# THE ROLE OF SELENOCYSTEINE LYASE IN PANCREATIC ISLET PHYSIOLOGY AND ITS SEX-SPECIFIC REGULATION OF ENERGY METABOLISM

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ABSTRACT

Obesity is a symptom of metabolic syndrome, a condition that increases lifetime risk of type 2 diabetes and cardiovascular disease. Proper maintenance of the insulin producing β-cells are necessary for glucose homeostasis. Although glucose is the most important nutrient factor involved in insulin secretion, we are increasingly becoming aware of the role of trace elements such as selenium (Se) in β-cell function. Previously, we reported that the putative Se recycling enzyme selenocysteine lyase (Scly) may be involved in regulating lipid and carbohydrate metabolism. Scly$^{-/-}$ mice develop a remarkable increase in fasting hyperinsulinemia, warranting further investigation into the role of Scly in β-cells. Moreover, we described possible sex differences in Scly regulation of energy metabolism. The experiments in the first aim focused on the role of Scly in pancreatic islet function. Immunofluorescent imaging studies were employed to confirm Scly expression in the pancreatic islet. Glucose-stimulated insulin secretion was increased in the absence of Scly$^{-/-}$ in the β-cell-like MIN6 cells but slightly impaired in isolated pancreatic islets, indicating Scly may influence islet paracrine signaling. Neither islet selenoprotein expression nor sulfur pathway enzyme gene expression were altered when Scly is deleted. While Scly is expressed in the islet and its deletion *in vivo* results in hyperinsulinemia, further studies are necessary to determine the mechanistic role of Scly in islet function. The second aim built upon previous observations that the role of Scly in energy metabolism may be sex-specific. Female Scly$^{-/-}$ mice were found to exhibit increased body fat composition when compared to their WT counterparts, but they were protected from impairments in insulin signaling. Castration was not sufficient to rescue adiposity or energy expenditure, but restored fasting insulin to WT levels in the male Scly$^{-/-}$ mice. Interestingly, reduced hypothalamic selenoprotein expression in male and female Scly$^{-/-}$ mice suggest Scly

may regulate lipid metabolism indirectly through maintaining hypothalamic function, indicating a novel role for Scly as a key regulator in the brain-fat axis.

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| | |
|---|---|
| MetS | Metabolic Syndrome |
| T2D | Type 2 diabetes |
| ER | Endoplasmic reticulum |
| GSIS | Glucose stimulated insulin secretion |
| GLUT | glucose transporter |
| PDH | pyruvate dehydrogenase |
| ATP | adenosine tri-phosphate |
| $K_{ATP}$ | ATP-sensitive $K^+$ channels |
| Pcx | pyruvate carboxylase |
| SOD | superoxide dismutase |
| GPX | glutathione peroxidase |
| ROS | reactive oxygen species |
| GLP-1 | glucagon-like peptide |
| Se | selenium |
| SELENOP | selenoprotein P |
| Sec | selenocysteine |
| Sec-tRNA$^{[Ser]Sec}$ | Sec-specific Trna |
| SECIS | Sec insertion sequence |
| NMD | nonsense-mediated decay |
| TXNRD1 | thioredoxin reductase 1 |
| Scly | selenocysteine lyase |
| Cys | cysteine |
| SPS1/2 | selenophosphate synthetase ½ |
| SELENOS | selenoprotein S |
| WT | wild-type |
| UBE2F | ubiquitin-conjugating enzyme E2F |
| LDL | low density lipoprotein |

| | |
|---|---|
| ApoB | apolipoprotein B |
| GPX1/4 | glutathione peroxidase ¼ |
| PTEN | phosphatase and tensin homolog |
| PTP | protein tyrosine phosphatase |
| PTPN2 | tyrosine-protein phosphatase non-receptro type 2 |
| STAT1 | signal transducer and activation of transcription 1 |
| Pdx1 | pancreatic duodenal homeobox-1 |
| PGC-1α | peroxisome proliferator-activated receptor gamma coactivator 1-alpha |
| ARE | antioxidant response element |
| FoxO1a | forkhead box protein O1 |
| HNF-4α | hepatocyte nuclear factor 4 alpha |
| PEPCK | phosphoenolpyruvate carboxykinase |
| G6Pase | glucose 6-phosphatase |
| AMPK | 5' adenosine monophosphate-activated protein kinase |
| SELENOM | selenoprotein M |
| Dio1-3 | iodothyronine deiodinase 1-3 |
| T4 | 3,5,3',5' tetraiodothyronine |
| T3 | 3,5,3' triiodothyronine |
| JNK | cJun N-terminal kinase |
| LXR | liver-X receptor |
| SELENOT | selenoprotein T |
| PACAP | pituitary adenylate cyclase activating polypeptide |
| NPY | neuropeptide Y |
| Agrp | agouti-related peptide |
| α-MSH | α-melanocyte stimulating hormone |
| SELENOS | selenoprotein S |
| ERAD | ER-associated degradation |
| Ipf1 | insulin promoter factor 1 |
| Cbs | cystathionine β-synthase |

| | |
|---|---|
| Cth | cystathionine γ-lyase |
| Mtr | methyltransferase |
| DMEM | Dulbecco’s Modified Eagle’s Medium |
| FBS | fetal bovine serum |
| FACS | fluorescence activated cell sorting |
| DAPI | 4',6-diamidino-2-phenylindole |
| KRB | Krebs-Ringer bicarbonate |
| Cphn2 | cyclophilin B |
| GS | glutathione synthase |
| $H_2S$ | hydrogen sulfide |
| NIH | National Institutes of Health |
| FAS | fatty acid synthease |
| ACC | acetyl-CoA carboxylase |
| TST | testosterone |
| $VO_2$ | volume oxygen |
| $VCO_2$ | volume carbon dioxide |
| RQ | respiratory quotient |
| GTT | glucose tolerance test |
| WAT | white adipose tissue |
| MafA | musculoaponeurotic fibrosarcoma oncogene family A |

# CHAPTER 1. INTRODUCTION

## 1.1 Background

Estimated to affect over 30% of the population of the United States alone, obesity is a growing world-wide epidemic. Although genetic predisposition has been found to be a contributing factor in a small percentage of the obese population[1], the current obesity epidemic can be attributed to a high caloric diet combined with physical inactivity. Obesity is one of the early stages of metabolic syndrome (MetS), a cluster of symptoms which significantly increase a person's risk of developing type 2 diabetes (T2D) and/or cardiovascular disease. In traditional views, the path to T2D begins with insulin resistance as a direct result of obesity. To combat insulin resistance, the β-cells of the pancreatic islets ramp up insulin production and secretion, leading to hyperinsulinemia. In most individuals, this compensatory mechanism by the β-cells is sufficient to delay the onset of T2D[2]. However, a fraction of individuals will eventually experience β-cell failure, leading to insulin deficiency and subsequently, hyperglycemia. Hyperglycemia is a collective result of diminished glucose uptake by insulin-sensitive tissues and the increase in hepatic gluconeogenesis, which is normally inhibited by the presence of insulin. Oxidative stress[3], endoplasmic reticulum (ER) stress[4], inflammation[5], glucotoxicity[6], and lipotoxicity[7] have all been found to play causative roles in the path to β-cell dysfunction.

Although hyperinsulinemia is a hallmark of T2D, its role in T2D development has largely been ignored as it was largely thought that insulin resistance is the chief driver of T2D pathophysiology. However, there is growing evidence that hyperinsulinemia can cause and/or exacerbate insulin resistance, contributing to a snowball effect during the insulin compensatory phase. For instance, transgenic mice containing extra copies of the human insulin gene develop insulin resistance[8]. Moreover *in vitro* studies have found that insulin directly causes insulin resistance as acute insulin exposure induced insulin resistance in dorsal root ganglion neurons[9].

Thus, a more comprehensive understanding of islet physiology will allow us to find new molecular targets to treat T2D.

### 1.2 Pancreatic Islet Physiology and Insulin Secretion

The pancreatic islet is a structure within the pancreas, which regulates glucose homeostasis. Several specialized cell types make up the islet, each producing and secreting a different set of hormones. The β-cell constitutes 60-80% of the islet, and is the main site of insulin production. Insulin stimulates glucose uptake in insulin-sensitive tissues as well as inhibiting gluconeogenesis, thereby reducing blood glucose levels. Working in opposition to insulin is glucagon, which is produced and secreted by the α-cell[10]. Glucagon is released when blood glucose levels fall below a certain threshold[11] and raises blood glucose levels via stimulation of hepatic gluconeogenesis[12]. Other cell types within the islet include the δ-cells that secrete somatostatin[13], ε-cells that secrete ghrelin[14], and PP-cells that secrete pancreatic polypeptide. These pancreatic hormones work in tandem to fine tune blood glucose levels.

Of the different cell types that make up the islet, the β-cell is perhaps the most well-studied, due to insulin being the first of the pancreatic hormones to be discovered. The classical insulin secretory pathway is known as glucose stimulated insulin secretion (GSIS), and the process begins with glucose entry into the β-cell via glucose transporters (GLUT2 in rodents and GLUT1 and 3 in humans). As glucose undergoes glycolysis to form pyruvate, ~50% of the pyruvate formed is oxidized to acetyl-CoA by pyruvate dehydrogenase (PDH) to produce NADH and $FADH_2$, which can be oxidized through the respiratory transport chain. As a result, intracellular adenosine tri-phosphate (ATP) can be synthesized through oxidative phosphorylation, and the rise in ATP/ADP closes ATP-sensitive $K^+$ channels ($K_{ATP}$)[15]. This results in membrane depolarization, stimulating influx of $Ca^{2+}$ through voltage-dependent L-type $Ca^{2+}$ channels. The increase in intracellular $Ca^{2+}$ concentration subsequently triggers exocytosis

of insulin granules[16]. In a parallel pathway, pyruvate is carboxylated by pyruvate carboxylase (Pcx) to participate in anaplerosis, through which additional second messengers and reducing equivalents required for insulin secretion are generated[17].

