sensitivity, and weight gain in $Ins1^{-/-}:Ins2^{+/+}$ male mice varied widely. Moreover, circulating insulin levels were hyper-variable across cohorts in $Ins1$-null male mice, pointing to sex-specific compensation of insulin homeostasis in these animals.

4.3 Results

4.3.1 Variability in insulin secretion between cohorts of male mice

We have recently studied the effects of reducing $Ins2$ gene dosage in $Ins1$-null female mice, and found consistent outcomes for insulin secretion and body composition across cohorts. While characterizing male siblings from the same cohorts, we noticed that a number of measured parameters showed dramatic inconsistencies between cohorts A and B, precluding us from pooling the male data from these two cohorts. For instance, we observed that in cohort A males, fasting insulin was significantly higher in HFD-fed mice than CD-fed mice at all measured time points across a year (Fig. 4.1b), and glucose-stimulated insulin secretion was higher in HFD-fed mice at 8, 15, and 52 weeks of age (Fig. 4.1c). However, there were unexpectedly no significant differences in insulin levels (either fasting or glucose-stimulated) between $Ins1^{-/-}:Ins2^{+/+}$ and $Ins1^{-/-}:Ins2^{+/+}$ mice, at any point up to one year in cohort A (Figs. 4.1b,c). In contrast, cohort B $Ins1^{-/-}:Ins2^{+/+}$ mice tended to have lower fasting insulin levels than their $Ins1^{-/-}:Ins2^{+/+}$ littermates (Fig. 4.1b), and at 27 weeks of age they had significantly reduced glucose-stimulated insulin secretion (Fig. 4.1d). In addition, $Ins1^{-/-}:Ins2^{+/+}$ mice were the only cohort B males showing more glucose-stimulated insulin secretion on HFD than CD at the early 8-week time point (Fig. 4.1d). However, the HFD-induced elevation of basal and glucose-stimulated insulin secretion was, in general, quite modest for most males in cohort B
(Figs. 4.1b,d). Notably, by 52 weeks all groups of cohort B male mice clearly had lower insulin levels than cohort A male mice (Fig. 4.1b).

To evaluate potential mechanisms underlying cross-cohort changes in circulating insulin levels in male mice, we assessed insulin mRNA, protein, and islet function in two distinct groups. Unfortunately, due to the longitudinal nature of the experiments we could not use islets from cohort A or B mice. However, a separate group of 25 week-old male mice tended to show a genotype effect for \textit{in vivo} fasting insulin ($p = 0.056$), as well as similar raw insulin values to those of cohort B mice (Figs. 4.1b, 2a). Islets from these 25 week-old \textit{Ins}1$^{-/-}$:\textit{Ins}2$^{+/+}$ mice had an expected reduction in \textit{Ins}2 mRNA compared to \textit{Ins}1$^{-/-}$:\textit{Ins}2$^{+/+}$ islets (Figs. 4.2b). Interestingly, the significant reduction in \textit{Ins}2 mRNA did not correspond to a significant reduction in islet insulin protein content (Fig. 4.2c), suggesting the involvement of post-transcriptional compensation. Consistent with the lack of a difference between genotypes for islet insulin content, dynamic secretion by islets from 25 week-old HFD-fed \textit{Ins}1$^{-/-}$:\textit{Ins}2$^{+/+}$ mice was not reduced compared to \textit{Ins}1$^{-/-}$:\textit{Ins}2$^{+/+}$ islets, and in fact 25 week-old \textit{Ins}1$^{-/-}$:\textit{Ins}2$^{+/+}$ islets appeared to have the capacity for a marginally increased 2\textsuperscript{nd} phase response to KCl stimulation (Fig. 4.2d).

We also evaluated 70 week-old islets from a group of HFD-fed mice that had shown no obvious genotype differences in fasting insulin levels at 52 weeks of age (Fig. 4.2e). This allowed us to confirm that the genetic manipulation also led to reduced \textit{Ins}2 mRNA in older HFD-fed \textit{Ins}1$^{-/-}$:\textit{Ins}2$^{+/+}$ male mice (Fig. 4.2f), with a similar capacity for compensation at the level of islet insulin content (Fig. 4.2g) as was evident at 25 weeks (Fig. 4.2c). The only detected differences in dynamic \textit{in vitro} islet secretion were minimal, showing that at 70 weeks, \textit{Ins}1$^{-/-}$:\textit{Ins}2$^{+/+}$ islets did not secrete quite as much insulin in the 2\textsuperscript{nd} phase of glucose stimulation as \textit{Ins}1$^{-/-}$:\textit{Ins}2$^{+/+}$ islets (Fig. 4.2h). Collectively, these data show that although the experimental
genetic manipulation did successfully reduce Ins2 expression in the male mice, there was evidence of compensation with respect to insulin protein content and islet secretory capacity. The capability for insulin production compensation may have accounted for the lack of consistent differences in circulating insulin levels between Ins1<sup>−/−</sup>:Ins2<sup>++</sup> and Ins1<sup>−/−</sup>:Ins2<sup>++</sup> male mice.

4.3.2 Variability in glucose homeostasis between cohorts of male mice

We also observed heterogeneity between cohorts in a longitudinal analysis of glucose homeostasis, as might be expected from cross-cohort variability in insulin levels. In conjunction with the sustained HFD-induced elevation of circulating insulin in cohort A mice, from 14 weeks onwards HFD-fed males from cohort A showed significant whole-body insulin resistance (i.e. minimal changes in blood glucose levels in response to insulin stimulation) compared to their CD-fed littermates (Fig. 4.3a). HFD-fed mice in cohort A also had a modest degree of glucose intolerance compared to mice on CD, at 8 and 50 weeks (Fig. 4.3b). In contrast, there were no statistically significant differences in insulin sensitivity or glucose tolerance observed between CD- and HFD-fed groups of cohort B mice, consistent with a limited response to HFD-feeding (Figs. 4.3c,d).

Reduced Ins2 dosage and the slight differences in circulating insulin levels observed between Ins1<sup>−/−</sup>:Ins2<sup>++</sup> and Ins1<sup>−/−</sup>:Ins2<sup>++</sup> cohort B mice did not appear to cause robust negative repercussions for glucose homeostasis in cohort B (Figs. 4.3c,d). However, in cohort A, Ins1<sup>−/−</sup>:Ins2<sup>++</sup> mice were slightly but significantly more glucose intolerant than their Ins1<sup>−/−</sup>:Ins2<sup>++</sup> littermate controls at each measured time point across a year. Closer examination of the responses to glucose stimulation shows a trend for a delayed or sustained peak in blood glucose in cohort A Ins1<sup>−/−</sup>:Ins2<sup>++</sup> male mice (Fig. 4.3b). This implies that although the cumulative glucose-stimulated insulin levels appeared nearly matched between genotypes in cohort A, at
least across the first 30 minutes post-stimulation (Figs. 4.1c), subtle differences in insulin secretory patterns (e.g. Fig. 4.2h, although this *in vitro* insulin secretion was assessed in different mice) could have affected glycemic control. Indeed, in addition to the long-term elevation of fasting glucose evident in HFD-fed mice compared to CD-fed mice in cohort A, there were periods where cohort A *Ins1⁺⁻:Ins2⁺⁻* males had higher fasting glucose levels than their *Ins1⁺⁻:Ins2⁺⁺* littermates (Fig. 4.4a). On the other hand, all cohort B mice had largely comparable fasting glucose levels throughout the year, which is further evidence for the minor impact of high fat feeding in this cohort (Fig. 4.4b).

### 4.3.3 Heterogeneity between cohorts in body mass of *Ins1⁺⁻:Ins2⁺⁻* mice

Perhaps one of the most striking differences noted between cohorts A and B was the differential impact of reducing *Ins2* gene dosage on body weights, particularly in HFD-fed mice. The loss of one *Ins2* allele did not notably affect either circulating insulin levels (Figs. 4.1b,c) or HFD-induced growth (Fig. 4.4c) in cohort A males, since all HFD-fed mice showed equivalent weight gain compared to CD-fed littermates, particularly from 20 weeks onward (Fig. 4.4c). Although young *Ins1⁺⁻:Ins2⁺⁻* mice were smaller than their *Ins1⁺⁻:Ins2⁺⁺* littermates for a limited period in cohort A, this did not persist (Fig. 4.4c). HFD-fed mice were heavier than CD-fed mice for a similar duration in cohort B as in cohort A, but the significantly smaller mass of cohort B *Ins1⁺⁻:Ins2⁺⁻* mice compared to their *Ins1⁺⁻:Ins2⁺⁺* littermates continued throughout most of the year (Fig. 4.4d). Notably, a difference in body mass between *Ins1⁺⁻:Ins2⁺⁺* and *Ins1⁺⁻:Ins2⁺⁻* mice of cohort B was detectable in male pups as young as 2 days of age (Fig. 4.4e), and it is possible that there could have also been similar size differences in neonatal pups of cohort A (not measured) that might have contributed to the genotype effect on body mass observed in young cohort A mice (Fig. 4.4c).
We attempted to evaluate different factors that could have contributed to phenotypic differences between cohorts A and B. We did not detect obvious means by which parental imprinting of the \( \text{Ins2} \) allele might have accounted for the observed variability, as both breeding pair options (i.e. either the dam or the sire donating the disrupted \( \text{Ins2} \) allele) contributed to cohort A and B experimental animals. There were no statistically significant differences between offspring of dams versus sires with the disrupted \( \text{Ins2} \) allele when the factor of “parental effect” was incorporated into analyses of body mass or fasting insulin levels (with both cohorts pooled); any patterns suggestive of a possible parental effect in one cohort were either not present or showed opposite trends in the other cohort (data not shown). There was also no apparent distinction in the mean or distribution of litter sizes between cohorts A and B (Fig. 4.4f).

As a limited indication of environmental stressors, we also measured 4 h-fasted corticosterone levels at 27 weeks. Interestingly, there tended to be a greater number of mice with elevated corticosterone levels in cohort B, and modest trends suggested that there were higher average corticosterone levels in HFD-fed \( \text{Ins1}^{-/-}:\text{Ins2}^{+/+} \) males from cohort B, compared to cohort A (Fig. 4.4g). Closer examination of HFD-fed animals showed that across both cohorts, fasting insulin levels in HFD-fed \( \text{Ins1}^{-/-}:\text{Ins2}^{+/+} \) male mice were inversely correlated to corticosterone levels \( (r^2 = 0.38, p < 0.01) \), and this relationship was not evident in HFD-fed \( \text{Ins1}^{-/-}:\text{Ins2}^{+/+} \) mice \( (r^2 = 0.00, p = 0.95) \). Interpretation of these data is limited, as it is based on using a single measurement of corticosterone for each individual animal as a marker of ‘stress’ during a time period. However, it appears that those HFD-fed \( \text{Ins1}^{-/-}:\text{Ins2}^{+/+} \) male mice that reached the highest fasting insulin levels at 27 weeks (predominantly individuals from cohort A) tended to have lower corticosterone levels, whereas increased stress may have dampened the capacity of HFD-fed \( \text{Ins1}^{-/-}:\text{Ins2}^{+/+} \) male mice to compensate for reduced \( \text{Ins2} \) dosage at the level of basal
insulin secretion. If mice in cohort B did experience more stressful conditions than cohort A animals (Fig. 4.4g), this could be one of potentially many contributing factors underlying phenotypic differences in circulating insulin levels between cohorts A and B.

Heterogeneity in our data precluded pooling cohorts A and B to generate averaged results. However, although experimental genetic and dietary manipulations did not have consistent effects in both cohorts, we did observe a comparable range of fasting insulin values and body masses for both cohorts (Figs. 4.1b, 4c,d). To indirectly evaluate whether differences in fasting insulin might be underlying body weight alterations in this model, we examined the relationship between these two variables across all year-old mice. There was a positive correlation between body mass and fasting insulin levels at this age ($r^2 = 0.55, p < 0.0001$; Fig. 4.4i). Therefore, while we did not observe consistent effects of reducing $Ins2$ gene dosage on circulating insulin and obesity in male mice, in general, these data support the concept that reduced insulin levels are associated with attenuated body weight and obesity.

4.3.4 Attenuated obesity and fat-free mass in cohort B $Ins1^{−/−}:Ins2^{+/−}$ males

We further characterized cohort B mice, as they showed a sustained divergence in body mass between $Ins1^{−/−}:Ins2^{+/−}$ and $Ins1^{−/−}:Ins2^{+/+}$ groups (Fig. 4.4d). First, we used metabolic cages to examine the in vivo energy balance of HFD-fed mice at 17 weeks, an age when both cohorts showed similar trends for body masses. Although HFD-fed $Ins1^{−/−}:Ins2^{+/−}$ mice exhibited a slight elevation in activity levels during the early hours of the dark period (Fig. 4.5a), it did not appear that there were genotype differences in whole-body energy expenditure (Fig. 4.5b), respiratory exchange ratio (Fig. 4.5c), or food intake (Fig. 4.5d) to account for the disparities in weight gain between HFD-fed $Ins1^{−/−}:Ins2^{+/−}$ and $Ins1^{−/−}:Ins2^{+/+}$ males.
We assessed body composition longitudinally with DEXA. Consistent with the evidence suggesting that lower Ins2 dosage led to generally reduced growth in male neonatal pups (Fig. 4.4e), reductions in both adiposity and fat-free mass contributed to the smaller size of cohort B Ins1⁻/⁺:Ins2⁺/+ male mice. Ins1⁻⁻:Ins2⁺/+ animals had significantly higher fat mass than Ins1⁻⁻:Ins2⁺/+ males on both diets, at all measured time points (Fig. 4.6a). A similar pattern was also evident for fat-free masses, particularly at the older ages (Fig. 4.6b). However, since reductions in fat mass were proportionally greater than reductions in fat-free mass for Ins1⁻⁻:Ins2⁺/+ versus Ins1⁻⁻:Ins2⁺/+ males up to 60 weeks of age (Figs. 4.6a,b), it is clear that an attenuation in adiposity contributed to the reduced body mass of cohort B Ins1⁻⁻:Ins2⁺/+ male mice, compared to their Ins1⁻⁻:Ins2⁺/+ controls. In the group of 25 week-old males with similar insulin levels as cohort B mice (Figs. 4.1b, 2a), both subcutaneous and visceral white adipose tissue depots were smaller in Ins1⁻⁻:Ins2⁺/+ versus Ins1⁻⁻:Ins2⁺/+ mice (Fig. 4.6c). Furthermore, the Ins1⁻⁻:Ins2⁺/+ mice had smaller interscapular brown adipose tissue depots than their Ins1⁻⁻:Ins2⁺/+ littermates, and a slight reduction in the size of a mixed triceps surae muscle group (Fig. 4.6c).

Despite the attenuated adiposity and fat-free mass of cohort B Ins1⁻⁻:Ins2⁺/+ males compared to their Ins1⁻⁻:Ins2⁺/+ littermates, we did not observe notable genotype differences in the fasting levels of circulating lipids and metabolic factors at 40 weeks of age. All HFD-fed mice had higher cholesterol and non-esterified fatty acids than CD-fed animals, without significant differences in triglycerides levels (Fig. 4.6d-f). In spite of differences in adipose tissue mass, leptin was similarly elevated in all cohort B HFD-fed mice compared to CD-fed mice (Fig. 4.6g), as was resistin (Fig. 4.6h). Based on levels of interleukin 6, there did not appear to be significant differences in inflammatory state between groups (Fig. 4.6i). Glucose-dependent
insulinotropic polypeptide levels were similarly elevated in all HFD-fed mice, compared to CD-fed animals (Fig. 4.6j), but no significant differences were detected in concentrations of peptide YY (Fig. 4.6k). These data suggest that while a combination of attenuated adiposity and reduced fat-free mass contributed to the smaller body weights of cohort B $\text{Ins}1^{-/-}:\text{Ins}2^{+/+}$ males compared to their $\text{Ins}1^{-/-}:\text{Ins}2^{+/+}$ littermate controls, cohort B HFD-fed $\text{Ins}1^{-/-}:\text{Ins}2^{+/+}$ males nonetheless displayed many of the expected characteristics of high fat feeding.

4.4 Discussion

The initial aim of our work was to test the hypothesis that reducing $\text{Ins}2$ gene dosage on an $\text{Ins}1$-null background would prevent HFD-induced hyperinsulinemia, and thereby protect against obesity in male mice. Contrary to our expectations, inactivating one $\text{Ins}2$ allele did not cause a consistent reduction of circulating insulin in $\text{Ins}1$-null male mice – not even the transient suppression of insulin hypersecretion that was consistently evident in their female $\text{Ins}1^{-/-}:\text{Ins}2^{+/+}$ littermates at a young age (Figs. 3.1, 3.2).\textsuperscript{274} We report that under some conditions, $\text{Ins}1$-null males with reduced $\text{Ins}2$ mRNA were capable of producing nearly equivalent circulating insulin levels as $\text{Ins}1^{-/-}:\text{Ins}2^{+/+}$ males across one year, albeit possibly with subtle differences in secretory patterns that could have contributed to modest glucose intolerance. This clearly distinguishes these $\text{Ins}1^{-/-}:\text{Ins}2^{+/+}$ males from the $\text{Ins}2$-null male mice with reduced dosage of the $\text{Ins}1$ gene, as $\text{Ins}1^{+/+}:\text{Ins}2^{-/-}$ male mice experienced a sustained suppression of hyperinsulinemia.\textsuperscript{102} Our findings show that $\text{Ins}1^{-/-}:\text{Ins}2^{+/+}$ male mice exhibit phenotypic hyper-variability across cohorts with respect to insulin levels, glucose homeostasis, and weight gain with chronic high fat feeding.
In the current study, all HFD-fed mice in the first experimental cohort, cohort A, showed notable insulin hypersecretion and weight gain, without significant effects of reduced Ins2 dosage. In contrast, cohort B tended towards a less pronounced degree of HFD-induced insulin hypersecretion and peripheral insulin resistance. In addition, in cohort B there seemed to be a sustained reduction in insulin levels and body mass in Ins1−/−:Ins2+/− mice compared to their Ins1−/−:Ins2+/+ littermate controls, without detected changes in food intake or energy expenditure. These two cohorts from the same colony were studied approximately one year apart, under consistent experimental conditions in a controlled SPF facility. Despite this, by one year of age the average differences in fasting insulin levels between the two cohorts were considerably more pronounced than the difference between having one or two functional Ins2 alleles.

It is important to note that pronounced phenotypic variability between different cohorts of Ins1−/−:Ins2+/− male mice, particularly with respect to body mass, was also observed within a separate animal facility during a different time period (A. Mehran, personal communication). Remarkably, in one of these previous experimental cohorts, Ins1−/−:Ins2+/− male mice actually exhibited greater HFD-induced weight gain than their Ins1−/−:Ins2+/+ controls (A. Mehran, personal communication), which is the opposite outcome to what was observed in cohort B of our current experiment (Fig. 4.4d). We cannot explain the widely diverse phenotypic responses to reduced Ins2 gene dosage in male mice. However, it is clear that cross-cohort phenotypic variability in Ins1−/−:Ins2+/− males has been observed in two distinct facilities to a degree that was not observed in their female littermates (Chapter 3),274 nor in Ins2-null male or female mice with full or partial Ins1 expression,102 despite the fact that these similar mouse models were studied by our group in the same time frames and under similar conditions.102,274 Therefore, in Ins1-null
male mice, the phenotypic outcomes of *Ins2* gene modulation appear to be susceptible to a wide range in variability.

Phenotypic variability, in general, is poorly understood, but likely affects many long-term animal studies. There is evidence that the *in utero* and neonatal environments, gut microbiome composition, and exposure to different stressors, including temperature, noise, social hierarchy, and even the sex of the researchers working with animal subjects, can have far-reaching effects on many physiological parameters. Additional considerations include animal background strain or sub-strain, and genetic drift within a colony. These variables can confound experimental results through such means as altering the endocrine milieu, or changing gene expression levels, directly or via the epigenome. Although we attempted to control for many of these potentially confounding variables to a reasonable level in our investigation, it is possible that these sorts of variables affected our results.

There are numerous other factors that may have played a part in the observed phenotypic heterogeneities in our investigation. For instance, the murine *Ins2* gene has been shown to be subject to developmental stage-dependent and tissue-specific genomic imprinting. We considered the possibility that the disrupted *Ins2* allele may have had variable effects depending on whether it was inherited from the maternal or the paternal side, particularly as our mice lacked the potential for compensatory *Ins1* expression. Although we did not observe obvious, consistent parental effects on the experimental animals, genomic imprinting is a complex system that has not yet been fully elucidated, and a potential role cannot be fully ruled out.

As the experimental cohorts were separated temporally, another potential explanation for cross-cohort variability is subtle environmental changes across the years (although the cohorts
were roughly matched for seasons since they were roughly one year apart). There were no differences between cohorts with respect to the average number of siblings sharing their in utero and neonatal environments, nor in numerous controlled parameters. However, in long-term experiments it is not always possible to avoid such environmental perturbations as earthquakes, minor construction periods around a facility, and so forth. At 27 weeks, an age when it was becoming clear that the two cohorts were diverging in their patterns of insulin secretion and weight gain, a single blood sample per mouse provided limited indication that levels of the stress hormone corticosterone might have been slightly higher in cohort B males, compared to mice from cohort A. Effects vary depending on duration or type of stressor, but there is evidence that chronic stress or glucocorticoid exposure itself can lead to reduced insulin secretion in rodents. Interestingly, the glucocorticoid receptor has been shown to bind to a negative regulatory element of the human INS gene. In our results, there was a negative correlation between basal insulin levels and corticosterone across both cohorts in HFD-fed Ins1−/−:Ins2+/− males. We suggest that reduced exposure to stress, signified by decreased plasma corticosterone, may have partially accounted for some HFD-fed Ins1−/−:Ins2+/− male mice having the ability to produce nearly equivalent amounts of fasting insulin as their Ins1−/−:Ins2+/+ littermates. However, multiple other contributing factors likely influenced these outcomes.

The hypothetically environmental- or stress-dependent ability to compensate for reduced Ins2 dosage at the level of insulin production and/or secretion was only observed in male Ins1−/−:Ins2+/+ mice across one year. Although the female littermates experienced largely consistent conditions under the same experimental time frame, they did not exhibit similar patterns in insulin levels. These findings thereby point to the possibility of sex-specific regulation of murine insulin 2 production or secretion. Both testosterone and estrogen have the
capacity to stimulate insulin production and secretion,\textsuperscript{299-301} so if the gonadal steroids have differential effects on \textit{Ins2} and or insulin 2 peptide, it might have contributed to the observed male-specific variability in insulin levels for \textit{Ins1}^{−/−}:\textit{Ins2}^{+/−} mice. However, there are numerous other sex-specific physiological differences that could have also played a role.

Overall, results from this investigation should serve to highlight the range of insulin’s physiological effects, and the phenotypic characteristics that can change in association with variability in insulin levels. We were unable to properly test the hypothesis that prevention of HFD-induced hyperinsulinemia would protect against obesity using this male mouse model, due to non-uniform effects of reducing \textit{Ins2} gene dosage on circulating insulin in \textit{Ins1}-null males. However, we did observe a positive correlation between body mass and fasting insulin, which supports the concept that mice with lower endogenously produced circulating insulin also tend to have reduced obesity.\textsuperscript{102,274} In addition, in cohort B experimental mice we show that reduced \textit{Ins2} expression and subtle reductions in insulin levels can be associated with a long-term attenuation of whole body growth, including both fat-free mass and adipose tissue. While it might be expected that differences in insulin levels would primarily affect circulating glucose and other metabolites, we found that anabolic repercussions could be detected in the absence of significant alterations to glucose homeostasis.

Our study demonstrates that circulating insulin levels are susceptible to wide variation in male mice, perhaps especially in mice lacking the potentially stabilizing effects of \textit{Ins1} expression. Indeed, measured insulin levels in humans and wild-type mice have been shown to be subject to considerable variation,\textsuperscript{242-244} and our results suggest that inadvertent stress exposure may be one factor underlying variability in insulin levels. In addition, we show that it is
important to look beyond insulin’s well-characterized effects on glucose homeostasis when evaluating the physiological effects of divergent insulin levels.
4.5 Figures

a

Cohort A: Born Oct 2011 - Dec 2011
Cohort B: Born Oct 2012 - Feb 2013

b

Fasting insulin (ng/mL)

8 weeks 15 weeks 27 weeks 52 weeks

Cohort A Cohort B Cohort A Cohort B Cohort A Cohort B Cohort A Cohort B

C

Insulin (ng/mL)

8 weeks 15 weeks 27 weeks 52 weeks

Time (min)

0 2 15 30

D

Insulin (ng/mL)

8 weeks 15 weeks 27 weeks 52 weeks

Time (min)

0 2 15 30
Figure 4.1 Hyper-variability in insulin secretion across two cohorts.

(a) Experimental design showing two cohorts (A and B) of $\text{Ins}1^{-/-}\cdot\text{Ins}2^{+/+}$ and $\text{Ins}1^{-/-}\cdot\text{Ins}2^{-/-}$ male littermates fed chow diet (CD) or high fat diet (HFD). (b) Periodic measurements of 4-h fasted insulin levels is shown for both cohorts, with scatter points to indicate individual values (cohort A: closed points, n = 10-11; cohort B: open points, n = 6-10). In addition, periodic measurements of glucose-stimulated insulin secretion in (c) cohort A (n = 10-11) and (d) cohort B (n = 6-8) is shown, with area under the curve (y-axis units of ng/mL•min) in panel insets. Data are means ± SEM. Dark blue and dark red represent CD- and HFD-fed $\text{Ins}1^{-/-}\cdot\text{Ins}2^{+/+}$ male mice, respectively; pale blue and orange represent CD- and HFD-fed $\text{Ins}1^{-/-}\cdot\text{Ins}2^{-/-}$ male mice, respectively. $p \leq 0.05$ denoted by * for CD vs HFD, $^{+/+}$ for CD- vs HFD-fed $\text{Ins}1^{-/-}\cdot\text{Ins}2^{+/+}$ mice, # for $\text{Ins}1^{-/-}\cdot\text{Ins}2^{+/+}$ vs $\text{Ins}1^{-/-}\cdot\text{Ins}2^{-/-}$, and § for cohort A vs cohort B.
Figure 4.2 Characterization of Ins\textsuperscript{1−}/Ins\textsuperscript{2+} and Ins\textsuperscript{1−}/Ins\textsuperscript{2−} islets.