In terms of protection from oxidative stress, the pancreatic islet is a unique environment as levels of antioxidant enzymes are kept at minimal levels. During glucose metabolism, the formation of NADH and $FADH_2$ stimulates superoxide anion production. Superoxide dismutase (SOD) converts superoxide anion to $H_2O_2$, which is quickly inactivated by $H_2O_2$ scavenging enzymes[18]. SOD activity in islets is less than 50% of that observed in the liver, and less than 10% of the activity levels of $H_2O_2$ scavenging enzymes, catalase and glutathione peroxidase (GPX)[19]. Interestingly, glucose stimulation was found to transiently increase intracellular reactive oxygen species (ROS) levels in the β-cell[20], leading to the hypothesis that ROS may be an important signal in insulin secretion. Strikingly, not only was mitochondrial ROS found to increase insulin secretion, the glucose-induced rise in mitochondrial ROS was found to be essential for insulin secretion[21]. Thus, the reason for tight regulation of antioxidant activity in the β-cell is likely to create a condition that is sensitive to slight changes in ROS. While reduced antioxidant expression in β-cells is advantageous for ROS signaling, it acts as a double-edged sword[19], potentially leaving β-cells more vulnerable to oxidative damage.

Other nutrient signals such as fatty acids[22] and amino acids[23], can also stimulate insulin release. In addition, gastrointestinal peptides such as glucagon-like peptide 1 (GLP-1) and the autonomic nervous system can potentiate insulin secretion. Understanding the mechanism of action of these additional insulinotropic agents is paramount to developing drugs to treat β-cell dysfunction. Moreover, while the contributions of macronutrients such as glucose or fats to metabolic diseases are well-studied, the influence of micronutrients such as Se is unclear.

### 1.3 Selenium

Dietary Selenium (Se) is critical for the synthesis of selenoproteins, which carry out the biological functions of Se. To date, 24 murine and 25 human selenoprotein genes have been identified[24]. Of these gene products, the glutathione peroxidases and thioredoxin reductases, which participate in redox reactions, are likely the most well-studied selenoprotein families. Other notable selenoproteins with known functions include the iodothyronine deiodinase family, which regulate thyroid hormone activation and Selenoprotein P (SELENOP), which transports Se through the serum. Thus, selenoproteins function in a wide variety of cellular processes (Table 1).

Selenoprotein synthesis involves several processes that are distinct from normal protein translation. Eukaryotic selenocysteine (Sec) biosynthesis is a unique process beginning with the synthesis of Sec directly on its tRNA (Sec-tRNA$^{[Ser]Sec}$)[25]. Perhaps one the most intriguing aspects of selenoprotein biosynthesis is the ability of Sec-tRNA[Sec]Ser to recognize the UGA codon, allowing for the recoding of what is conventionally a stop codon into a Sec insertion site. Consequently, intricate mechanisms have evolved to prevent premature translation termination. One of these is the unique secondary structure known as the Sec Insertion Sequence (SECIS) element in the 3' untranslated region of the selenoprotein mRNA[26] which recruits several trans-acting elements that are necessary for selenoprotein translation.

The presence of Se regulates selenoprotein synthesis. It was recently demonstrated through ribosome profiling that Se influence over selenoprotein synthesis occurs mainly through changes in translational efficiency[27]. Reduced Sec incorporation causes the UGA codon to be read as a premature stop codon, activating the nonsense-mediated decay (NMD) pathway[28]. Nevertheless, it is important to note that selenoproteins are not affected equally by this pathway.

**Table 1. List of selenoproteins and their known functions.**

| Selenoprotein Gene | Abbreviation | Function(s) |
|---|---|---|
| 15 kDa-selenoprotein | SELENOF | Protein folding |
| Iodothironine Deiodinase 1–3 | DIO1–3 | Thyroid hormone activity regulation |
| Glutathione Peroxidase 1–4, 6 | GPX1–6 | Hydroperoxide/phospholipid peroxide reduction |
| Methionine-R-Sulfoxide Reductase 1 | MSRB1 | Reduces oxidized methionine residues |
| Selenoprotein H | SELENOH | Genome maintenance |
| Selenoprotein I | SELENOI | Unknown |
| Selenoprotein K | SELENOK | ER-associated degradation; inflammation |
| Selenoprotein M | SELENOM | $Ca^{2+}$ homeostasis |
| Selenoprotein N | SELENON | Muscle development |
| Selenoprotein O | SELENOO | Unknown |
| Selenoprotein P | SELENOP | Selenium transport |
| Selenoprotein S | SELENOS | ER-associated degradation; inflammation |
| Selenophosphate Synthetase 2 | SEPHS2 | Selenoprotein biosynthesis |
| Selenoprotein T | SELENOT | $Ca^{2+}$ homeostasis; neuroendocrine secretion |
| Thioredoxin Reducase 1–3 | TXNRD1–3 | Disulfide bond reduction |
| Selenoprotein V | SELENOV | Unknown |
| Selenoprotein W | SELEONOW | Redox reaction |

Housekeeping selenoproteins such as TXNRD1 and glutathione peroxidase 4 (GPX4) are more resistant to changes in Se levels and are essential for life, as evidenced by the fact that whole-body knockout of these selenoproteins is embryonic lethal[29,30]. On the other hand, "stress-response" selenoproteins are more reactive to fluctuating Se status and, while crucial for the molecular mechanisms they are involved in, are not considered to be essential to life.

### 1.4 Selenocysteine Lyase and Se Metabolism

Sec decomposition occurs through selenocysteine lyase (Scly), a pyridoxal 5'-phosphate-dependent enzyme that was initially purified from pig liver[31]. Scly has the ability to specifically distinguish Sec from the structurally similar amino acid, cysteine (Cys)[32]. In the presence of cofactor pyridoxal 5-phosphate, Scly cleaves Sec to form alanine and selenide. The released selenide can be further metabolized and excreted or it can be re-purposed for selenoprotein synthesis, implicating a role for Scly as a Se recycling enzyme when Se supply is low (Figure 1). *In vitro* immunoprecipitation studies demonstrated Scly interaction with selenophosphate synthetase 1 (SPS1) and SPS2, suggesting that these enzymes work in a complex where Sec donates Se to SPS to form selenophosphate[33]. Moreover, Scly and

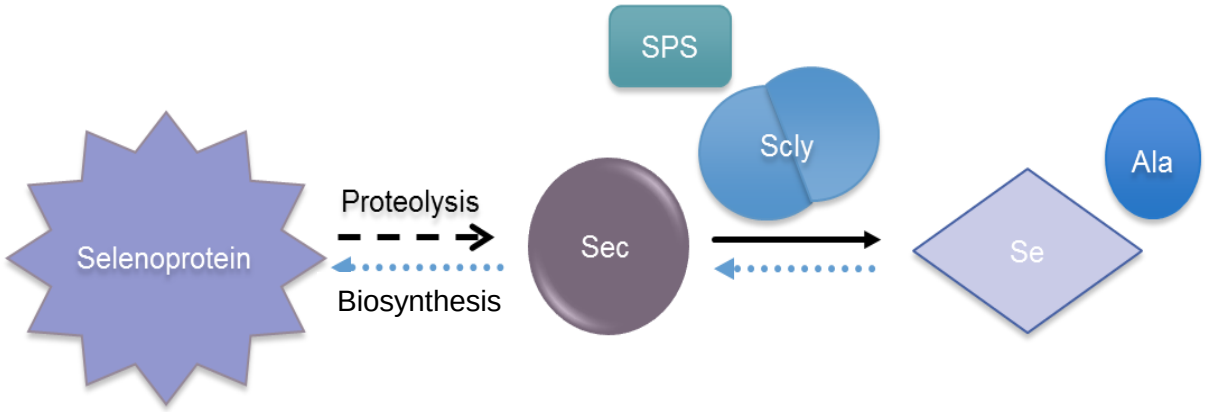


**Figure 1. Schematic representation of Scly in Sec degradation and Se recycling.**
Selenoproteins undergo proteolysis to release Sec. Scly further metabolizes Sec to produce selenide and alanine (Ala). Through a potential interaction with SPS, Scly possibly salvages Se to be repurposed in selenoprotein biosynthesis.

SELENOP have been suggested to act in tandem, with SELENOP delivering Sec to tissues where Scly can cleave and recycle Se[34]. In support of this, mice lacking both Scly and SELENOP develop severe neurological deficits, surpassing the neurological phenotypes observed when only one gene is absent[35].

Unlike classical amino acids, free Sec is kept at minimal levels, likely because its high reactivity is capable of producing harmful free radicals. Moreover, there is a possibility that Sec can compete with Cys and become misincorporated into proteins, resulting in misfolded proteins[36]. Strikingly, a study involving transgenic expression of Scly in *Arabidopsis* plants highlighted a role for Scly in mitigating Sec toxicity through its degradation[37]. Yet, we found that Scly deletion in mice does not appear to generate symptoms indicative of severe toxicity due to Sec accumulation[38]. Therefore, an alternate pathway that is capable of decomposing Sec must exist. One candidate pathway is the consumption of Sec through sulfur metabolism. Several studies have confirmed that Se and its derivatives can be metabolized via sulfur pathways, albeit many of these studies were conducted in plants and must be verified in vertebrates[39].