(a) In vivo 4-h fasted insulin is shown from 25 week-old mice prior to collection of islets to assess (b) Ins\textsuperscript{2} mRNA, corrected against β-actin and normalized to CD-fed Ins\textsuperscript{1−}/Ins\textsuperscript{2+} mice, (c) islet insulin content, and (d) insulin secretion by 150 islets perfused with 3 mM glucose (basal), 15 mM glucose (Glu), and 30 mM KCl, with area under the curve (y-axis units of ng/mL•min) depicted for phases I/II of glucose and KCl stimulation, minus basal secretion. (e) in vivo 4-h fasted insulin is shown from 52 week-old mice prior to collection of islets at 70 weeks to assess (f) Ins\textsuperscript{2} mRNA, corrected against β-actin and normalized to HFD-fed Ins\textsuperscript{1−}/Ins\textsuperscript{2+} mice, (g) islet insulin content, and (h) insulin secretion by 150 islets perfused with 3 mM glucose (basal), 15 mM glucose (Glu), and 30 mM KCl, with area under the curve (y-axis units of ng/mL•min) depicted for phases I/II of glucose and KCl stimulation, minus basal secretion. n = 3-7. Data are means ± SEM, with scatter points to indicate individual values. Dark blue and dark red represent CD- and HFD-fed Ins\textsuperscript{1−}/Ins\textsuperscript{2+} male mice, respectively; pale blue and orange represent CD- and HFD-fed Ins\textsuperscript{1−}/Ins\textsuperscript{2−} male mice, respectively. p ≤ 0.05 denoted by * for CD vs HFD, and # for Ins\textsuperscript{1−}/Ins\textsuperscript{2+} vs Ins\textsuperscript{1−}/Ins\textsuperscript{2−}. (#) indicates p = 0.056 for Ins\textsuperscript{1−}/Ins\textsuperscript{2+} vs Ins\textsuperscript{1−}/Ins\textsuperscript{2−}.
Figure 4.3 Variability in glucose homeostasis across two cohorts.

Periodic measurements of blood glucose response to intraperitoneal delivery of (a) an insulin analog (n = 10-19) in cohort A mice, and (b) glucose (n = 11-19) in cohort A mice, as well as (c) an insulin analog (n = 7-11) in cohort B mice, and (d) glucose (n = 7-11) in cohort B mice. Area under or over the curve (y-axis units of a,c percent•min, b,d mmol/L•min) is shown in panel insets. Data are means ± SEM. Dark blue and dark red represent CD- and HFD-fed $Ins1^{+/−}:Ins2^{+/+}$ male mice, respectively; pale blue and orange represent CD- and HFD-fed $Ins1^{+/−}:Ins2^{+/−}$ male mice, respectively. $p \leq 0.05$ denoted by * for CD vs HFD, and # for $Ins1^{+/−}:Ins2^{+/+}$ vs $Ins1^{+/−}:Ins2^{+/−}$. 

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Figure 4.4 Cross-cohort variation in fasting glucose, body mass, and corticosterone.

Periodic measurements of 4-h fasted blood glucose in (a) cohort A (n = 12-18, most time points) and (b) cohort B (n = 8-10, most time points) is shown, in addition to weekly body mass in (c) cohort A (n = 12-18, most time points) and (d) cohort B (n = 8-10, most time points). (e) Body mass was also tracked in a subset of pre-weaned male pups from cohort B (n = 12-22). (f) Litter sizes were compared between cohorts, with scatter points indicating the number of pups per individual litter (n = 12-16). (g) 4-h fasted corticosterone levels, measured in plasma collected during early afternoon from 27 week-old mice, were assessed between cohorts. Data are means ± SEM. (h) The relationship between corticosterone and insulin levels across cohorts in 27 week-old HFD-fed males is shown (cohort A: closed points, n = 10; cohort B: open points, n = 9-10), with r² = 0.38 and p < 0.01 for Ins1⁻⁻:Ins2⁺⁺ male mice. Data are means ± SEM. In addition, (i) The relationship between body mass and 4-h fasted insulin levels at one year of age is shown, with r² = 0.55 and p < 0.0001 (cohort A: closed points, n = 9-12; cohort B: open points, n = 6-7). Dark blue and dark red represent CD- and HFD-fed Ins1⁻⁻:Ins2⁺⁺ male mice, respectively; pale blue and orange represent CD- and HFD-fed Ins1⁻⁻:Ins2⁺⁻ male mice, respectively. p ≤ 0.05 denoted by * for CD vs HFD, and # for Ins1⁻⁻:Ins2⁺⁺ vs Ins1⁻⁻:Ins2⁺⁻.
Figure 4.5 *In vivo* energy homeostasis of HFD-fed cohort B males.

In HFD-fed 17 week-old mice (n = 6-7), (a) 24-h activity, with inset showing mean beam breaks across the first 4 h of the dark period (y-axis units of beam breaks), as well as (b) energy expenditure (adjusted for covariates of lean and fat mass), (c) respiratory exchange ratio, and (d) food intake were averaged across 48-84 h, with grey indicating dark cycles. Energy expenditure is shown as estimated marginal means ± SEM, adjusted for covariates of lean and fat mass; other data are simple means ± SEM. Dark red represents HFD-fed *Ins1<sup>−/−</sup>:Ins2<sup>+/+</sup>* male mice; orange represents HFD-fed *Ins1<sup>−/−</sup>:Ins2<sup>+/−</sup>* male mice. *p* ≤ 0.05 denoted by # for *Ins1<sup>−/−</sup>:Ins2<sup>+/+</sup>* vs *Ins1<sup>−/−</sup>:Ins2<sup>+/−</sup>*.
Figure 4.6 Attenuated adiposity and fat-free mass in cohort B $Ins1^{-/-}:Ins2^{+/+}$ males.

Periodic DEXA-measured (a) fat mass and (b) fat-free mass is shown in cohort B male mice ($n = 5-7$). This corresponded to (c) wet masses of inguinal, epigonal, and mesenteric WAT depots, the interscapular BAT depot, whole liver, and the triceps surae hindlimb mixed muscle group, with all tissues from a group of 25 week-old male mice ($n = 3-7$) that was discrete yet similar in phenotype to cohort B mice. In addition, 4-h fasted (d) cholesterol, (e) triglycerides, (f) non-esterified fatty acids (NEFAs), (g) leptin, (h) resistin, (i) interleukin 6, (j) glucose-dependent insulinotropic polypeptide (GIP), and (k) peptide YY is shown in 40 week-old mice from cohort B ($n = 7-9$). Data are means ± SEM, with scatter points to indicate individual values. Dark blue and dark red represent CD- and HFD-fed $Ins1^{-/-}:Ins2^{+/+}$ male mice, respectively; pale blue and orange represent CD- and HFD-fed $Ins1^{-/-}:Ins2^{+/+}$ male mice, respectively. $p \leq 0.05$ denoted by * for CD vs HFD, and # for $Ins1^{-/-}:Ins2^{+/+}$ vs $Ins1^{-/-}:Ins2^{+/+}$. 
Chapter 5: Modest reductions in circulating insulin can extend lifespan in mammals

5.1 Chapter preface and acknowledgements

For the data presented in this chapter, the veterinarian and animal technician staff assisted with animal care. In addition, Jana Hodasova and the Animal Care Services Diagnostic Laboratory assisted with necropsies and prepared tissues for histopathology. Nick Nation was the primary veterinary pathologist, and he kindly provided very comprehensive information.

5.2 Introduction

Long-living organisms tend to share common distinguishing characteristics, including reduced insulin and IGF-1 levels, enhanced insulin sensitivity, smaller body sizes, and reduced oxidative damage. This suggests that there is evolutionary conservation of mechanisms that regulate aging and lifespan. Many of the genes identified as having major effects on post-maturation aging and longevity operate in the regulation of metabolism and energy homeostasis. For example, in the invertebrates Caenorhabditis elegans and Drosophila melanogaster, significant lifespan extension results from mutating several intermediates in the insulin/IGF-1-like signaling pathway, including reduction-of-function mutations of genes encoding the transmembrane tyrosine kinase receptor, the receptor substrate, and the effector phosphatidylinositol 3-kinase. In both C. elegans and D. melanogaster, one key contributor to extending lifespan with these manipulations seems to be a homologue of the
mammalian class O forkhead box (FOXO) family, a transcription factor which is phosphorylated and excluded from the nucleus in response to insulin/IGF-1-like signaling.

Strikingly, genetic variation within the FOXA3 gene has also been repeatedly associated with long lifespan in humans.\textsuperscript{154,156-159} In addition, genetic manipulations to moderately downregulate insulin/IGF-1 signaling in mice through reducing IGF-1 receptors in the whole animal,\textsuperscript{144,145} deleting insulin receptors in adipose tissue,\textsuperscript{143} deleting insulin receptor substrate (IRS)-1,\textsuperscript{141,147} decreasing IRS-2 in some models,\textsuperscript{148} or transgenically overexpressing the negative repressor phosphatase and tensin homolog PTEN\textsuperscript{149} can lead to lifespan extension, although more extreme reductions in insulin/IGF-1 signaling tend to be detrimental.\textsuperscript{139-141} Even so, the precise roles of insulin versus IGF-1 can be unclear when components of this signaling network are genetically disrupted in mice.\textsuperscript{44,164} At the ligand level, reduced cumulative IGF-1 exposure has been repeatedly associated with lifespan extension in mammals, particularly in mutant mouse models with disruptions to the somatotropic axis.\textsuperscript{135,136,163,165-174} However, somatotropic genetic disruptions are associated with a range of endocrine and physiological changes, and it appears that reducing IGF-1 signaling alone may not always be sufficient to impart the robust lifespan effects observed with more extensive somatotropic disruptions in mice.\textsuperscript{127,172,173}

The role of insulin in mammalian longevity has remained controversial and not directly tested. This is probably in part because insulin is the ligand most strongly linked to the metabolic effects of insulin/IGF-1 signaling, and drastic reductions in insulin levels are widely associated with diabetes and lethality.\textsuperscript{91,92,302} Interpreting the impact of insulin on mammalian aging and longevity is further complicated by the interrelatedness of circulating insulin levels and systemic insulin responsiveness. Although genetically reducing intermediates of the insulin/IGF-1 signaling network can increase mammalian longevity, reduced insulin signaling in the form of
peripheral insulin resistance is paradoxically a deleterious feature of mammalian aging which is a risk factor for a number of age-related diseases. However, all branches of insulin signaling in target tissues are not equally affected in the impaired insulin-stimulated glucose uptake that characterizes peripheral insulin resistance, and insulin levels escalate when there is inadequate insulin-stimulated glucose uptake in peripheral tissues. Many long-lived mammals share the attribute of low circulating insulin, but this could simply be auxiliary to an enhancement of insulin sensitivity. It had not yet been determined if a moderate lowering of the insulin ligand itself could have a causal role in extending mammalian healthspan and longevity.

To test whether a modest reduction of circulating insulin levels could affect aging and lifespan in mammals, we used a mouse model in which the rodent-specific insulin gene *Ins1* was completely inactivated, and compared mice with either full or partial expression of the ancestral *Ins2* gene, under two distinct diet regimes. Inactivating one *Ins2* allele did not cause a consistent reduction of circulating insulin in *Ins1*-null male mice (Fig. 4.1). However, female *Ins1*⁻/⁻:*Ins2*⁺/⁻ mice showed a reduction in insulin secretion and circulating insulin levels compared to their *Ins1*⁺/⁺:*Ins2*⁺/⁺ littermates, particularly evident in young mice on an obesogenic, high-energy high fat diet (Figs. 3.1, 3.2). This did not have lasting adverse effects on glucose homeostasis across one year (Fig. 3.2), making the female mice an ideal model for our investigation. In this study, we found that that a slight reduction in circulating insulin levels was sufficient to promote healthier aging and extend lifespan in these female *Ins1*⁻/⁻:*Ins2*⁺/⁻ mice.
5.3 Results

5.3.1 Glucose homeostasis in aged \( \text{Ins}1^{-/-}\cdot\text{Ins}2^{+/+} \) and \( \text{Ins}1^{-/-}\cdot\text{Ins}2^{+/+} \) female mice

To continue our evaluation of the effects of reducing \( \text{Ins}2 \) gene dosage across murine lifespan, we assessed insulin levels and glucose homeostasis in aged, 1.5 year-old mice. At 1.5 years, \( \text{Ins}1^{-/-}\cdot\text{Ins}2^{+/+} \) female mice showed a modest reduction in fasting circulating insulin levels compared to \( \text{Ins}1^{-/-}\cdot\text{Ins}2^{+/+} \) littermate controls, under both diets (Fig. 5.1a). Indeed, since absolute insulin levels were already low in \( \text{Ins}1^{-/-}\cdot\text{Ins}2^{+/+} \) females fed a moderate-energy chow diet, the effects of halving \( \text{Ins}2 \) dosage were particularly slight: the 24% decrease in the aged \( \text{Ins}1^{-/-}\cdot\text{Ins}2^{+/+} \) mice versus \( \text{Ins}1^{-/-}\cdot\text{Ins}2^{+/+} \) littermates (Fig. 5.1a) was the maximum difference in average fasting insulin levels detected on CD at all measured time points across their lifespan (Fig. 3.1). With chronic consumption of a distinct high-energy high fat diet, \( \text{Ins}1^{-/-}\cdot\text{Ins}2^{+/+} \) females suppressed HFD-induced hyperinsulinemia while young and growing, but reached an equivalent degree of insulin hypersecretion as their \( \text{Ins}1^{-/-}\cdot\text{Ins}2^{+/+} \) littermates by one year of age (Figs. 3.1, 3.2). However, by 1.5 years HFD-fed \( \text{Ins}1^{-/-}\cdot\text{Ins}2^{+/+} \) female mice again had lower fasting insulin levels than \( \text{Ins}1^{-/-}\cdot\text{Ins}2^{+/+} \) controls (Fig. 5.1a). Notably, the experimental manipulation of reducing \( \text{Ins}2 \) gene dosage did not affect total circulating IGF-1 in young or aged female mice, on either diet (Fig. 5.1b). Alterations to aging and lifespan in these mice were therefore not due to changes in total IGF-1 levels in circulation, but were instead likely caused by the observed slight, physiologically relevant reduction in circulating insulin.

Reducing \( \text{Ins}2 \) gene dosage and circulating insulin did not have lasting adverse effects on glucose homeostasis in the first year of murine life (Fig. 3.2). However, there was potential for long-term detrimental consequences of this experimental manipulation, since aging is often associated with insulin secretory defects further compounded by increased demand on pancreatic...
\(\beta\) cells due to such conditions as peripheral insulin resistance and elevated adiposity.\textsuperscript{245-247} Unexpectedly, compared to their \(\text{Ins1}^{-/-}:\text{Ins2}^{+/+}\) littermates the \(\text{Ins1}^{-/-}:\text{Ins2}^{+/+}\) female mice began to exhibit lower fasting glucose levels with the advancement of age (Fig. 5.1c), and at 80 weeks had a decreased mean HOMA-IR score (Fig. 5.1d), a simplified measure of insulin resistance based on fasting glucose and insulin. Enhanced insulin analog-stimulated glucose disposal was also evident in aged \(\text{Ins1}^{-/-}:\text{Ins2}^{+/+}\) female mice on either diet (Fig. 5.1e), confirming that their peripheral insulin sensitivity was superior to that of the \(\text{Ins1}^{-/-}:\text{Ins2}^{+/+}\) littermates. Despite modest changes in fasting insulin and glucose levels, there were no statistically significant effects of genotype detected for the insulin secretory response (Fig. 5.1f) or blood glucose response (Fig. 5.1g) to an intraperitoneal delivery of glucose in 1.5 year-old mice. Collectively, these data indicate that rather than having negative repercussions for glucose homeostasis, reduced \(\text{Ins2}\) gene dosage and slightly lowered basal insulin levels could lead to a healthier metabolic state in aged mice.

5.3.2 Effects of chronic HFD in aged female mice

At 1.5 years, HFD-fed mice showed elevated basal and glucose-stimulated insulin secretion compared to mice on CD (Figs. 5.1a,f), in addition to diminished glucose-lowering responsiveness to an insulin analog (Fig. 5.1e) and higher fasting glucose levels (Fig. 5.1c) leading to slight glucose intolerance (Fig. 5.1g). We previously found that lower insulin levels in young, growing \(\text{Ins1}^{-/-}:\text{Ins2}^{+/+}\) female mice provided protection from obesity that persisted in the face of a late-onset emergence of hyperinsulinemia at one year (Fig. 3.3).\textsuperscript{274} At 85 weeks of age, \(\text{Ins1}^{-/-}:\text{Ins2}^{+/+}\) female mice still had reduced body mass (Fig. 5.2a) and adiposity (Fig. 5.2b), compared to their \(\text{Ins1}^{-/-}:\text{Ins2}^{+/+}\) littermates. Despite differences in adiposity between aged \(\text{Ins1}^{-/-}:\text{Ins2}^{+/+}\) and \(\text{Ins1}^{-/-}:\text{Ins2}^{+/+}\) female mice, plasma leptin had reached similarly elevated levels.
in all HFD-fed mice compared to animals on CD by 90 weeks of age, without a statistically significant difference between genotypes (Fig. 5.2c). Otherwise, largely similar patterns for plasma lipids and metabolic factors were evident at 90 weeks (Figs. 5.2c-k) as had been observed in 40 week-old female mice (Fig. 3.4). Specifically, in addition to increased leptin (Fig. 5.2c), HFD-fed mice had higher resistin (Fig. 5.2d) and glucose-dependent insulinotropic polypeptide (Fig. 5.2f) levels compared to CD-fed mice, with no differences between genotypes. At 90 weeks of age, unlike at 40 weeks, glucagon was also elevated in HFD-fed mice, regardless of genotype (Fig. 5.2h). No differences were observed between groups in levels of circulating interleukin 6 (Fig. 5.2e) or peptide YY (Fig. 5.2g). Total cholesterol was elevated in all HFD-fed mice compared to mice on CD (Fig. 5.2i), although there were no differences in circulating triglycerides (Fig. 5.2j) or non-esterified fatty acids (Fig. 5.2k). Therefore despite their reduced obesity, 90 week-old Ins1\textsuperscript{−/−}:Ins2\textsuperscript{−/−} female mice had many similarities to Ins1\textsuperscript{−/−}:Ins2\textsuperscript{+/+} controls with respect to the expected effects of high fat feeding on circulating lipids and metabolic factors.

Nonetheless, Ins1\textsuperscript{−/−}:Ins2\textsuperscript{−/−} female mice did experience a reduced degree of obesity-associated sequelae. For instance, histopathological post-mortem analyses revealed that while 12.5% (4 out of 32 mice) of analyzed HFD-fed Ins1\textsuperscript{−/−}:Ins2\textsuperscript{−/−} female mice had abnormal hepatic lipid accumulation that was rated as moderate or severe, none of the analyzed HFD-fed Ins1\textsuperscript{−/−}:Ins2\textsuperscript{+/+} female mice (29 mice) showed evidence of this degree of lipidosis ($p \leq 0.05$). Hematoxylin and eosin-stained liver sections from randomly selected 2 year-old HFD-fed mice also confirmed fewer signs of hepatic steatosis in Ins1\textsuperscript{−/−}:Ins2\textsuperscript{−/−} female mice (Fig. 5.3).
5.3.3 Lean mass, bone mineral density, and skeletal muscle aging in female mice

As insulin is known to be an anabolic hormone,79 we felt it was important to closely follow the body composition and skeletal muscular strength of mice with reduced insulin levels. Few statistically significant differences were detected between groups, which is notable considering that a cohort of male $\text{Ins1}^{-/-}:\text{Ins2}^{+/+}$ mice had shown reduced size from neonatal pups to at least one year of age, with reductions in both adiposity and fat-free mass compared to their $\text{Ins1}^{-/-}:\text{Ins2}^{+/+}$ littermates (Fig. 4.6). 17 week-old $\text{Ins1}^{-/-}:\text{Ins2}^{+/+}$ female mice did have reduced fat-free mass compared to $\text{Ins1}^{-/-}:\text{Ins2}^{+/+}$ females, and trends suggested this might have continued to be the case on HFD at all measured time points (Fig. 5.4a). However, it is clear for mice on the CD regime that reduced $\text{Ins2}$ gene dosage and circulating insulin levels did not significantly alter whole body fat-free mass of fully grown mice (Fig. 5.4a). In addition, across both diets the reduction in $\text{Ins2}$ dosage had very slight if any effects on bone mineral density in aged animals (Fig. 5.4b), and did not significantly affect neuromuscular health as evaluated by forelimb grip strength (Fig. 5.4c). Interestingly, the two diets had differential effects on these parameters, with middle-aged HFD-fed mice showing increased lean mass, bone mineral density, and skeletal muscle strength compared to mice on the particular chow diet used in our study (Figs. 5.4a-c). This may be indicative of divergent health impacts of these two diets, distinct from obesogenic effects of high fat content of the HFD.

5.3.4 Lifespan, oxidative damage, and histopathological assessment of female mice

Unlike the extensive endocrine disruption of many long-lived mouse models, reducing $\text{Ins2}$ gene dosage had only modest effects on circulating insulin. Remarkably, our data show that $\text{Ins1}^{-/-}:\text{Ins2}^{+/+}$ female mice had a significant increase in lifespan, compared to $\text{Ins1}^{-/-}:\text{Ins2}^{+/+}$ littermate controls (Fig. 5.5a). This was validated using both the Kaplan-Meier method with a
log rank test stratified by the factor of diet, and a Cox proportional hazards regression analysis incorporating covariates of diet and cohort. The maximum lifespan also tended to be higher in $\text{Ins}1^{-/-}:\text{Ins}2^{+/+}$ females compared to their $\text{Ins}1^{-/-}:\text{Ins}2^{+/+}$ littermate controls, across both diet regimes ($p = 0.059$; Fig. 5.5b). $\text{Ins}1^{-/-}:\text{Ins}2^{+/+}$ mice had a 3% extension of median lifespan over their $\text{Ins}1^{-/-}:\text{Ins}2^{+/+}$ littermate controls on the HFD, as compared to the 11% median lifespan extension evident with CD. Since the degree of lifespan extension afforded by reduced $\text{Ins}2$ dosage was similar for female mice on both CD and HFD, or even blunted on HFD, it seems that a lifelong attenuation of HFD-induced obesity did not impart an additive extension of lifespan beyond the other lifespan-extending effects of reducing circulating insulin.

One evolutionarily conserved feature of aging is increased production of reactive oxygen species and oxidative damage, concurrent with declining mitochondrial function, and many long-lived organisms exhibit resistance to oxidative stress.\textsuperscript{15} 8-isoprostan e is a compound produced from the non-enzymatic peroxidation of tissue phospholipids by oxygen radicals, and can therefore be a circulating marker of oxidative stress. Although there were no differences in 30 week-old mice, aged 90 week-old $\text{Ins}1^{-/-}:\text{Ins}2^{+/+}$ female mice likely experienced less cumulative oxidative damage than their $\text{Ins}1^{-/-}:\text{Ins}2^{+/+}$ littermates, as indicated by reduced plasma levels of 8-isoprostan e (Fig. 5.5c).

Histopathological evaluations of post-mortem tissues revealed some interesting trends related to the causes of morbidity or mortality for these mice. For instance, in HFD-fed $\text{Ins}1^{-/-}:\text{Ins}2^{+/+}$ female mice compared to $\text{Ins}1^{-/-}:\text{Ins}2^{+/+}$ littermates, there tended to be a delayed occurrence of malignant cancer-associated mortality (Fig. 5.5d), combined with a tendency for a slight reduction in the percentage of mice noted to be bearing tumours (Fig. 5.5e) and a lower number of tumours detected per animal (Fig. 5.5f). There was also a very modest tendency for
delayed occurrence of death due to pathologies related to cardiac dysfunction in
\textit{Ins1}^{-/-}:\textit{Ins2}^{+/+} females (Fig. 5.5g). Interestingly, there was a significant delay in mortality
associated with renal degeneration and kidney diseases in \textit{Ins1}^{-/-}:\textit{Ins2}^{+/+} female mice compared to
their \textit{Ins1}^{-/-}:\textit{Ins2}^{+/+} littermates (Fig. 5.5h).

The particular “chow diet” used in this study seems to have caused disease in
middle-aged female mice, as suggested by the tendency for a relatively constant mortality rate
past 1 year of age in females on CD (Fig. 5.5a). In contrast, the survival curve of the female mice
on the HFD has a more rectangular shape with rapid decline in survival late in life, characteristic
of multiple age-dependent causes of mortality (Fig. 5.5a). Unfortunately, macroscopic and
histopathological assessments did not reveal the specific CD-associated disease-type experienced
by CD-fed mice. The lesions that were detected in major organs did not seem sufficiently severe
to cause the observed early onset mortality and morbidity for a large number of CD-fed mice
(Fig. 5.5i), although there is a pattern indicating earlier occurrence of cardiac-associated
mortality in some of the CD-fed female mice, compared to HFD-fed females (Fig. 5.5g).

Regardless, since trends suggest that the relative proportion of mice experiencing each of these
major murine pathologies was largely comparable between \textit{Ins1}^{-/-}:\textit{Ins2}^{+/+} and \textit{Ins1}^{-/-}:\textit{Ins2}^{+/+} mice
(Figs. 5.5d-e,g-i), the experimental manipulation likely provided a general extension of
healthspan and lifespan, rather than alleviating a specific disease.