### 1.5 Physiological Role of Selenocysteine Lyase

Until recently, much of the work on Scly utilized *in vitro* methods[31], and so the physiological role Scly has remained elusive. We generated Scly knockout ($Scly^{-/-}$) mice, uncovering a potential relationship for Scly in regulating energy metabolism. The $Scly^{-/-}$ mice develop a metabolic syndrome-like phenotype under a Se adequate diet, displaying hyperinsulinemia, increased body weight, dyslipidemia, and reduced glucose tolerance[38]; however, a mechanistic link has yet to be determined. Under Se adequate conditions, hepatic selenoprotein levels are unchanged[38], suggesting that Scly regulation of glucose metabolism may be independent from its known function in selenoprotein biosynthesis.

On the other hand, under Se deficient conditions, the metabolic phenotype of Scly$^{-/-}$ mice is exacerbated[38]. Additionally, hepatic expression of GPX1 and SELENOS, and serum SELENOP levels were diminished, strengthening the hypothesis that Scly$^{-/-}$ is important in Se recycling when Se supply is low. Thus, it appears that Scly directly regulates selenoprotein expression in Se deficiency, and reduced selenoprotein expression contributes to altered energy metabolism in Scly$^{-/-}$ mice.

High fat diet studies demonstrated that Scly$^{-/-}$ mice are more susceptible to diet-induced obesity than their wild-type (WT) counterparts as gluconeogenic and lipogenic enzymes were upregulated in Scly$^{-/-}$ mice [40]. Altogether, this study suggests Scly may serve as a regulatory point connecting energy metabolism with Se pathways. Strengthening the idea that Scly is involved in lipid metabolism, a recent genome wide association study involving Mexican-Americans revealed an intronic variant rs201606363, located between Scly and its conjoined gene, ubiquitin-conjugating enzyme E2F (UBE2F)[41], to be positively associated with low density lipoprotein (LDL), apolipoprotein B (ApoB), and total cholesterol levels. Further studies are necessary to determine the mechanistic relationship between cholesterol and Scly.

### 1.6 Selenium and T2D: Clinical Trials

Se was long touted for its cytoprotective properties, due to its ability to upregulate antioxidant selenoenzymes. Thus, it was believed that Se supplementation could prevent the onset of metabolic diseases, such as T2D by counteracting oxidative stress. Indeed, Se in the form of selenate, was found to act as an insulin mimetic, displaying anti-diabetic effects[42]. In support of this, two cross-sectional studies reported lower baseline Se levels to be associated with T2D incidence among elderly French men[43], as well as in samples taken from a population in southeastern Spain[44]. A more recent, longitudinal study conducted in the United States, reported higher toenail Se to be associated with lower T2D risk[45]. However, other cross-

sectional studies, namely the National Health and Nutrition Examination Survey (NHANES) III[46] and NHANES 2003–2004[47], revealed an association between high Se intake and an increased risk for metabolic diseases. Moreover, increased T2D risk was found to be a secondary outcome in the Nutritional Prevention of Cancer (NPC) trial, a randomized, controlled trial assessing the efficacy of Se supplementation in the form of Se yeast (200 μg/day) in preventing skin cancer[48]. The Selenium and Vitamin E Cancer Prevention Trial (SELECT), testing the effects of selenomethionine (SeMet, 200 μg/day) and/or Vitamin E in preventing prostate cancer was curtailed as it became apparent Se supplementation was not beneficial in the prevention of prostate cancer, and a nonsignificant trend towards T2D in the experimental group was reported[49,50]. Yet, other epidemiological studies and clinical trials failed to find a correlation between increased Se and T2D susceptibility[51]. One reason for the discrepancies in the human trials may be due to differences in baseline Se levels. For instance, the mean baseline serum Se levels in SELECT[49] subjects were already high (136 μg/L) whereas only the NPC[51] subjects in the upper third tertile of baseline Se (>122 μg/L) demonstrated higher incidence of T2D. Strengthening the idea of a narrow beneficial window of Se dose, it is likely that with regards to T2D, Se supplementation may be advantageous in populations with low Se status, but detrimental in Se-replete populations. In fact, randomized, controlled trials of Se supplementation in elderly patients[52] and pregnant women[53] from the UK, who have lower baseline Se than US subjects, did not result in increased T2D risk, as determined by serum adiponectin concentration. Another source of the inconsistencies might be attributable to differences in Se source, as different Se forms vary in their bioavailability and biological effects. Thus, it is difficult to delineate a clear-cut relationship between Se status and T2D based on evidence from the current human clinical trials and epidemiological studies.

### 1.7 Selenium and T2D: Animal Models

Although the association between Se supplementation and T2D in humans is considered controversial, studies in animal models may provide physiological and molecular insights into some of the inconsistencies reported in human clinical trials. In a study comparing three different concentrations of Se in the diet, it was found that mice on a relatively high 0.4 ppm selenite diet developed insulin resistance, a hallmark of T2D[54]. This concentration of Se is comparable to the 200 µg Se regimen that was administered to humans in the NPC trial. High Se exposure also led to insulin resistance in rats, which was attributed paradoxically to both ROS production and attenuated ROS[55]. Moreover, 16 weeks of Se supplementation in pigs on an already Se adequate diet resulted in a trend towards increased body weight and HOMA-IR score, a measure of insulin resistance[56]. This was accompanied by alterations in glucose and lipid metabolic pathways in insulin-sensitive tissues. With the exception of the skeletal muscle, GPX activity and TXNRD activities were largely unchanged in insulin-sensitive tissues in response to Se supplementation, suggesting selenoprotein expression was already saturated under Se adequate conditions. These results suggest the proclivity towards metabolic diseases in pigs receiving supranutritional Se doses may be a result of nonspecific incorporation of selenomethionine, rather than a consequence of increased selenoprotein activity. It is important to note that most of the parameters measured in this study only trended towards significance, and thus it was concluded that supranutritional Se contributes to but does not cause T2D. However, as the duration of the study was relatively short, it is premature to speculate whether significance would have been achieved under long-term supplementation.

*In vitro* studies in pancreatic islets and the mouse insulinoma derived β-cell line, MIN6, have demonstrated increased insulin content and secretion in response to Se treatment, leading to the hypothesis that Se protects islet function[57]. Higher levels of plasma Se were indeed found

to be associated with elevated serum insulin in mice[54], but it is not known whether this is protective or detrimental. However, selenate treatment conferred protection to rat insulinoma cells, INS1, against streptozotocin-induced β-cell death[58], suggesting the antioxidant properties of Se may be protective in the late stages of T2D.

These studies demonstrate that both over-supplementation and deficiency of Se can be associated with T2D risk, following a U-shaped curve that is consistent with the narrow range of upper and lower tolerable doses of Se. It appears that Se over-supplementation may promote T2D in an otherwise healthy animal. However, in diabetic animals which may have suboptimal Se status, Se appears to have beneficial effects by preventing further development of T2D complications. Additional studies will be useful in determining the appropriateness of Se supplementation with respect to T2D.

### 1.8 Selenoproteins in T2D

Selenoproteins are important products that fulfill the catalytic effects of dietary Se. Thus, in order to clarify the discrepancies in the association between Se supplementation and T2D, it is necessary to understand the role of each selenoprotein in maintaining glucose homeostasis. This section will review what is currently known about the roles of selenoproteins that have been connected to metabolic diseases.

#### *1.8.1 Glutathione Peroxidase 1*

Glutathione Peroxidase 1 (GPX1) is the primary cytosolic peroxide scavenger, reducing peroxides to water, and protecting the cell from free radical damage. Considered to be a stress responsive selenoprotein, GPX1 expression is sensitive to levels of Se intake. Overexpression of GPX1 in mice yielded surprising results, leading to reduced glucose clearance, hyperinsulinemia, hyperglycemia, and diminished insulin signaling[59]. Diet restriction alleviated

all metabolic symptoms except hyperinsulinemia, suggesting the functional role of GPX1 lies in regulating insulin production[60]. In this study, it was found that the H3 and H4 histones in the proximal promoter region of the insulin gene were hyperacetylated, ultimately leading to hyperinsulinemia. Although not shown directly, this hyperacetylation was speculated to be due to enhanced $H_2O_2$ scavenging. The importance of $H_2O_2$ in cellular signaling was established further when it was demonstrated that GPX1 null mice have increased insulin sensitivity in the muscle, resulting in a high fat diet-resistant phenotype[61]. The absence of GPX1 allowed for the oxidation of phosphatase and tensin homolog (PTEN), a member of the protein tyrosine phosphatase (PTP) family. Oxidation of PTEN inhibits its activity, sensitizing insulin signaling.

In contrast, several studies suggest that GPX1 might play a protective role against T2D. GPX1 overexpression in HIT-T15 cells protects against β-cell dysfunction induced by ribose treatment[62]. As ribose was found to induce oxidative stress in human islets, it is possible that GPX1 promotes β-cell survival through its ability to scavenge peroxides. Although constitutive $GPX1^{-/-}$ mice appear to preserve insulin signaling in response to an obesogenic diet, in the context of insulin secretion, GPX1 deficiency can be detrimental[63]. In the absence of GPX1, excess ROS in the pancreatic islets oxidize tyrosine-protein phosphatase non-receptor type 2 (PTPN2), promoting signal transducer and activation of transcription 1 (STAT1) signaling which results in downregulating key enzymes of the insulin production and secretory pathway, such as pancreatic duodenal homeobox-1 (Pdx1). Inactivation of hepatic PTPs due to excess ROS also appears to promote obesity and T2D disease progression. In hepatocytes isolated from $GPX1^{-/-}$ mice, the presence of excess ROS inactivates PTPN2, which negatively regulates STAT5-induced lipid synthesis[64]. Thus, hepatic GPX1 may prevent hepatic steatosis indirectly by regulating ROS levels. Further supporting the protective function of GPX1, the GPx mimetic, ebselen, was found to restore islet function in $GPX1^{-/-}$ mice through a peroxisome proliferator-

activated receptor gamma coactivator 1-alpha (PGC-1α) dependent mechanism[65]. Nrf2 is a transcription factor that controls the transcription of antioxidant enzymes by interacting with an antioxidant response element (ARE). Several selenoproteins and selenoprotein synthesis factors were found to contain an ARE in their promoters, and are thus Nrf2-responsive[66]. GPX1 levels were found to be suppressed when mice were fed a high fat diet due to 12-lipoxygenase activity, which was found to inhibit Nrf2 nuclear translocation[67]. Pancreatic islet specific 12-lipoxygenase deletion resulted in GPX1 upregulation in response to high fat feeding. The resulting reduction in oxidative stress was found to be beneficial in preserving islet β-cell function under high fat diet consumption in mice. To corroborate these studies, a genetic polymorphism in the GPX1 gene which results in lower GPx activity, was found to correlate with increased incidence of metabolic syndrome in a cohort of Japanese men[68].

Both GPX1 overexpression and deficiency appear to have negative effects in metabolic diseases. These findings are aligned with the U-shaped therapeutic dose effect of Se intake. However, the function of GPX1 is tissue dependent, exhibited by the differences in outcome of GPX1 deficiency in the muscle[61], liver[64], and pancreatic islets[63]. Increased understanding of tissue-specific functions of GPX1 will improve our knowledge of the role GPX1 plays in metabolic diseases. It is also important to note the GPX1 response may also have a timing specific component in that excess GPX1 in the pre-disease state may promote disease pathogenesis. The disease state, however, may suppress GPX1 expression.

*1.8.2 Selenoprotein P*

In humans, a positive correlation between hepatic SELENOP expression and T2D has been reported[69]. In this same study, increased hepatic SELENOP mRNA expression was also associated with reduced glucose tolerance and higher fasting glucose levels, which are indicative of insulin resistance. However, it is important to note that serum SELENOP levels

become saturated at high Se intake[70]. One limitation of this study[69] is that the patients' Se intake levels were not reported. Likely, the usefulness of SELENOP as a biomarker is limited to subjects who do not receive an optimal Se intake. Additionally, since Se deficiency has been reported in T2D patients[43], it is possible that SELENOP mRNA expression is elevated in the diseased state, as Se transport will be in higher demand. Thus, the elevation of SELENOP may be a secondary effect of T2D, rather than a cause. Nevertheless, adiponectin, an adipokine with anti-diabetic effects[71], was found to be inversely correlated with serum SELENOP levels in T2D patients[72]. Moreover, elevated serum SELENOP levels were positively associated with carotid intima-media thickness and C-reactive protein, both of which are predictors for cardiometabolic disease[73]. Studies of SELENOP genetic variants reveal SELENOP polymorphisms to be associated with fasting insulin and the acute insulin response[74]. Taken together, SELENOP appears to be involved in glucose homeostasis, although direct conclusions cannot be made due to the correlative nature of these studies.

Mouse models and cell lines have been used to delineate the mechanistic relationship between SELENOP and carbohydrate metabolism. For example, studies in HepG2 cells revealed that hepatic SELENOP mRNA and promoter activity are under the control of insulin and a supramolecular complex composed of PGC1α, forkhead box protein O1 (FoxO1a), and hepatocyte nuclear factor 4 alpha (HNF-4α), which also regulates gluconeogenic enzymes phosphoenolpyruvate carboxykinase (PEPCK) and glucose 6-phosphatase (G6Pase)[75]. Thus, an increase in SELENOP expression is likely positively correlated with hepatic gluconeogenesis. Additionally, SELENOP was found to negatively regulate insulin signaling in the liver, through 5' adenosine monophosphate-activated protein kinase (AMPK) inactivation in female mice[69]. SELENOP also downregulates insulin signaling in the muscle, although the mechanism remains unclear. Mice with SELENOP deletion ($SELENOP^{-/-}$) were found to be

protected from diet induced obesity and insulin resistance. In a follow-up study, SELENOP$^{-/-}$ mice were found to be protected from the drop in serum adiponectin levels in response to a high-sucrose, high-fat diet, although adiponectin levels were not equal to those observed in untreated wild-type controls. This implicates a partial, but direct role for SELENOP in regulating adiponectin[72]. Because SELENOP is associated with gluconeogenic enzymes and downregulation of the insulin signaling pathway, it was proposed as a potential drug target. In fact, the commonly prescribed glucose lowering drug, metformin, suppresses SELENOP expression[76]. Further investigation demonstrated that metformin-induced inhibition of SELENOP expression occurs in an AMPK and FoxO3 dependent pathway[77].

*1.8.3 Selenoprotein M*

Selenoprotein M (SELENOM) is localized to the ER and is thought to participate in thiol-disulfide exchange through its thioredoxin-like domain[77]. *In vitro*, SELENOM has been shown to regulate calcium signaling and protect against oxidative stress[78]. Because of its high expression levels in the brain, it was initially hypothesized that SELENOM offered neuroprotective properties, although simply deleting SELENOM in mice does not result in learning or memory deficits under a Se adequate diet[79]. Interestingly, SELENOM deletion in mice results in body weight gain and increased adiposity, suggesting SELENOM may play a role in energy metabolism. Immunohistochemistry further supported this hypothesis, as SELENOM was revealed to be highly expressed in the paraventricular nucleus and the arcuate nucleus of the hypothalamus, regions that are implicated in energy homeostasis. Leptin is an adipocyte-derived peptide which activates receptors on the arcuate nucleus, allowing for crosstalk between the brain and fat depots[80]. The downstream effects of the leptin receptor are carried out via the Jak2-Stat3 pathway. Leptin resistance in the hypothalamus leads to alterations in energy homeostasis as the brain is no longer able to detect satiety. Although a direct

mechanistic relationship remains to be tested, SELENOM may regulate energy metabolism through regulating leptin signaling. Whole body SELENOM deletion in mice results in elevated circulating leptin levels and diminished phosphorylated Stat3 levels in the hypothalamus, which are indicative of leptin resistance[79]. Furthermore, ER stress has been implicated in hypothalamic leptin resistance[80]. As SELENOM is an ER-resident protein, there is a possibility SELENOM may promote leptin signaling by protecting against ER stress. Currently, it is unknown whether SELENOM contributes to human obesity. Given the possibility that SELENOM may promote leptin signaling by mitigating ER stress, further investigation into this relationship is warranted.

*1.8.4 Iodothyronine Deiodinase Family*

The iodothyronine deiodinase family is composed of types 1-3 deiodinases (Dio1, Dio2, and Dio3), a group of selenoenzymes involved in thyroid hormone regulation. The thyroid prohormone 3,5,3',5' tetraiodothyronine (T4) is produced and secreted by the thyroid, but binding of thyroid hormone to its receptor requires the action of Dio1 or Dio2, which catalyzes deiodination of T4 to its active form, 3,5,3' triiodothyronine (T3). In contrast to Dio1 and Dio2, Dio3 inactivates T3 and T4.

The role of thyroid hormone in energy expenditure has been well-documented[81]. It is therefore unsurprising that deiodinases are also implicated in energy metabolism. Indeed, genetic polymorphisms in the human DIO2 gene are associated with greater insulin resistance in T2D patients[82]. In mouse studies, Dio2 activation by cJun N-terminal kinase (JNK) in the anterior pituitary was found to be a critical component in diet-induced obesity[83]. On the other hand, whole-body Dio2 knockout mice (D2KO) develop insulin resistance on a standard laboratory chow diet and are more susceptible to diet-induced obesity[84]. Moreover, D2KO mice on a high fat diet preferentially utilize carbohydrates as a fuel source and have lower serum free

fatty acid and β-hydroxybutyrate levels, suggesting that Dio2 is involved in fatty acid utilization. Additionally, the liver-X receptor (LXR), which is activated by 22(R)-OH-cholesterol, negatively regulates Dio2, further strengthening the case for Dio2 involvement in lipid metabolism[85].

Thyroid hormone deactivation is also involved in energy homeostasis. Dio3 is expressed in pre-natal pancreatic tissue and appears to be important in β-cell development as Dio3 knockout mice (D3KO) have reduced islet size and insulin content[86,87]. Dio3 activation has also been found to be important in GLP-1 mediated insulin potentiation[88]. Thus, tight regulation by deiodinases contribute to the maintenance of glucose homeostasis by ensuring tissue and time appropriate thyroid hormone activation.