\textbf{5.3.5 Aging and longevity of \textit{Ins1}^{-/-}:\textit{Ins2}^{+/+} and \textit{Ins1}^{-/-}:\textit{Ins2}^{+/+} male mice}

As observed in the animals at a younger age, inactivating one \textit{Ins2} allele did not cause a
consistent reduction of circulating insulin in aged \textit{Ins1}-null male mice. Up to 1 year of age, we
had observed hyper-variability and strong cohort-dependent effects in circulating insulin levels
of \textit{Ins1}^{-/-}:\textit{Ins2}^{+/+} male mice, pointing to sex-specific compensation of insulin homeostasis in these
animals (Fig. 4.1). At 1.5 years of age, cohort A male mice on HFD had elevated fasting levels of insulin (Fig. 5.6a) and glucose (Fig. 5.6b), with a corresponding increase in HOMA-IR scores (Fig. 5.6c) compared to CD-fed mice. Interestingly, although Ins1−/−:Ins2+/− male mice in cohort A had shown similar fasting insulin levels as their Ins1−/−:Ins2+/+ littermates up to 1 year, coupled with a frequent tendency for elevated fasting glucose (Figs. 4.1, 4.4), by 1.5 years cohort A Ins1−/−:Ins2+/− males had lower fasting insulin levels (Fig. 5.6a) and HOMA-IR scores (Fig. 5.6c) than their Ins1−/−:Ins2+/+ littermates, and similar fasting glucose levels (Fig. 5.6b). In contrast, although cohort B Ins1−/−:Ins2+/− male mice had tended to have lower insulin levels than their Ins1−/−:Ins2+/+ littermates up to 1 year of age (Fig. 4.1), by 1.5 years this trend is no longer evident (Fig. 5.6d). Consistent with the diminished overall effect of high fat feeding previously observed in cohort B males (Figs. 4.1, 4.3, 4.4), HFD was associated with a moderate elevation in fasting insulin (Fig. 5.6d) with no effect on fasting glucose (Fig. 5.6e), leading to a moderately elevated HOMA-IR score in cohort B HFD-fed mice compared to males on CD (Fig. 5.6f). Both cohorts showed similar patterns of glucose homeostasis (Figs. 5.6g-l), with a trend for reduced insulin analog-stimulated glucose disposal in HFD-fed mice (Figs. 5.6g,j), which appears to have been somewhat balanced by increased glucose-stimulated insulin secretion (Figs. 5.6h,k) to provide seemingly normal glucose tolerance on HFD (Figs. 5.6i,l). However, there were no genotype effects evident for these metabolic parameters (Figs. 5.6g-l). All in all, male Ins1−/−:Ins2+/− mice did not seem to be a suitable model for testing whether moderately reducing circulating insulin could affect aging and lifespan.

It is notable that across both cohorts and both diets, aged 85 week-old Ins1−/−:Ins2+/− male mice had smaller body masses than their Ins1−/−:Ins2+/+ littermates (Figs. 5.7a,b). In the aged cohort B male mice, the difference in body mass between genotypes no longer seemed to result
primarily from attenuated adiposity in $Ins1^{-/-}:Ins2^{+/+}$ males (Fig. 5.7c); instead, there was a tendency for a slight reduction in fat-free mass in $Ins1^{-/-}:Ins2^{+/+}$ mice (Fig. 5.7d; detailed body composition not assessed in cohort A mice). Indeed, unlike what was seen in the female mice (Fig. 5.4b), middle-aged and old $Ins1^{-/-}:Ins2^{+/+}$ males showed a reduction in bone mineral density compared to $Ins1^{-/-}:Ins2^{+/+}$ littermates (Fig. 5.7e), pointing to potential importance of $Ins2$ gene contributions to bone health of male mice. Aside from a slight divergence at 17 weeks that correspond to dissimilar body sizes (Fig. 4.4), there were no lasting differences between groups with respect to forelimb grip strength (Fig. 5.7f).

As might be expected based on the lack of an obvious, consistent effect of reducing $Ins2$ dosage on circulating insulin in male $Ins1^{-/-}:Ins2^{+/+}$ mice, there were no clear effects of genotype on median or maximum lifespan for the males in this experimental model, whether considering all male mice pooled (Fig. 5.8a), or separated into cohorts that were studied a year apart (Figs. 5.8b,c). Interestingly, in accordance with the wide phenotypic variability observed between the two cohorts, there was also a trend suggesting cross-cohort variability in their survival curves, with male mice on the CD diet showing a pattern for earlier mortality in cohort B than in cohort A (Figs. 5.8b,c), although the covariate of cohort did not have a statistically significant impact in the Cox regression analyses. It is clear that there was no effect of reducing $Ins2$ dosage on lifespan in the male mice, in contrast to what was seen in the females.

Intriguingly, $Ins1^{-/-}:Ins2^{+/+}$ male mice tended to experience a delayed occurrence of mortality associated with malignant cancers compared to $Ins1^{-/-}:Ins2^{+/+}$ males (Fig. 5.8d, $p = 0.058$), with additional trends suggesting that the CD-fed $Ins1^{-/-}:Ins2^{+/+}$ males also had a reduced percentage of mice bearing tumours (Fig. 5.8e) and a lower tumour burden (Fig. 5.8f). Therefore, while there was were no clear signs of lifespan extension (Figs. 5.8a-c) or changed incidences of
other pathologies (Figs. 5.8g-i) in $\text{Ins}1^{-/}:\text{Ins}2^{+/-}$ male mice compared to their $\text{Ins}1^{-/}:\text{Ins}2^{+/+}$ littermates, reducing $\text{Ins}2$ gene dosage may have imparted some protection against cancers in the males, perhaps relating to the attenuation of general growth in $\text{Ins}1^{-/}:\text{Ins}2^{+/-}$ male mice (Figs. 5.7, 4.4, 4.6). However, given the small samples sizes of animals that experienced mortality associated with specific causalities, the conclusions that can be made for these observations are limited.

5.4 Discussion

Unlike the extensive endocrine disruption of many long-lived mouse models, such as dwarf mice with mutations affecting the somatotropic axis,\textsuperscript{165-171} our experimental manipulation of reducing $\text{Ins}2$ gene dosage had very modest effects on the outcome of circulating insulin. Indeed, after 15 weeks of age there were no statistically significant genotype differences detected in the amount of glucose-stimulated insulin secretion in female mice, and basal plasma insulin levels were only slightly lowered in young and aged $\text{Ins}1^{-/}:\text{Ins}2^{+/-}$ females, compared to $\text{Ins}1^{-/}:\text{Ins}2^{+/+}$ controls. Yet, compared to $\text{Ins}1^{-/}:\text{Ins}2^{+/-}$ littermates, female $\text{Ins}1^{-/}:\text{Ins}2^{+/-}$ mice exhibited many of the hallmark characteristics of long-lived model organisms, including enhanced insulin sensitivity, smaller body sizes, and reduced oxidative stress. In addition, $\text{Ins}1^{-/}:\text{Ins}2^{+/-}$ female mice had an increased lifespan compared to $\text{Ins}1^{-/}:\text{Ins}2^{+/+}$ littermate controls, under two distinct diet regimes.

We did not observe a significant effect of lowering $\text{Ins}2$ gene dosage on lifespan for male mice on either diet in this model, whether the evaluation was performed for all males pooled or for each cohort separately. Insulin/IGF-1 signaling might affect longevity in a sex-specific manner, as a significant increase in survivorship has been observed only in the female $D$.\textsuperscript{100}
melanogaster with a mutated insulin-like receptor\textsuperscript{62} or insulin-like receptor substrate,\textsuperscript{63} and only in the female mice with partially inactivated IGF-1 receptors.\textsuperscript{144,145} In addition, a study in humans showed that lower composite insulin/IGF-1 signaling scores were associated with reduced mortality in women but not men.\textsuperscript{162} Even when there are apparent longevity effects for both sexes of a mouse model with mutations within the somatotropic axis or insulin/IGF-1 signaling network, there can be bias for greater lifespan extension in females,\textsuperscript{141,172,213} so we cannot discount the possibility that reducing insulin/IGF-1 signaling might have sex-specific effects on aging and lifespan in mammals. However, it is important to emphasize that our experimental manipulation did not have the intended effect of consistently lowering plasma insulin levels in males, so we were unable to ascertain whether moderate reductions in circulating insulin could lengthen lifespan in male mice. Interestingly, we nonetheless observed a tendency for male $\text{Ins}1^{-/-}:\text{Ins}2^{+/+}$ mice to show reduction in general growth and a limited protection from cancers, compared to their $\text{Ins}1^{-/-}:\text{Ins}2^{+/+}$ littermates, indicating that Ins2 may play a key anabolic role in male mice.

In this investigation, we wished to study the effects of circulating insulin levels on longevity independent of the complex and varied influences of macronutrient or micronutrient composition, or total caloric intake.\textsuperscript{205} Although the use of distinct, unmatched diets meant that we were unable to pinpoint the components of the CD that may have directly led to impaired health in the female mice, a strength of this design is that we found robust effects of reduced Ins2 dosage and insulin levels on several parameters of aging and lifespan in female mice, irrespective of diet composition. Indeed, the greater cumulative reduction of insulin levels, as well as the lifelong alleviation of high fat-induced obesity and such obesity-associated sequelae as hepatic steatosis in HFD-fed $\text{Ins}1^{-/-}:\text{Ins}2^{+/+}$ female mice compared to $\text{Ins}1^{-/-}:\text{Ins}2^{+/+}$ littermates
might have been expected to impart a greater extension of lifespan with the HFD, but that did not appear to be the case. Interestingly, dietary restriction, the experimental intervention most consistently associated with promoting healthy aging and extending lifespan, has been shown to be most effective for extending lifespan in mouse strains that are resistant to losing adiposity with the intervention. This points to physiological benefits of dietary restriction beyond reduced white adipose tissue. In addition, chronically HFD-fed C57BL/6 mice have comparable survival curves regardless of whether they exhibit notably increased adiposity or are resistant to HFD-induced obesity. Our study suggests that a slight reduction in circulating insulin levels, without altered food intake (Fig. 3.5), can have positive effects for healthspan and lifespan across multiple diet compositions.

Many long-lived mammals share the attribute of low circulating insulin, sometimes supplementary to genetic reductions in components of insulin/IGF-1 signaling. However, since insulin levels are intimately related to the ability of peripheral target tissues to lower blood glucose in response to insulin stimulation, it was unclear if these lowered insulin levels were simply ancillary to diminished peripheral insulin resistance. Interestingly, it has been suggested that enhancement of insulin sensitivity and the concurrent reduction in insulin is one of the central mediators of longevity that could be key for extending lifespan in mice with reduced growth hormone signaling. The pronounced degree of lifespan extension evident in mice with disrupted somatotropic signaling could therefore result from a combination of lowered IGF-1 levels and modulated circulating insulin and/or peripheral insulin sensitivity. Importantly, we report here that modestly reducing circulating insulin levels through partial inactivation of the ancestral insulin gene in mice can itself lead to enhanced peripheral insulin sensitivity with aging. In addition, female mice with lowered insulin levels
show evidence for reduced oxidative damage, and a significant extension of lifespan. While there may be additional beneficial effects of limiting cumulative exposure to IGF-1 bioactivity in mammals, dietary and lifestyle interventions can result in modestly decreased circulating insulin levels. To our knowledge, this is the first study to demonstrate that a targeted, moderate reduction of the insulin ligand may be sufficient to promote healthier aging and extend lifespan in mammals.
5.5 Figures

**Figure 5.1** Insulin and glucose homeostasis in aged \( \text{Ins1}^{-/-}\text{Ins2}^{+/+} \) and \( \text{Ins1}^{-/-}\text{Ins2}^{+/-} \) females.

4-h fasted (a) insulin at 80 weeks of age (n = 16-26), (b) IGF-1 in young and old mice (n = 5-9), and (c) blood glucose (n = 13-33) is shown, with the added evaluation of (d) HOMA-IR scores, based on fasting glucose and insulin levels at 80 weeks (n = 16-26). (e) Blood glucose response to intraperitoneal delivery of an insulin analog, and as well as (f) glucose-stimulated insulin secretion and (g) blood glucose response to intraperitoneal glucose is also shown for 1.5 year-old mice (n = 15-33), with area under or over the curve (y-axis units of e percent•min, f ng/mL•min, g mmol/L•min) in panel insets. Data are means ± SEM, with scatter points indicating individual values. Dark blue and dark purple represent CD- and HFD-fed \( \text{Ins1}^{-/-}\text{Ins2}^{+/-} \) female mice, respectively; pale blue and pale purple represent CD- and HFD-fed \( \text{Ins1}^{-/-}\text{Ins2}^{+/+} \) female mice, respectively. \( p \leq 0.05 \) denoted by * for CD vs HFD, # for \( \text{Ins1}^{-/-}\text{Ins2}^{+/-} \) vs \( \text{Ins1}^{-/-}\text{Ins2}^{+/+} \), and \( \#_{\text{CD}} \) for CD-fed \( \text{Ins1}^{-/-}\text{Ins2}^{+/-} \) vs Ins1^{-/-}Ins2^{+/-} mice.
Figure 5.2 Obesity and plasma metabolic factors and lipids in aged female mice.

85 week-old (a) body mass (n = 17-30) and (b) DEXA-measured fat mass (n = 7-8) is shown, in addition to 4-h fasted (c) leptin, (d) resistin, (e) interleukin 6, (f) glucose-dependent insulinoetric polypeptide (GIP), (g) peptide YY, (h) glucagon, (i) cholesterol, (j) triglycerides, and (k) non-esterified fatty acids (NEFAs), in 90 week-old mice (n = 6-10). Data are means ± SEM, with scatter points indicating individual values. Dark blue and dark purple represent CD- and HFD-fed $\text{Ins}1^{-/-}:\text{Ins}2^{+/+}$ female mice, respectively; pale blue and pale purple represent CD- and HFD-fed $\text{Ins}1^{-/-}:\text{Ins}2^{-/-}$ female mice, respectively. $p \leq 0.05$ denoted by * for CD vs HFD, and # for $\text{Ins}1^{-/-}:\text{Ins}2^{+/+}$ vs $\text{Ins}1^{-/-}:\text{Ins}2^{-/-}$. 
Figure 5.3 Liver histology in aged HFD-fed $\text{Ins}1^{-/-} \cdot \text{Ins}2^{+/+}$ and $\text{Ins}1^{-/-} \cdot \text{Ins}2^{+/+}$ females.