*1.8.5 Selenoprotein T*

Selenoprotein T (SELENOT) was first identified *in silico*, using an algorithm to identify SECIS elements in the human dbEST[89]. Containing a thioredoxin-like fold, SELENOT was proposed to possess redox activity[90], but its precise function remains unknown. Bioinformatics analysis revealed SELENOT to be localized to the ER, possibly being trafficked to the plasma membrane[91]. SELENOT expression in mice appears to be highest during development, with SELENOT mRNA expression in most tissues decreasing in adulthood, except in endocrine tissues such as the thyroid, pituitary, testis, and thymus[92]. The pituitary adenylate cyclase activating polypeptide (PACAP) is a neuropeptide which increases cAMP through adenylate cyclase stimulation, having implications in a variety of cellular processes, including cell survival and secretory function. SELENOT was recently identified as a target of PACAP[93]. In differentiated rat pheochromocytoma PC12 cells, SELENOT is necessary for PACAP-dependent neuroendocrine secretion by regulating intracellular $Ca^{2+}$ levels. Many neuropeptides regulate energy homeostasis, such as neuropeptide Y (NPY)[94], agouti-related peptide (Agrp)[95], α-melanocyte stimulating hormone (α-MSH)[96], among others. Identifying the neuropeptides

under SELENOT regulation could provide greater understanding of the connection between dietary Se and energy metabolism. Immunofluorescence demonstrated SELENOT to be highly expressed in the adult pancreatic β and δ-cells, indicating SELENOT may be involved in glucose homeostasis. Conditional knockout of SELENOT in the β-cell resulted in defective insulin secretion, suggesting SELENOT is critical to β-cell function. Studies in the glucose responsive murine β-cell line, MIN6, determined that PACAP-induced insulin secretion depends on SELENOT expression. This indicates that SELENOT may regulate blood glucose at multiple levels. Although SELENOT is expressed in other metabolic tissues such as the pituitary and thyroid[92], the molecular function of SELENOT in these tissues is unknown.

*1.8.6 Selenoprotein S*

Like SELENOT, Selenoprotein S (SELENOS) was first identified *in silico*, and was shown to localize to the plasma membrane[24]. Functionally, SELENOS has implications in ER-associated degradation (ERAD)[97], inflammation[98], and the transport of multi-protein complexes[99]. In 2003, a novel protein, Tanis, was characterized as a glucose-regulated protein in *Psammomys obesus*, an animal model for T2D[100]. Tanis was found to be expressed in insulin-sensitive tissues such as adipose tissue, liver, and skeletal muscle. Through yeast two-hybrid screening, Tanis was found to interact with serum amyloid A, a family of proteins associated with the acute-phase inflammatory response, which is typically elevated in T2D patients. Potentially, Tanis acts as a receptor for serum amyloid A. Tanis was later identified to be a SELENOS homolog[24], leading to the hypothesis that SELENOS links inflammation to T2D. In support of this, a positive correlation between serum amyloid A levels and SELENOS expression in the skeletal muscle and adipose tissue of T2D patients was reported[101]. SELENOS appears to be dysregulated in the disease state, as hyperinsulinemia increases SELENOS mRNA expression in the adipocytes of T2D subjects but not healthy subjects.

Conversely, a different study found subcutaneous adipocyte SELENOS mRNA expression to increase in response to insulin in both obese and lean subjects[102]. This study also failed to find a correlation between serum amyloid A and SELENOS expression. However, SELENOS expression was found to be higher in obese subjects, with increased subcutaneous SELENOS expression in obese subjects associated with BMI, sagittal diameter, serum HDL, triglycerides, insulin, and insulin resistance. Additionally, SELENOS polymorphisms were correlated with higher diastolic blood pressure and circulating insulin. These individuals were also at a higher risk for cardiovascular disease. Taken together, these studies support a role for SELENOS in metabolic diseases, however, the specific mechanism remains to be elucidated.

One possibility is that SELENOS plays a protective role. For instance, SELENOS has been shown to be upregulated in the hepatoma-derived HepG2 cells in response to glucose deprivation[103], albeit the physiological relevance is debated, as the low glucose concentration tested was 2 mM, well below the range of normal blood glucose levels in humans. However, in HepG2 cells, SELENOS was also found to increase in response to ER stress, while overexpression of SELENOS conferred protection against oxidative stress in the mouse-insulinoma derived MIN6 cells. Thus, SELENOS may play a protective role, counteracting oxidative stress in T2D development.

### 1.9 Sex Differences in Se and T2D

Sex differences in Se uptake and selenoprotein expression patterns have been described and are reviewed elsewhere[104]. Given the sexual dimorphism in Se regulation, it is unsurprising that human clinical trials hint at the possibility that the relationship between Se and T2D is sex-specific. In the NPC trial, the increased T2D risk in response to Se supplementation was limited to males[49], although one limitation of this study is that females were severely underrepresented, comprising only 25% of the subjects. Nevertheless, the NHANES III, found a

correlation between T2D risk and high Se in males, but not females[46], a finding which was supported by NHANES 2003–2004[47]. In contrast, but still supporting the hypothesis that the Se and T2D interrelationship is sex-specific, Akbaraly *et al*.[43] reported a correlation between lower baseline Se and T2D incidence among elderly French men, but not women. Understanding sex differences in the biological function and regulation of selenoproteins may explain the sexually dimorphic results of Se and T2D. Unfortunately, studies involving sex differences in the contribution of individual selenoproteins to metabolic diseases are limited. This section will highlight some of the known sex differences reported in selenoprotein regulation of energy metabolism.

GPX1 polymorphisms were correlated with increased MetS incidence in Japanese men, but not women[68]. The initial studies that investigated obesity and hyperinsulinemia in GPX1 overexpressing mice only utilized male mice, thus it is unknown whether the effect of GPX1 overexpression in mice is sex-specific[59,60]. Serum SELENOP levels were elevated in diabetic men and women compared to healthy subjects[69]. However, subsequent studies investigating SELENOP and insulin resistance were restricted to female mice. Whether SELENOP directly induces insulin resistance through AMPK in male mice is unknown. In the same study, SELENOP deficiency was found to produce an obesity-resistant phenotype in male mice, but female mice were left out due to inconsistencies in the results. Although sex differences in the association between SELENOS and metabolic diseases have not yet been described, there are sex differences in the amount of Se necessary to reach maximal murine hepatic SELENOS expression[105]. This discrepancy in SELENOS expression may contribute to the differences observed in the effectiveness of Se supplementation in a model of septic shock. We must not exclude the possibility that the sex differences in the regulation of SELENOS expression might result in sex-specific outcomes in metabolic diseases.

The studies in Scly$^{-/-}$ mice focused primarily on males as it was observed that female Scly$^{-/-}$ tended to display a mild metabolic phenotype, whereas the differences in males were more pronounced[38]. A plausible explanation is that the Se metabolic pathway does not interfere with energy metabolism in females. Recent evidence from mice with combined Scly and SELENOP deletion (Scly$^{-/-}$SELENOP$^{-/-}$) demonstrates that female mice are less dependent on the Se recycling pathway for neurological function, as female Scly$^{-/-}$SELENOP$^{-/-}$ mice do not exhibit the severe neurological deficits reported in male Scly$^{-/-}$SELENOP$^{-/-}$ mice[35]. As the primary Se source for the brain and testes, SELENOP is particularly critical for these tissues, supplying Se via the ApoER2 receptor[106,107]. Absence of either SELENOP or ApoER2 results in a similar phenotype, consisting of behavioral deficits and male infertility. Strikingly, castration of male Scly$^{-/-}$SELENOP$^{-/-}$ mice was shown to attenuate the neurological dysfunction present in uncastrated mice, offering a novel representation of the competition between the brain and testes for Se supply[79]. In the context of metabolic disease, it is conceivable that sequestration of available Se by the testes occurs at the expense of metabolic tissues, leading to altered glucose homeostasis in males.

### 1.10 Introductory Highlights

Se undoubtedly has numerous benefits to human health, with implications in T2D[51], cancer[108], male fertility[109], and neurological function[110], among others. However, the association of high Se intake and T2D in human epidemiological studies and clinical trials raises concerns regarding the practicality of Se supplements. Animal studies have offered mechanistic insight, identifying potential links between Se and T2D, namely oxidative stress. In addition, because the effects of Se occur through the enzymatic actions of selenoenzymes, the individual selenoproteins (GPX1, SELENOP, SELENOM, DIO2, SELENOT, SELENOS) that have been connected to T2D provide important information on the details of Se in T2D. Moreover, the Se

metabolic pathway has been suggested to influence carbohydrate and lipid metabolism, strengthening the idea that Se does in fact play a role in T2D. The aforementioned sexually dimorphic association between Se and metabolic diseases reveals the complexity of Se processes. In order to reduce potentially undesirable effects of Se supplementation, and to further improve dietary guidelines for Se, a more comprehensive understanding of Se metabolism and selenoproteins in both male and female subjects is essential.

### 1.11 Hypothesis

Scly regulates insulin secretion and whole-body glucose homeostasis in male mice, but not females.

### 1.12 Specific Aims

Specific Aim 1: Characterize the role of Scly in pancreatic islet physiology.

Working hypothesis: Scly is present in the insulin producing β-cells and its regulation of selenoprotein expression is critical in pancreatic islet function.

Rationale: We previously reported that Scly$^{-/-}$ mice develop a metabolic syndrome-like phenotype, with remarkably high fasting serum insulin levels[38]. This suggests that alterations in Sec decomposition may lead to pancreatic islet dysfunction. While selenoproteins such as GPX1, SELENOT, Dio3 and SELENOS have been shown to play important roles in insulin secretion, the impact of Se metabolism remains unexplored. Therefore, the goal of this aim is to determine whether Scly-dependent regulation of selenoprotein expression is necessary for glucose-stimulated insulin secretion (GSIS).

Specific Aim 2: Investigate the sex differences in Scly regulation of energy metabolism and determine the contributing role in male reproduction.

Working hypothesis: Scly$^{-/-}$ mice display sex differences in their metabolic phenotype, and these differences can be accounted for by the Se demands of the male reproductive system.

Rationale: Scly$^{-/-}$ mice appear to develop metabolic syndrome in a sex-specific manner, and thus may serve as a model to understand sex differences in Se metabolism and the onset of metabolic syndrome in humans. In a previous study, castration was found to rescue neurological effects in Scly$^{-/-}$SELENOP$^{-/-}$ mice, elucidating a mechanism in which the testes act as a Se sink, worsening the outcome for male mice. Therefore, the effects of castration were tested on Scly$^{-/-}$ mice to determine whether Se sequestration by the testes negatively impacts glucose homeostasis.

### 1.13 Significance

The studies in this dissertation were aimed at establishing a novel role for Scly in islet function and to clarify the potential sex-specific regulation that was previously reported. Overall, these studies improve our understanding of Se in the development of metabolic diseases, which will aid improving general guidelines for appropriate Se intake. The significance of this project on public health is that the obtained knowledge could facilitate identification of undesirable effects mediated by the Se metabolic pathway during drug discovery.

# CHAPTER 2. THE ROLE OF SELENOCYSTEINE LYASE IN PANCREATIC ISLET FUNCTION

## 2.1 Introduction

Regulation of blood glucose levels relies heavily on insulin secretion and its action on target tissues. In the early stages of T2D, insulin production is increased to compensate for insulin resistance in order to maintain regulation of glucose levels. The main site of insulin production is the β-cell, one of several cell types present in the pancreatic islets. As the disease progresses, β-cell mass and function decline, and hyperglycemia ensues. Thus, maintaining normal islet function is critical to reversing or slowing the progression to T2D. While the main insulinotropic agent is glucose, many other nutrient signals stimulate insulin secretion by enhancing vesicle release.

Se is an essential dietary micronutrient whose physiological functions are dependent upon its incorporation into selenoproteins. In epidemiological studies, higher serum Se levels have been associated with elevated fasting insulin[47]. Additionally, treatment of the mouse insulinoma-derived MIN6 cells and rat islets with selenite improves insulin production and secretion through the upregulation of insulin promoter factor 1 (Ipf1), a key transcription factor in insulin synthesis[57]. To corroborate these findings, the overexpression of GPX1, a selenoenzyme whose expression is particularly sensitive to Se levels, results in primary hyperinsulinemia in mice, a condition in which hyperinsulinemia precedes insulin resistance. It was proposed that enhanced $H_2O_2$ scavenging by GPX1 resulted in hyperacetylation in the insulin promoter PDX1, leading to increased insulin synthesis[60]. On the other hand, the expression of selenoproteins DIO3 and SELENOT are necessary for β-cell development and GSIS, as their respective knockout mice present with decreased β-cell mass and impaired insulin secretion[86,111]. Additionally, SELENOS overexpression protected mouse insulinoma-derived MIN6 cells against

$H_2O_2$ induced cell death, implicating a role for SELENOS in protecting β-cells against oxidative damage. Further investigation is necessary to clarify the role of Se and selenoproteins in islet function.

In the β-cell, nutrients are metabolized and participate in pathways which produce second messengers that enhance insulin vesicle release[112]. Scly is an important component of Se metabolism as it is involved in Sec decomposition, cleaving Sec to produce alanine and selenide. Moreover, Scly may be involved in the Se salvage pathway as the produced selenide can re-enter the Sec pool to be repurposed for selenoprotein synthesis. We previously generated and characterized mice with whole-body Scly deletion which provided insight to the role of Scly in hepatic carbohydrate and lipid metabolism[38,40]. Of particular interest, $Scly^{-/-}$ mice exhibit remarkably elevated fasting insulin levels. Thus, we hypothesized that Scly is a critical factor that couples Se and/or selenoproteins to insulin secretion.

Unlike Scly, sulfur pathway enzymes lack the ability to distinguish sulfur molecules from their Se analogs. In the glutathione synthesis pathway, Cys/Sec can be converted to glutathione through the rate limiting enzyme glutathione cysteine ligase, which is composed of the regulatory and the catalytic subunits Gclm and Gclc, respectively. Cysteine degradation involves the cysteine desulfurase, Nfs1. The enzymes cystathionine β-synthase (Cbs) and cystathionine γ-lyase (Cth), act sequentially to convert homocysteine to Cys in the reverse trans-sulfuration pathway. In the methionine cycle, methyltransferase (Mtr) converts homocysteine to methionine. It is unknown whether sulfur pathways can compensate for Scly deletion.

The main goal of this study was to investigate the role of Scly in pancreatic islet physiology. We found that Scly is highly expressed in the pancreatic islet and we therefore

tested whether Scly influences GSIS. We also measured selenoprotein expression to determine if any effects Scly has on islet function occurs through a selenoprotein-dependent mechanism. To determine whether sulfur pathways could compensate for Scly deletion, we employed qPCR to measure gene expression changes that could explain alterations in the insulin secretory pathway.

## 2.2 Methods

### *2.2.1 Animals*

Whole-body male $Scly^{-/-}$ mice were generated and backcrossed to WT C57BL/6N mice as previously described[38]. All mice were bred and housed in the animal facility at University of Hawaii. Mice were weaned onto a special diet containing adequate (0.25 ppm) or low (0.08 ppm) levels of Se in the form of sodium selenite (Research Diets Inc., New Brunswick, NJ) and maintained on a 12-h light/dark cycle. Unless otherwise noted, animals were euthanized via $CO_2$ asphyxiation. All procedures were approved by the Institutional Animal Care and Use Committee of the University of Hawaii.

### *2.2.2 Cell culture*

MIN6 cells are a murine pancreatic β-cell line that is useful in studying insulin secretion, as it maintains β-cell characteristics, and has been shown to secrete insulin in response to glucose treatment[113]. MIN6 cells were purchased from AddexBio (San Diego, CA) and cultured in Dulbecco's Modified Eagle's Medium (DMEM; Cellgro, Manassas, VA) with 15% fetal bovine serum (FBS; Invitrogen, Carlsbad, CA), 1X Penicillin-Streptomycin-Glutamine (Cellgro, Manassas, VA) at 37°C and 5% $CO_2$. FBS was tested for total Se content and found to contain 320 nM Se.

*2.2.3 Scly knockdown*

For Scly knockdown, CRISPR/Cas9 constructs were designed and purchased from the Gene Editing Institute at the University of Delaware. Scly-targeting gRNA was cloned into px458, which contains an eGFP tag. MIN6 cells were seeded and grown in a T-75 flask. Constructs were transfected with Lipofectamine 2000 (Invitrogen, Carlsbad, CA). MIN6 cells were collected and fluorescence-activated cell sorting (FACS) was performed with the FACSAria II (BD Biosciences, San Jose, CA) to sort for all GFP+ cells 48 hours post-transfection. Cells were allowed to recover and grow for one week prior to experimentation. Knockdown was confirmed using Western Blot.

*2.2.4. Scly activity*

Scly activity was assessed as previously described[31], by measuring the absorbance of selenide that has been precipitated by lead acetate.

*2.2.5 Immunofluorescence*

Pancreases were fixed in 4% paraformaldehyde, paraffin embedded, and sectioned at 5 µm thickness. Antigen unmasking was completed with boiling in 0.1M citrate buffer, pH 6 (0.1M anhydrous citric acid, 0.05% Tween-20) for 4 min. Endogenous mouse antigen was blocked using 20 µg/ml unconjugated affinity pure Fab fragment goat anti-mouse IgG (Jackson ImmunoResearch Inc., West Grove, PA) and triple-labeled in parallel using specific primary antibodies and Alexafluor conjugated secondary antibodies (Jackson ImmunoResearch Inc., West Grove, PA). Coverslips were mounted using mounting media with the nuclear marker 4',6-diamidino-2-phenylindole (DAPI, Vector Laboratories, Burlingame, CA). Fluorescence was detected using the Zeiss LSM 5 Pascal Confocal microscope (Oberkochen, Germany). DAPI

was detected using the Zeiss Axioskop 2 (Oberkochen, Germany)

*2.2.6 Subcellular fractionation*

Nuclear and cytosolic separation was performed in whole pancreas as described previously[114]. Briefly, tissue was minced over ice, re-suspended in a hypotonic solution (STM buffer: 250mM Sucrose, 50mM Tris-HCl pH 7.4, 5mM $MgCl_2$, 1X protease/phosphatase inhibitor) and homogenized using a GT21-18 heavy duty laboratory stirrer (G.K. Heller Company, Floral Park, NJ) to disrupt cell membrane. Homogenate was centrifuged and the supernatant (cytosolic fraction) was precipitated with acetone. The pellet was washed, resuspended in nuclear extraction buffer (20mM HEPES pH 7.9, 1.5mM $MgCl_2$, 0.5M NaCl, 0.2 mM EDTA, 20% glycerol, 1% Triton X-100, 1X protease/phosphatase inhibitor) and sonicated to disrupt the nuclear membrane. Fractionation was confirmed via Western Blot using anti-GAPDH (Abcam, Cambridge, MA) as the cytosolic control and anti-Histone H3 (CST, Danvers, MA) as the nuclear control.

*2.2.7 Islet isolation*

Pancreatic islets were isolated using a modified version of the collagenase method as previously described[115]. Briefly, after euthanasia, a collagenase and neutral protease solution (CIzyme RI, VitaCyte, LLC, Indianapolis, IN) was perfused into the pancreas via the common bile duct and excised. Pancreases were kept on ice for not more than one hour before digestion. Digestion was performed in a water bath at 37°C for 18 min and terminated with the addition of RPMI 1640 (Cellgro, Manassas, VA) with 10% newborn calf serum (Gibco, Waltham, MA). Islets were separated with a density gradient using lymphocyte separation medium (Cellgro, Manassas, VA) and handpicked for purity.