Representative images of liver sections stained with hematoxylin and eosin are shown, from 2 year-old HFD-fed female mice ($n = 3$) that reached humane endpoint without signs of gross liver lesions. Scale bar = 48 µm.
Figure 5.4 Effects of reduced \textit{Ins2} on female body composition and neuromuscular function

Periodic DEXA-measured (a) fat-free mass and (b) bone mineral density is shown (n = 7-11), in addition to (c) forelimb grip strength in aging female mice (n = 7-17). Data are means ± SEM, with scatter points indicating individual values. Dark blue and dark purple represent CD- and HFD-fed \textit{Ins1}\(^{-/-}\):\textit{Ins2}\(^{+/-}\) female mice, respectively; pale blue and pale purple represent CD- and HFD-fed \textit{Ins1}\(^{-/-}\):\textit{Ins2}\(^{+/-}\) female mice, respectively. \(p \leq 0.05\) denoted by * for CD vs HFD, and # for \textit{Ins1}\(^{-/-}\):\textit{Ins2}\(^{+/-}\) vs \textit{Ins1}\(^{-/-}\):\textit{Ins2}\(^{+/-}\).
Figure 5.5 Reduced Ins2 extends lifespan and reduces oxidative damage in female mice.

(a) Survival curves with a significant ($p \leq 0.05$) extension of lifespan in $Ins1^{-/-}:Ins2^{+/-}$ female mice compared to $Ins1^{-/-}:Ins2^{+/+}$ littermate controls, as assessed using either the Kaplan-Meier method with a log rank test stratified by diet, or a Cox proportional hazards regression analysis incorporating covariates of diet and cohort. $n = 40$-43, with ticks indicating censored individuals. For (b) the lifespan of the longest-lived decile, which indicates the maximum lifespan, and (c) 4-h fasted 8-isoprostane levels, measured at 30 and 90 weeks of age ($n = 7$-9), data are means ± SEM, with scatter points indicating individual values. (d-i) Histopathological post-mortem assessments were also performed on the majority of mice from lifespan studies. (e) Tumour incidence shows the percentage of mice in each group detected to have malignant tumours (hatched), benign tumours (solid), or both (grey) in post-mortem analysis, and (f) tumour load shows the means ± SEM of the number of distinct tumour-types identified per mouse. Survival curves for (d) malignant cancers, (g) cardiac-associated pathologies, and (h) kidney degeneration indicate the timelines of causative contributions by severe lesions in these categories to mortality or morbidity, with (i) unidentified pathologies indicating the timeline of deaths where cause could not be identified based on macroscopic changes or histopathology. # denotes $p \leq 0.05$ and (#) denotes $p = 0.059$ for $Ins1^{-/-}:Ins2^{+/-}$ vs $Ins1^{-/-}:Ins2^{+/+}$, and * denotes $p \leq 0.05$ for CD vs HFD. Dark blue and dark purple represent CD- and HFD-fed $Ins1^{-/-}:Ins2^{+/-}$ female mice, respectively; pale blue and pale purple represent CD- and HFD-fed $Ins1^{-/-}:Ins2^{+/+}$ female mice, respectively.
Figure 5.6 Insulin and glucose homeostasis in aged \textit{Ins1}^{−/−}:\textit{Ins2}^{+/−} and \textit{Ins1}^{−/−}:\textit{Ins2}^{+/+} males.

4-h fasted (a,d) insulin at 80 weeks of age, (b,e) blood glucose, and (c,f) HOMA-IR scores, based on fasting glucose and insulin levels at 80 weeks, are shown for cohort A (a-c, n = 5-15) and cohort B (d-f, n = 3-10). (g,j) Blood glucose response to intraperitoneal delivery of an insulin analog, as well as (h,k) glucose-stimulated insulin secretion and (i,l) blood glucose response to intraperitoneal glucose is also shown for 1.5 year-old male mice from cohort A (g-i, n = 6-13) and cohort B (j-l, n = 3-10), with area under or over the curve (y-axis units of g,j percent•min, h,k ng/mL•min, i,l mmol/L•min) in panel insets. Data are means ± SEM, with scatter points indicating individual values. Dark blue and dark red represent CD- and HFD-fed \textit{Ins1}^{−/−}:\textit{Ins2}^{+/+} male mice, respectively; pale blue and orange represent CD- and HFD-fed \textit{Ins1}^{−/−}:\textit{Ins2}^{+/−} male mice, respectively. \( p \leq 0.05 \) denoted by * for CD vs HFD, \(+/−\) for CD- vs HFD-fed \textit{Ins1}^{−/−}:\textit{Ins2}^{+/−}, and \# for \textit{Ins1}^{−/−}:\textit{Ins2}^{+/+} vs \textit{Ins1}^{−/−}:\textit{Ins2}^{+/−}.
Figure 5.7 Effects of reduced Ins2 on male body composition and neuromuscular function.

85 week-old body mass in (a) cohort A male mice (n = 6-14) and (b) cohort B male mice (n = 4-10) is shown, in addition to DEXA-measured (c) fat mass and (d) fat-free mass in 85 week-old males from cohort B (n = 4-8). (e) Periodic DEXA-measured bone mineral density is also shown from cohort B males (n = 4-8), as well as (f) forelimb grip strength (n = 3-16). Data are means ± SEM, with scatter points indicating individual values. Dark blue and dark red represent CD- and HFD-fed Ins1+/–:Ins2+/+ male mice, respectively; pale blue and orange represent CD- and HFD-fed Ins1+/–:Ins2+/– male mice, respectively. p ≤ 0.05 denoted by * for CD vs HFD, # for Ins1+/–:Ins2+/+ vs Ins1+/–:Ins2+/– and #HFD for HFD-fed Ins1+/–:Ins2+/+ vs Ins1+/–:Ins2+/– mice.
Cardiac-associated pathologies

Kidney degeneration

Unidentified pathologies

Malignant cancers

All mice

Cohort A

Cohort B

$p = 0.058$

$\text{Ins}^1/-:\text{Ins}^2\text{+/+}$ vs $\text{Ins}^1/-:\text{Ins}^2\text{+-}$

Tumour incidence (% of group)

Tumour load (number / mouse)

$\text{Ins}^1/-:\text{Ins}^2\text{+/+}$ CD

$\text{Ins}^1/-:\text{Ins}^2\text{+-}$ HFD

$\text{Ins}^1/-:\text{Ins}^2\text{+/+}$ HFD
Figure 5.8 Survival curves of $Ins1^{-/-} : Ins2^{+/+}$ and $Ins1^{-/-} : Ins2^{+/+}$ male mice.

Survival curves for (a) all male mice pooled (n = 29-41), (b) the first large cohort of male mice (n = 13-19), and (c) a second large cohort of male mice, studied one year later (n = 15-22) do not show significant effects of genotype on lifespan, evaluated using both the Kaplan-Meier method with a log rank test stratified by diet, and a Cox proportional hazards regression analysis incorporating covariates of diet and cohort. Ticks indicate censored individuals. (d-i) Histopathological post-mortem assessments were also performed on the majority of mice from lifespan studies. (e) Tumour incidence shows the percentage of mice in each group detected to have malignant tumours (hatched), benign tumours (solid), or both (grey) in post-mortem analysis, and (f) tumour load shows the means ± SEM of the number of distinct tumour-types identified per mouse, with * denoting $p \leq 0.05$ for CD vs HFD. Survival curves for (d) malignant cancers, (g) cardiac-associated pathologies, and (h) kidney degeneration indicate the timelines of causative contributions by severe lesions in these categories to mortality or morbidity, with (i) unidentified pathologies indicating the timeline of deaths where cause could not be identified based on macroscopic changes or histopathology. Dark blue and dark red represent CD- and HFD-fed $Ins1^{-/-} : Ins2^{+/+}$ male mice, respectively; pale blue and orange represent CD- and HFD-fed $Ins1^{-/-} : Ins2^{+/+}$ male mice, respectively.
Chapter 6: Discussion

6.1 General summary

In this investigation, we explored the effects of reducing insulin gene dosage on obesity, metabolic health, and other physiological parameters from early neonatal growth to advanced age, for the first time in a mammalian model. This culminated in an evaluation of how moderately lowered insulin levels can affect mammalian lifespan. Through these studies, we uncovered a series of noteworthy findings that both advanced our understanding of insulin’s functional role in mammals and provoked further questions that have yet to be resolved.

Our first research aim was to examine the effects of reducing Ins2 dosage on high fat diet-induced hyperinsulinemia and obesity. In these experiments we found that young female \( \text{Ins1}^{+/\text{−}}:\text{Ins2}^{+/\text{−}} \) mice experienced a transient repression of insulin hypersecretion on a high fat diet, but reached equivalently elevated insulin levels as HFD-fed \( \text{Ins1}^{+/\text{−}}:\text{Ins2}^{+/\text{+}} \) females by one year of age, corresponding to mid-life adulthood. Reducing insulin levels attenuated obesity in the growing female \( \text{Ins1}^{+/\text{−}}:\text{Ins2}^{+/\text{−}} \) mice, and importantly, a significant difference in body weight and adiposity was maintained despite the considerable rise in insulinemia later in life. This study supports the concept that preventing or limiting insulin hypersecretion can protect against obesity, particularly if insulin is repressed in young, still-growing mice.

We conducted a similar study comparing \( \text{Ins1}^{+/\text{−}}:\text{Ins2}^{+/\text{+}} \) and \( \text{Ins1}^{+/\text{−}}:\text{Ins2}^{+/\text{−}} \) male mice, but found, unexpectedly, that reducing Ins2 gene dosage did not have a consistent effect on circulating insulin levels in \( \text{Ins1}^{-}\)-null male mice. Rather, high fat-fed \( \text{Ins1}^{+/\text{−}}:\text{Ins2}^{+/\text{−}} \) male mice were capable of producing nearly equivalent amounts of circulating insulin as \( \text{Ins1}^{+/\text{−}}:\text{Ins2}^{+/\text{+}} \) males under some conditions, perhaps at least partially dependent on stress levels. When reduced
Ins2 dosage did result in slightly lowered circulating insulin in male mice, this was associated with smaller body size and a long-term reduction in both fat-free mass and adipose tissue. Hyper-variability in insulin levels and physiological responses to high fat feeding was observed across cohorts of Ins1-null male mice, both in these experiments and in previously performed experiments in a separate animal facility. Since this degree of phenotypic variability was not evident in the female littermates, nor in Ins2-null mice with full or partial Ins1 expression, it seems that levels of the murine insulin 2 peptide is particularly susceptible to modulation in male mice (an effect which might have been readily observed here since the animals also lacked potentially compensatory Ins1 expression). Furthermore, our observations suggest the possibility of sex-specific regulation of insulin production or secretion under certain conditions.

To fulfill the final experimental aim, we tracked most of these same male and female mice for the duration of their lifespan. Our objective was to determine how reducing Ins2 gene dosage and moderately lowering circulating insulin levels would affect mammalian aging and lifespan. Intriguingly, instead of having negative repercussions for glucose homeostasis, a lifelong reduction in endogenous insulin led to enhanced insulin sensitivity and lowered fasting glucose levels in female mice after the age of 1.5 years. Moreover, chronically reducing fasting insulin levels in female mice – even the very slight absolute reduction that was experienced by Ins1\(^{+/−}\):Ins2\(^{+/−}\) female mice on CD – was sufficient to significantly extend lifespan. High fat-fed Ins1\(^{+/−}\):Ins2\(^{+/−}\) female mice experienced a lifelong attenuation of HFD-induced obesity, but this did not impart a greater degree of lifespan extension than that afforded by reducing insulin levels on CD. Reducing Ins2 gene dosage did not significantly affect lifespan in male mice, but since Ins2 inactivation had inconsistent effects on circulating insulin in the male mice we were unable
to address the question of whether there is a sex-specific effect of moderately lowering insulin levels on mammalian lifespan.

### 6.2 Insulin and obesity

Our long-term characterization of female *Ins1*<sup>−/−</sup>:*Ins2*<sup>+/−</sup> mice demonstrated that early repression of insulin hypersecretion could lead to a prolonged attenuation of obesity. Whole-body adiposity levels were abated well beyond one year of age, a point by which high fat-fed female *Ins1*<sup>−/−</sup>:*Ins2*<sup>+/−</sup> mice had reached a degree of fasting and glucose-stimulated insulin secretion that was indistinguishable from their *Ins1*<sup>−/−</sup>:*Ins2*<sup>+/+</sup> littermates, at least based on measured parameters. Although this adult-onset period of matching insulinemia was not sustained in aging *Ins1*<sup>−/−</sup>:*Ins2*<sup>+/−</sup> mice, it also did not result in *Ins1*<sup>−/−</sup>:*Ins2*<sup>+/−</sup> females matching the adiposity of their high fat-fed *Ins1*<sup>−/−</sup>:*Ins2*<sup>+/+</sup> littermates at any point, despite a lifetime of high fat feeding. Thus, this study joins the body of evidence that supports an important role of hyperinsulinemia in the etiology of obesity<sup>102,228-231</sup> as well as pointing to certain life history stages, such as adolescence and young adulthood, when it may be critical to suppress insulin levels in order to achieve a lasting protection against obesity.