*2.2.8 Glucose-stimulated insulin secretion (GSIS), insulin content, and DNA concentration*

Handpicked islets from 8-week-old male mice were cultured overnight in RPMI 1640, 10% FBS (Invitrogen, Carlsbad, CA), and 1% penicillin-streptomycin (Cellgro, Manassas, VA) at 37°C with 5% $CO_2$ and 80% humidity. GSIS was performed using a static incubation method as previously described[116]. Islets were transferred to Krebs-Ringer bicarbonate (KRB) buffer with low glucose (2.8 mmol/L) and incubated sequentially for 1 hour at 37°C in the following order: twice in KRB buffer with low glucose and once with KRB buffer with high glucose (16.8 mmol/L). Islets were washed twice with KRB buffer with low glucose between each incubation. Media was collected after the second low glucose incubation to determine basal insulin secretion and after the high glucose incubation for GSIS, which was measured using a mouse ELISA kit (ALPCO, Salem, NH). Islets were collected and resuspended in 500 µl of PBS and sonicated with 3x 10s pulses over ice. 250 µl of the supernatant was used to determine insulin content via ELISA while the other half was used to determine DNA concentration. DNA was extracted and concentration was determined using the Quant-iT Picogreen kit (ThermoFisher, Grand Island, NY) according to manufacturer's instructions.

*2.2.9 Western blot*

Handpicked islets were washed twice with ice cold PBS, re-suspended in Sigma CelLytic™ MT (St. Louis, MO), incubated over ice for 20 min, and vortexed at maximum speed for 15s. Protein concentration was measured using the Bradford method with the Bio-Rad Protein Assay Reagent (Hercules, CA). 20 µg of total protein were separated on a 4-20% Tris-Glycine polyacrylamide gel (Bio-Rad, Hercules, CA), transferred to an Immobilon-FL membrane Biosciences, Lincoln, NE) and incubated in primary antibody overnight at 4°C. Blots were washed 3x 5 min in 0.1% Tween-20 in PBS and incubated in the appropriate IRDye secondary

antibody (Li-Cor Biosciences, Lincoln, NE) for 30 min. An Odyssey IR imaging system (Li-Cor Biosciences, Lincoln, NE) was used for detection and analysis was complete on the Image Studio v4.0 software (Li-Cor Biosciences, Lincoln, NE).

### *2.2.10 qPCR*

Synthesis of cDNA was completed using a High Capacity cDNA Reverse Transcription kit (Applied Biosystems, Waltham, MA) according to manufacturer's instructions. SYBR green (Invitrogen, Carlsbad, CA) and Roche LightCycler 480II were used to detect mRNA levels. Results were analyzed via the ΔCt method using a reference gene, which is a variation of the $2^{-\Delta\Delta Ct}$ method. All samples were normalized to the housekeeping gene cyclophilin B (Cphn2), which was unaffected by genotype. Primer efficiencies between 1.95 and 2.05 were considered to be acceptable. Primer sequences are listed in Table 2.

**Table 2. Quantitative PCR primer sequences.**

| Gene | Forward (5'>3') | Reverse (5'>3') |
|---|---|---|
| *Cphn2* | GGAGATGGCACAGGAGGAA | GCCCGTAGTGCTTCAGCTT |
| *Gclc* | CTCCAGTTCCTGCACATCTACC | AACATGTACTCCACCTCGTCAC |
| *Gclm* | CCACCAGATTTGACTGCCTTTG | AGAGCAGTTCTTTCGGGTCATT |
| *Nfs1* | TCACAACCCAGACAGAACACAA | GATGGCAGCCTCTAGTTCCTTT |
| *Mtr* | GCAACAAGAGTCATTAAGGAAA | AGGAGAAGGATAAGTTGGAAAG |
| *Cbs* | ATGAGTATGGAGAAGGTGGAT | GAATGTGAGAATTGGGGATTTC |
| *Cth* | TGCCACCATTACGATTACCCAT | TTGGTGCCTCCATACACTTCAT |

### *2.2.11 Statistical analysis*

Unless otherwise noted, data were analyzed and graphed with GraphPad Prism 5 (GraphPad Software Inc., La Jolla, CA) using the appropriate statistical tests. The statistical tests are referred in figure legends.

## 2.3 Results

### *2.3.1 Scly is present in the β-cells of the pancreatic islets*

Scly$^{-/-}$ mice have been shown to develop remarkable fasting hyperinsulinemia[38]. Thus, we hypothesized that Scly regulates insulin secretion and that alternations in β-cell function contribute to the metabolic dysfunction observed in Scly$^{-/-}$ mice. To test this hypothesis, we first used immunofluorescence to investigate Scly expression within β-cells of the pancreatic islet, the main site of insulin production and secretion. Our images revealed that Scly expression largely overlaps with insulin expression in the islet (Figure 2**Error! Reference source not found.**), although not exclusively, as we also observed overlap with glucagon expression, which is produced and secreted by the α-cell. Nevertheless, our images suggest that Scly is localized where it can possibly interact with components of the insulin synthesis and/or secretion pathway.

### *2.3.2 Subcellular localization of Scly*

Based on the established role of Scly, it has been predicted that Scly is localized in the cytosol. However, in our confocal images (Figure 3), Scly appeared to mimic a nuclear staining pattern. Using the nuclear marker DAPI as a counterstain, we checked for localization of Scly within the nucleus. To our surprise, Scly was co-localized with DAPI within the islet (Figure 3A). To further confirm this Scly subcellular localization, we separated nuclear and cytosolic extracts from whole pancreata. In contrast to our immunofluorescence images, Scly was restricted to the cytosolic fraction (Figure 3B).

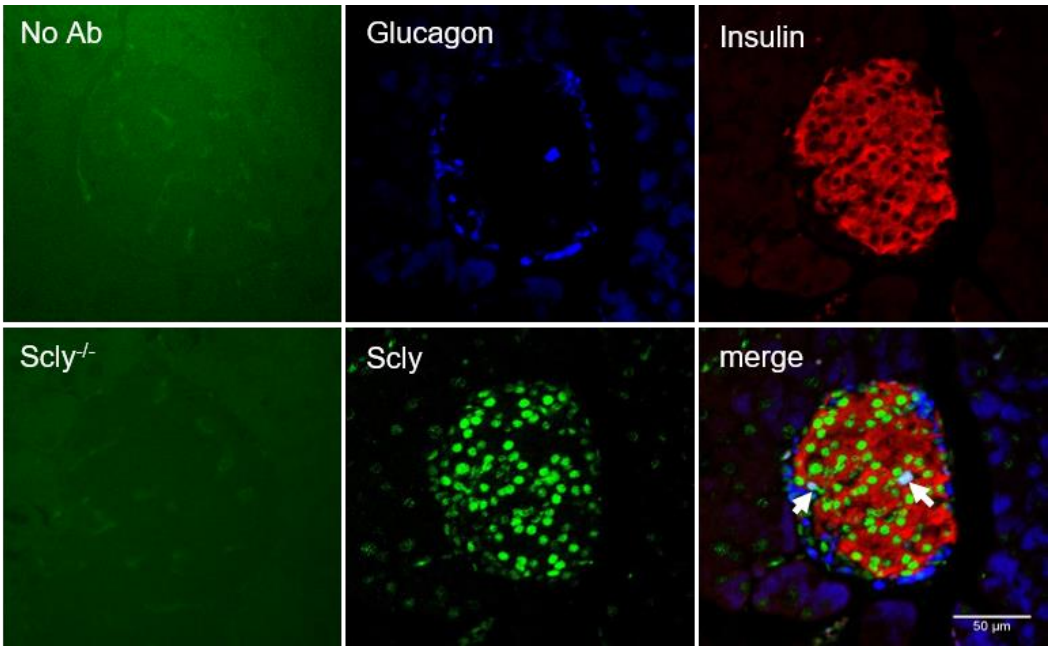


**Figure 2. Scly is highly expressed in the pancreatic islet.** Representative confocal image depicting an islet from 10-week-old male WT mice fed a Se adequate diet. As controls, staining procedure was performed without primary antibody, as well as with sections from $Scly^{-/-}$ mice. Scly appears to be most strongly expressed within the pancreatic islet. Glucagon is indicated in blue, insulin in red, and Scly in green. White arrows indicate artifacts. Image representative of n=3.

*2.3.3 Scly has opposing effects on GSIS in MIN6 cells versus pancreatic islets*

Following confirmation that Scly is expressed in the pancreatic islet, we next tested the effects of Scly deletion on GSIS. To verify that MIN6 is an appropriate model to study Scly function in β-cell physiology, Scly activity was measured. In accordance with previous studies[117], Scly activity was detected under low Se conditions and significantly decreased under Se adequate conditions ($p<0.0001$) (Figure 4A). Scly expression was knocked down using CRISPR/Cas9 and confirmed via Western Blot (Figure 4B). Because all GFP+ cells were sorted together, low levels of Scly expression can be explained by the fact that CRISPR/Cas9 does not induce a mutation at 100% efficiency.