These findings have implications for strategies to limit weight gain and obesity in humans. There are physiological mechanisms that contribute to maintenance of body mass and composition by defending against loss of weight and adiposity.<sup>82,306-308</sup> This is demonstrated by the propensity for a limited period of food restriction in mammals to be followed by obesity rebound, for instance.<sup>309</sup> Therefore, it is likely that preventative measures would be more successful at restricting the worldwide rise in obesity incidence, as opposed to interventions to reverse existent obesity.<sup>216,224</sup> It is clear that early-life factors can play an important role in
determining future adipose tissue characteristics, since prenatal and neonatal conditions have been shown to influence obesity and metabolic health later in life.\textsuperscript{310} Being overweight or obese during childhood is a risk factor for adulthood obesity;\textsuperscript{256,257} strikingly, our results indicate that the converse can also be true. Pubescence is associated with a transient period of reduced insulin sensitivity and elevated insulin secretion,\textsuperscript{270,271,311} during a developmental stage which may be key for determining white adipose tissue characteristics such as stable adipocyte cell number.\textsuperscript{220} We suggest that limiting insulin hypersecretion during this period could have lasting beneficial effects.

\textbf{6.2.1 Implications for longevity}

Our study provides evidence that lifespan extension can be dissociated from obesity. We expected that effects of reduced $\text{Ins2}$ gene dosage on lifespan would be more pronounced with high fat feeding, considering that female $\text{Ins1}^{+/+}$:$\text{Ins2}^{+/+}$ mice with attenuated adiposity exhibited long-term protection from such obesity-associated conditions as hepatic steatosis. In addition, obesity is a risk factor for numerous age-associated disease states and pathologies,\textsuperscript{2,8,312} and female $\text{Ins1}^{-/-}$:$\text{Ins2}^{+/+}$ mice showed a tendency for delayed occurrence of mortality associated with cancers, cardiac dysfunction, and renal degeneration, compared to $\text{Ins1}^{-/-}$:$\text{Ins2}^{+/+}$ female mice on HFD. Instead, the degree of lifespan extension resulting from lowered insulin levels in female mice appeared quite consistent between the two distinct diet regimes.

It is important to consider that protection from a more severe form of obesity than that observed in our study could have led to differential effects on lifespan. For instance, the high fat diet-induced obese phenotype is often observed to be relatively mild in female rodents compared to male rodents, with respect to such detrimental effects as impaired glucose homeostasis and chronic inflammation.\textsuperscript{313-317} However, there is further evidence to support the concept that
lifespan extension can be dissociated from the reduction in adiposity which is often a feature of long-lived organisms.\textsuperscript{11,204} For instance, across 41 inbred mouse strains that range from shortening to extending lifespan in response to a 40\% dietary restriction\textsuperscript{203}, minimizing the loss of fat mass during the dietary restriction intervention is positively correlated with lifespan extension.\textsuperscript{204} Long-lived mutant mice with disrupted somatotropic signaling, such as mice with inactivated GH receptors, can also have an increased proportional adiposity compared to their controls, due to a preservation of absolute fat mass (especially subcutaneous WAT) in the dwarf mice.\textsuperscript{318,319} In addition, high fat-fed wild-type C57BL/6 mice have equivalent survival curves regardless of whether they are obese or resistant to HFD-induced obesity.\textsuperscript{303}

Our results show that although female $\text{Ins1}^{-/-}:\text{Ins2}^{+/+}$ mice had a lifelong attenuation of adiposity with chronic high fat feeding, they experienced a similar degree of lifespan extension on both CD and HFD, compared to their $\text{Ins1}^{-/-}:\text{Ins2}^{+/+}$ littermate controls. Therefore, we suggest that while limiting circulating insulin levels can both protect against HFD-induced obesity and extend lifespan, the former effect is likely independent from the latter.

6.3 Circulating insulin levels and peripheral insulin sensitivity

The relationships between systemic insulin sensitivity, insulin hypersecretion and longevity have not been fully elucidated, although it is often assumed that circulating insulin levels become elevated in a compensatory response to prevent hyperglycemia, when there is insufficient insulin-stimulated glucose uptake in the periphery. While tracking the female experimental mice into adulthood, we observed that chronic high fat feeding led to hyperglycemia and an impairment of whole-animal insulin-stimulated glucose disposal that was neither worsened nor improved by the attenuated obesity and lowered circulating insulin levels.
of Ins1−/−:Ins2+/- females, at least up to one year of age. Remarkably, the aged 1.5 year-old Ins1−/−:Ins2+/- female mice showed enhanced insulin sensitivity and lowered fasting glucose levels compared to their Ins1+/-:Ins2+/- littermates, regardless of diet.

Indeed, persistent exposure to the insulin ligand, due to conditions such as continuous rather than pulsatile insulin stimulation, can lead to target tissue desensitization, likely due to changes at the receptor or post-receptor level. In addition, alleviating insulin hypersecretion in obese mammals has been shown to lead to improvements in insulin signaling and sensitivity. Since biological aging in mammals is often associated both with worsening insulin responsiveness and the emergence of insulin secretory defects, a lifetime of reducing peripheral stimulation by insulin and lessening the demand of insulin production at the level of the β cell could have contributed to the improved metabolic health of Ins1−/−:Ins2+/- female mice. It has also been proposed that insulin resistance could be a protective mechanism in response to tissue accumulation of excess nutrients or increased oxidative stress from the associated overactive mitochondrial respiration. By this reasoning, perhaps it is also significant that Ins1−/−:Ins2+/- female mice appeared to have reduced overall oxidative damage, based on the plasma levels of a marker of lipid peroxidation.

One of the most intriguing aspects of our insulin sensitivity findings lies in the fact that the reduced insulin levels of Ins1−/−:Ins2+/- female mice were only associated with improvements to glucose homeostasis late in life. In our model, repressing insulin hypersecretion did not have notable effects on the elevation of fasting glucose or the impairment of blood glucose response to intraperitoneal insulin that resulted from chronic high fat feeding across one year. Rather, it seems that the enhancement of insulin sensitivity and glucose homeostasis experienced by Ins1−/−:Ins2+/- female mice was either specific to the particular changes underlying age-associated
insulin resistance, or it was a cumulative effect requiring a long-term repression of insulin levels. Either way, these observations strengthen the concept that moderately reducing insulin levels is protective with aging. We have thereby demonstrated that lowering or limiting insulin secretion can lead to enhanced peripheral insulin sensitivity in aged mammals.

6.3.1 Implications for longevity

Improved insulin sensitivity in conjunction with reduced circulating insulin have been repeatedly detected in many long-living mammals,\textsuperscript{148,149,152,168,169,183-185} often in addition to genetically reduced insulin/IGF-1 signaling components. It has even been suggested that of all the widespread physiological and endocrine alterations associated with diminished somatotropic signaling, enhanced insulin sensitivity and concomitant lowered insulin levels may be one of the more important mediators of lifespan extension for mice with reduced GH signaling.\textsuperscript{211,304,305} This concept is supported by the findings that additive extension of lifespan under dietary restriction only occurs in those models that show a further enhancement of insulin-stimulated glucose disposal.\textsuperscript{211,304,305} Since GH both stimulates the production and secretion of insulin\textsuperscript{90} and generally antagonizes its peripheral effects,\textsuperscript{131} reducing GH signaling would lead to lowered insulin secretion and enhanced insulin sensitivity via multiple mechanisms, both direct and indirect, that are distinct from lowering IGF-1.\textsuperscript{131,323} Although the existence of some long-living mouse models with hyperinsulinemia and peripheral insulin resistance, such as mice with deficient IRS-2 levels in the brain,\textsuperscript{148} implies that improving peripheral insulin sensitivity and lowering circulating insulin may not be a requirement of lifespan extension, it is also possible that reducing the strength of insulin signaling may be particularly important for specific tissues (such as the brain, in this case).\textsuperscript{148,211}
When characterizing mice with mutated components of insulin/IGF-1 signaling, it seems that there are inherent limitations attached to the broad terminology of ‘insulin resistance,’ which is often used interchangeably in these studies to describe an impairment of whole-animal insulin-stimulated glucose disposal or a reduction in insulin/IGF-1 signaling. Illogically, by these definitions many of the long-lived mouse models are simultaneously ‘insulin sensitive’ (in the first sense) and ‘insulin resistant’ (in the second sense). The additional complication of circulating insulin levels being intimately related to peripheral insulin-stimulated glucose disposal makes it even more challenging to untangle which of these characteristics has a crucial impact on the physiological decline of aging. Importantly, our study points to a reduction in the insulin ligand as being an upstream condition that can lead to improved whole-animal insulin sensitivity. Nonetheless, there are still many unresolved questions regarding ‘insulin resistance’ in general, and how it contributes to aging in particular.

6.4 Sex differences in metabolic homeostasis and insulin regulation

Male Ins1+/−:Ins2+/− mice did not show evidence for lifespan extension compared to their Ins1+/−:Ins2+/+ littermates. However, a crucial proviso of this result is the observation that reducing Ins2 expression also did not have clear, consistent effects on circulating insulin levels in Ins1-null males.

There are notable differences between males and females relating to many aspects of obesity, metabolic homeostasis, and aging. For instance, obese males appear to be more susceptible to developing glucose intolerance, impaired insulin-stimulated glucose disposal, and chronic inflammation, even when compared to similarly obese females. Divergence in white adipose tissue distribution likely influence sex-specific differences in metabolic
Males exhibit a greater tendency for central deposition of visceral WAT, which, compared to subcutaneous WAT, is associated with larger adipocytes, more inflammatory and immune cells, a distinct profile of secreted adipokines, increased innervation and blood supply that drains directly to the liver, and greater lipolytic activity coupled with reduced insulin responsiveness. In contrast, while females often have increased proportional adiposity overall, it is primarily due to a greater enlargement of subcutaneous WAT depots. The gonadal steroids play a major role in this divergence in WAT distribution, as indicated by the increased tendency for central adiposity in post-menopausal women with lowered estrogen levels. However, the mechanisms underlying sex-specific differences are not fully understood, and additional variables, such as dosage of genes expressed on the X chromosome, are also thought to contribute to these effects.

Interestingly, our results raise the possibility of sex-specific differences in the regulation of insulin production and/or secretion in mice. Hyper-variability in circulating insulin levels was evident in male but not female $Ins1^{+/-}:Ins2^{+/-}$ mice. Other experiments have also shown sex-specific impacts of changing $Ins2$ gene dosage. In the converse model of murine insulin gene manipulation, $Ins2$-null male mice with partial inactivation of rodent-specific $Ins1$ show a marked reduction in insulin levels, and in fact have a significant probability of experiencing escalating hyperglycemia and death due to diabetes around 10 weeks of age, likely due to insufficient insulin production (unpublished observations). The probability of frank diabetes in male $Ins1^{+/-}:Ins2^{-/-}$ mice is affected by environmental factors, since it could be detected more frequently in the SPF facility used in our current experiments, compared to the incidence in a different animal facility (A. Mehran, personal communication). Moreover, we did not observe any incidence of diabetes in female $Ins1^{+/-}:Ins2^{-/-}$ mice, in either facility. Similarly, on a
non-obese diabetic mouse background, all Ins1\footnote{}\footnote{}\footnote{}--:Ins2\footnote{}\footnote{}\footnote{}-- male mice exhibit diabetes by 10 weeks of age, associated with reduced insulin content as opposed to lymphocyte infiltration of the islets.\footnote{}\footnote{}\footnote{}\footnote{} Diminished islet insulin is not associated with diabetes in Ins1\footnote{}\footnote{}\footnote{}--:Ins2\footnote{}\footnote{}\footnote{}-- female mice on the non-obese diabetic background, thus closely corroborating our observations.\footnote{}\footnote{}\footnote{}\footnote{} Collectively, these results suggest that male mice may place a greater reliance than female mice on the capacity to modulate Ins2 expression or insulin 2 production in response to environmental conditions. Also, it is clear that inactivation of Ins1 can affect insulin levels in male mice. In contrast, while we have shown here that young and aged female Ins1\footnote{}\footnote{}\footnote{}--:Ins2\footnote{}\footnote{}-- mice have consistently lowered insulin levels compared to their Ins1\footnote{}\footnote{}\footnote{}--:Ins2\footnote{}\footnote{}-- littermates, particularly on a HFD, reducing Ins1 gene dosage on an Ins2-null background only slightly affects circulating insulin in female mice (D. Dionne, personal communication), even with chronic high fat feeding.\footnote{}\footnote{}\footnote{}\footnote{} This implies that reducing Ins1 gene dosage has minimal effects on circulating insulin in female mice.

Sex-specific differences in metabolic homeostasis, including possible differences in insulin production and/or secretion, remain an understudied area that is poorly understood. It is possible that the gonadal steroids themselves might exert differential effects on the two murine insulin genes and peptides, since testosterone and estrogen can both stimulate insulin production and secretion.\footnote{}\footnote{}\footnote{} Despite the high degree of sequence homology between the murine Ins1 and Ins2,\footnote{}\footnote{}\footnote{} the murine insulin genes differ with respect to some key regulatory elements controlling their transcription, such as the number of identified enhancer boxes.\footnote{}\footnote{}\footnote{} Moreover, there are disparities in the number of introns in these genes, as well as modest changes in the amino acid sequences of the C-peptides and B-chains of the encoded murine preproinsulins.\footnote{} We found that despite lower levels of Ins2 mRNA, islets of male Ins1\footnote{}\footnote{}\footnote{}--:Ins2\footnote{}\footnote{}-- mice could contain comparable
insulin levels as $\text{Ins1}^{+/+}:\text{Ins2}^{+/+}$ islets. This implies that post-translational compensatory mechanisms may have played a role in the observed hyper-variability of circulating insulin. Unfortunately, in the vast majority of previous studies exploring murine insulin regulation and differences between the two murine insulin genes, experiments were performed solely in male mice\textsuperscript{111,114} or female mice,\textsuperscript{112} or without sex being specified.\textsuperscript{97-99,101-108,110,111,113,325} Our understanding of murine insulin production and secretion might benefit from further investigation in light of potential sex-specific effects. It could also be worthwhile to explore whether these observed sex-specific differences in mice have any implications for insulin regulation in humans, or other mammals with a single insulin gene.

6.4.1 Implications for longevity

It is tempting to speculate that sex-specific differences in metabolic health could also underlie some of the dissimilarities between males and females with respect to aging and lifespan. As a rule, women live longer than men, for reasons that are yet unclear.\textsuperscript{326} Interestingly, genetic manipulations to reduce insulin/IGF-1 signaling often show a bias for greater lifespan extension in female model organisms,\textsuperscript{62,63,141,144,145,172,213} and in humans, lower composite insulin/IGF-1 signaling scores could only be associated with reduced mortality in women.\textsuperscript{162} In contrast, various pharmacological treatments often favour greater lifespan extension in male mice.\textsuperscript{326} For instance, treatment with acarbose, a drug which limits postprandial hyperglycemia by inhibiting $\alpha$-glucosidases in the intestine, significantly extends lifespan in male mice while exerting no notable effect on female mice.\textsuperscript{327} Interestingly, although the acarbose treatment was associated with a greater decline in IGF-1 in females rather than males, only the male mice treated with acarbose showed reduced insulin levels.\textsuperscript{327} It is therefore possible that lifespan may
be influenced by sex-specific differences with respect to insulin/IGF-1 production, secretion, and signaling, as well as metabolic homeostasis in general.

However, there are clearly other factors to consider, such as the gonadal steroids themselves. Androgens and estrogens have widespread physiological effects, and their age-related decline contributes to the physical deterioration of aging. Moreover, an inverse relationship between fecundity and lifespan can often be detected in nature, perhaps in part because of a compromise between reproductive fitness and somatic maintenance. Many lifespan-extending manipulations, such as dietary restriction or reducing insulin/IGF-1–like signaling, can also negatively impact fecundity, even though a reproductive trade-off is not always necessary for increasing lifespan. Therefore, divergent reproductive requirements between males and females could also influence sex differences in longevity. Although our study highlights some key features that diverge between male and female mice, we could not determine whether moderately lowering insulin has sex-specific effects on mammalian lifespan.

6.5 Study strengths and limitations

The studies outlined in this body of work were tempered by both experimental limitations and design strengths. For instance, in our mouse model we were unfortunately unable to properly test whether reducing circulating murine insulin 2 could attenuate high fat diet-induced obesity or extend lifespan in male mice, due to the inconsistent effects of partially inactivating \( \text{Ins2} \) gene dosage on circulating insulin levels in \( \text{Ins1} \)-null male mice. However, this drawback was outside of our control, and a more detailed exploration of the observed sex-specific effects was beyond the scope of these experiments.
An additional consideration for these investigations is that all mice were on an *Ins1*-null background. Although this prevented any interference by the rodent-specific *Ins1* gene in our examination of the effects of reducing the ancestral *Ins2* gene and lowering endogenous insulin production, it also necessitated manipulating the basal insulin production of the *Ins1*<sup>−/−</sup>:*Ins2*<sup>+/+</sup> littermate control mice. While young *Ins1*-null and *Ins2*-null mice have pancreatic insulin content and circulating insulin levels that are equivalent to wild-type mice, to our knowledge longer-term comparisons under a range of conditions have not been performed. Indeed, in our current experiments we notably did not observe any incidence of profound glucose intolerance or diabetes with age, even in mice that were fed HFD across their lifetime. Therefore, the distinct likelihood of a baseline suppression of circulating insulin levels in all *Ins1*-null mice should be borne in mind for our obesity and longevity studies (e.g. 15 week-old *Ins1*<sup>−/−</sup>:*Ins2*<sup>+/+</sup> female mice had mean 4 h-fasted insulin levels ranging from 0.46-0.61 ng/mL, depending on diet, whereas 0.72-1.81 ng/mL has been detected in wild-type C57/BL6 mice of the same age<sup>329</sup>). This could have limited the phenotypic divergence between *Ins1*<sup>−/−</sup>:*Ins2*<sup>+/+</sup> and *Ins1*<sup>−/−</sup>:*Ins2*<sup>+/+</sup> groups.

Furthermore, there are a number of caveats inherent in the use of unmatched diets for these experiments. Firstly, the specific CD used in our study was, at 26%, relatively high in fat content compared to the ~4-11% fat chow diets that are more commonly used.<sup>329,330</sup> It is important to note that the body mass values, growth curves, and the weight divergence between CD-fed and HFD-fed female mice in our study were closely comparable to a high-powered characterization of wild-type C57BL/6 female mice under a similar high fat feeding experimental regime,<sup>330</sup> suggesting that our experiments were at least successful in promoting HFD-induced weight gain in female mice. Nonetheless, a major drawback to our studies lies in the experimental diets having an unmatched composition with respect to ingredients, macronutrients,
or micronutrients, thus limiting interpretation of differences in lifespan and pathologies between CD- and HFD-fed mice. For instance, the mice on HFD were, to some degree, protein-restricted compared to CD-fed mice, and this could have contributed to the visible differences in survival curves between these two diets. On the other hand, using unmatched diets could be considered one of the strengths of our longevity study. It allowed us to demonstrate that reducing $Ins2$ expression and moderately lowering insulin levels has consistent, robust effects on lifespan in female mice, regardless of the clearly divergent diet compositions.

Our studies were distinguished by following a large sample size of both male and female mice, throughout growth and development and to the extent of their lifetime. Thus, we performed long-term evaluations of the effects of reduced endogenous insulin production, to the degree of statistical power that is rare in many metabolic and/or physiology experiments, since we were evaluating longevity in these mice. The necessity of using the same experimental animals for both phenotype and lifespan analyses meant that the animals were exposed to many stresses that are perhaps undesirable in longevity studies, but also potentially more analogous to the stresses that are experienced by mammals in uncontrolled environments, such as humans. It is also clear that we were restricted in our capacity for invasive studies or molecular analyses of $Ins1^{+/+}:Ins2^{+/+}$ and $Ins1^{+/+}:Ins2^{+/+}$ mice, due to the need for directing our resources towards longevity analyses. However, the trade-off allowed us to perform a large-scale in vivo assessment of the effects of modulating endogenous insulin production. All in all, we believe that these studies provided fundamental insights into insulin’s functional role in mammals.
6.6 Significance and future directions

The findings reported here point to numerous directions for prospective experiments. We have highlighted several understudied but intriguing areas of research, such as the potential for sex-specific regulation of murine insulin production or secretion, metabolic homeostasis, and aging. Additional concepts that beg further investigation include the relevant impact of obesity and/or peripheral insulin resistance on mammalian longevity. In addition, considering the improvements in insulin sensitivity and glucose homeostasis that were observed in female $\text{Ins}1^{-/-}:\text{Ins}2^{+/+}$ mice of advanced age, future work should determine the direct effects of reducing $\text{Ins}2$ and moderately lowering circulating insulin on tissue-level insulin/IGF-1 signaling, particularly in aging female mice. It would also be worthwhile to further dissect the temporal aspect of our results through such tools as inducible genetic manipulations. Since our experiments suggest that preventing or limiting elevated insulin secretion during certain life stages can have lasting effects for obesity, it is likely that insulin levels affect other health parameters in an age- and developmental stage-dependent manner. This promising area of research could be particularly relevant in the context of influencing parameters of aging and longevity. Lastly, if we wish to pursue the concept that the benefits of moderately reducing levels of the insulin ligand can be translated to humans, the effects of lifestyle alterations on circulating insulin levels, healthspan, and lifespan should be further investigated.

Importantly, the outcomes we observed in our studies took place within a normal physiological range of endogenous insulin production, suggesting that manipulations that moderately lower circulating insulin levels could be sufficient to impart beneficial effects. For instance, a variety of specialized dietary interventions$^{206,331}$ and exercise regimes$^{332,333}$ can be associated with improvements to insulin sensitivity and lowered circulating insulin. Although
there are still many holes in our understanding of the mechanisms underlying the beneficial effects of these manipulations, our current work highlights the relevance of targeting the insulin ligand itself to extend mammalian healthspan and lifespan through modulation of insulin/IGF-1 signaling.

There is a tendency for insulin levels to rise in aging individuals, or be maintained at a relatively consistent level despite insulin secretory defects.\textsuperscript{245-247} In contrast, the amplitude of GH pulses and circulating levels of IGF-1 decline with age in both humans and rodents, and these hormonal changes are believed to perpetuate some aspects of the physiological deterioration of aging, such as the decline in skeletal muscle mass and reduction in bone mineral density.\textsuperscript{11,323,334,335} This raises the question of whether it would be desirable to specifically reduce IGF-1 levels in aging humans, and has even prompted some advocates to tout exogenous GH as a potential ‘anti-aging’ treatment, despite the large body of evidence showing that exogenous GH treatment is associated with a range of adverse effects that include insulin resistance and metabolic deterioration, and increased risk of cancer incidence.\textsuperscript{11,323,334,335} These outcomes should illustrate the importance of evaluating the widespread basis of biological aging, rather than solely addressing specific age-related forms of deterioration. Moreover, it is crucial to consider the varied effects that manipulations may have on different individuals, dependent on such aspects as the specific environmental, genetic, or physiological conditions, additional inherent risk factors, and the potential for dose-specific, sex-specific, or age- and developmental stage-dependent effects.

In summary, our studies suggest that while murine insulin levels and metabolic homeostasis can be influenced in a sex-specific, environmentally-dependent manner, moderately lowering circulating insulin or limiting insulin hypersecretion has the potential to: 1) confer
long-term protection against obesity; 2) improve glucose homeostasis and peripheral insulin sensitivity in advanced age, and lastly, 3) lead to generally healthier aging and extension of lifespan in mammals. Importantly, these effects could be induced in our experiments by very modest reductions in circulating insulin levels. Therefore, further explorations of the impact of modulating the insulin ligand in mammals have potentially far-reaching implications, particularly in light of the burgeoning worldwide obesity epidemic, and the shift towards a greater ‘aged’ demographic proportion in the human population.\(^1,^{216}\)
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Appendix

Appendix A. Supplemental experimental data

A.1 Figure

Figure A.1 Longitudinal effects of HFD and reduced Ins2 on fasting insulin in female mice.

Periodic measurements of 4-h fasted insulin levels in female mice from 8 to 80 weeks of age, for (a) all mice pooled (n = 16-26), (b) the first large cohort of female mice (n = 8-12), and (c) the second large cohort of female mice, studied one year later (n = 8-14). Data are means ± SEM. Dark blue and dark purple represent CD- and HFD-fed Ins1−/−:Ins2+/+ mice, respectively; pale blue and pale purple represent CD- and HFD-fed Ins1−/−:Ins2+/− mice, respectively. * p ≤ 0.05 denoted by * for CD vs HFD, and # for Ins1−/−:Ins2+/− vs Ins1−/−:Ins2+/−.
# A.2 Table

Table A.1 Proportion of mice euthanized before natural endpoint in lifespan study.

<table>
<thead>
<tr>
<th>Group</th>
<th>Euthanized mice (n; proportion of population)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Female mice</strong></td>
<td></td>
</tr>
<tr>
<td>CD-fed</td>
<td></td>
</tr>
<tr>
<td>$Ins1^{-/-}:Ins2^{+/+}$</td>
<td>9; 22%</td>
</tr>
<tr>
<td>$Ins1^{-/-}:Ins2^{+/-}$</td>
<td>10; 26%</td>
</tr>
<tr>
<td>HFD-fed</td>
<td></td>
</tr>
<tr>
<td>$Ins1^{-/-}:Ins2^{+/+}$</td>
<td>15; 39%</td>
</tr>
<tr>
<td>$Ins1^{-/-}:Ins2^{+/-}$</td>
<td>12; 30%</td>
</tr>
<tr>
<td><strong>Male mice</strong></td>
<td></td>
</tr>
<tr>
<td>CD-fed</td>
<td></td>
</tr>
<tr>
<td>$Ins1^{-/-}:Ins2^{+/+}$</td>
<td>8; 21%</td>
</tr>
<tr>
<td>$Ins1^{-/-}:Ins2^{+/-}$</td>
<td>7; 25%</td>
</tr>
<tr>
<td>HFD-fed</td>
<td></td>
</tr>
<tr>
<td>$Ins1^{-/-}:Ins2^{+/+}$</td>
<td>22; 55%</td>
</tr>
<tr>
<td>$Ins1^{-/-}:Ins2^{+/-}$</td>
<td>9; 33%</td>
</tr>
</tbody>
</table>