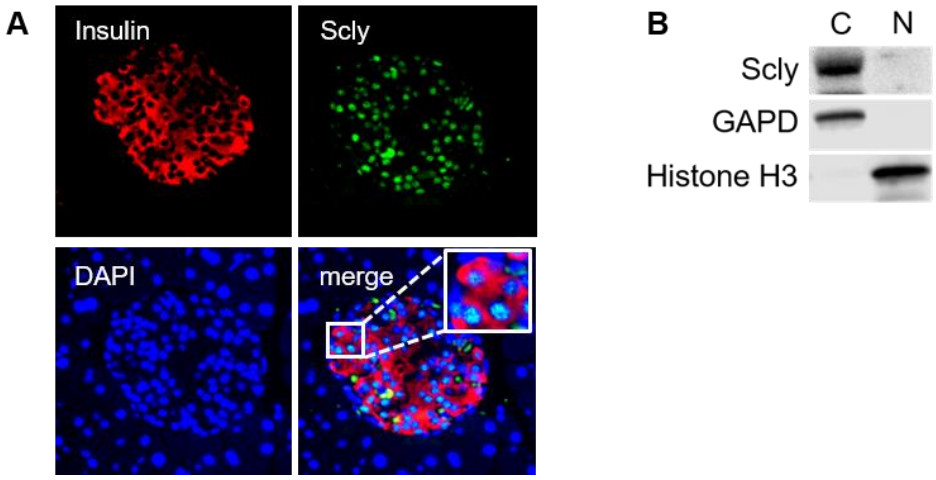


**Figure 3. Contrasting cellular localization of Scly in immunofluorescence images and subcellular fractionation.** (A) Representative immunofluorescent image of an islet from a 10-week-old male mouse. Scly is indicated in green, insulin in red, and DAPI in blue. Scly appears to colocalize with DAPI (cyan). (B) Western Blot of cytosolic, C and nuclear, N fractions from whole pancreas of 10-week old mice. Scly expression was detected only in the cytosolic fraction. Detection of GAPDH and histone H3 were used as controls for cytosolic and nuclear purity, respectively, n=3.

Next, we measured GSIS in MIN6 cells with diminished Scly expression. While there was a main effect of glucose treatment in GSIS, there was no effect of genotype (Figure 4C). Nevertheless, the insulin response was approximately 1.8 times greater when Scly was knocked down. Surprisingly, there were no differences in total insulin content (Figure 4D).

Because differences in GSIS among MIN6 and primary cells have been reported[118], we repeated GSIS in islets isolated from WT and Scly$^{-/-}$ mice. In contrast to our results in MIN6 cells, WT islets had nearly a two-fold greater insulin response to glucose than Scly$^{-/-}$ islets (Figure 4E). Insulin content among the WT and Scly$^{-/-}$ remained unchanged after GSIS (Figure 4F), indicating insulin synthesis and production was unaffected by Scly$^{-/-}$ deletion in pancreatic islets.

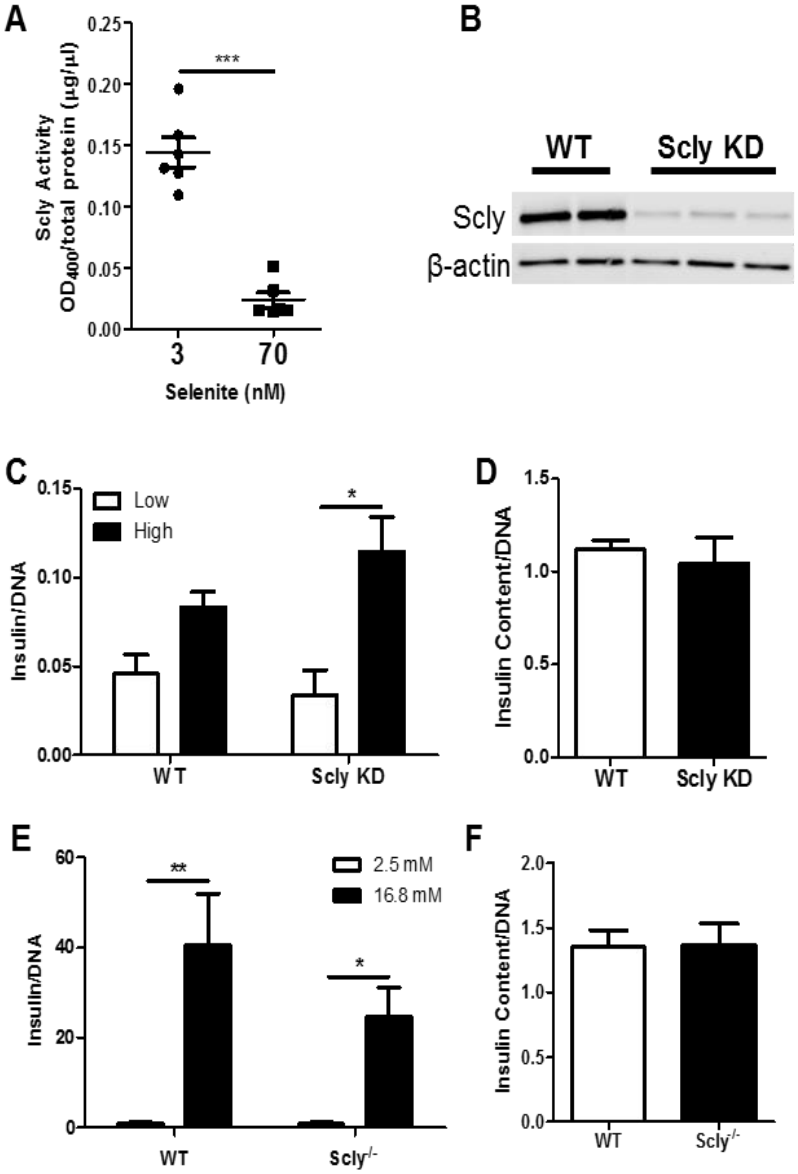


**Figure 4. GSIS in MIN6 cells and isolated islets.** (A) Scly activity under Se deficient (3 nM Se) and adequate (70 nM Se) conditions for 24 hours, t-test: ***$p$<0.001, n=6. (B) Western Blot confirming successful knock-down using CRISPR/Cas9. The low level of Scly in the knock-down is a result of differences in homologous recombination efficiencies. Different lanes are replicates for each condition. (C) Insulin secretion at baseline (2.5 mM glucose) and after high glucose (16.7 mM) conditions. Two-way ANOVA showed a main effect of glucose ($F_{1,6}$=20.15, $p$=0.004), but not knockdown ($F_{1,6}$=0.41, $p$=0.54), or interaction ($F_{1,6}$=2.70, $p$=0.15), n=4, and (D) insulin content, t-test: *ns* (not significant). (E) GSIS in isolated islets from 8-week-old male WT and Scly$^{-/-}$ mice. 2-WA showed a main effect for glucose ($F_{1,11}$=25.34 $p$=0.0004) but not genotype ($F_{1,11}$=1.61 $p$=0.23) or interaction ($F_{1,11}$=1.59 $p$=0.23). Bonferroni's correction: *$p$<0.05 **$p$<0.01, n=7. (F) Insulin content, *ns*. All data are represented as means ± S.E.M.

*2.3.4 Scly does not affect selenoprotein expression under Se adequate conditions*

We previously reported that hepatic selenoprotein expression is largely unaffected by the absence of Scly when Se supply is adequate[38,40]. However, Se distribution among tissues and selenoprotein expression regulation occur in a hierarchical manner[104]. Thus, it is possible that the hepatic selenoprotein profile does not reflect the conditions in the pancreatic islet. We therefore measured selenoprotein levels by Western Blotting in isolated islets from 8-week-old WT and Scly$^{-/-}$ mice. We measured GPX1 and SELENOS levels based on their confirmed roles in islet function, but we did not detect differences between WT and Scly$^{-/-}$ islets (Figure 5A-C). Additionally, we measured SEPHS2 levels because it has been identified as a potential interactor of Scly and hypothesized to work as part of a complex in selenoprotein biosynthesis[33].

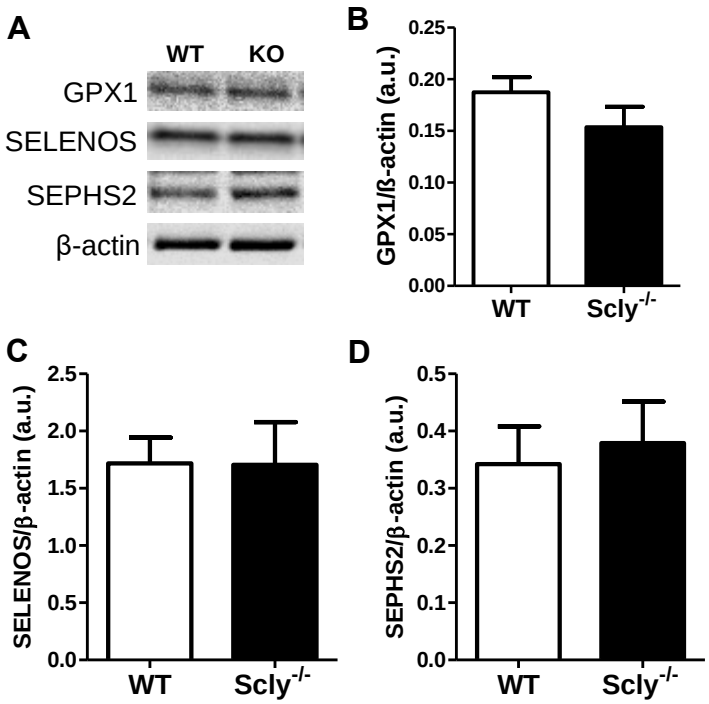


**Figure 5. Islet selenoprotein expression in 8-month-old WT versus Scly-/- mice.**
(A) Representative Western Blot image showing indicated selenoprotein expression. (B-D) Quantification of the expression of selenoproteins normalized to β-actin levels. All data are represented as means ± S.E.M., t-test was used to calculate *p* values, n=4.

Nevertheless, no observable changes were detected in SEPHS2 protein expression (Figure 5A,D). Taken together, our data suggest that when Se demands are fulfilled, Scly does not influence selenoprotein expression in the pancreatic islet.

*2.3.5 Sulfur pathway enzyme mRNA expression is unchanged in response to Scly deletion*

We next determined whether glutathione synthesis and cysteine degradation pathways can compensate for the absence of Scly because of their ability to further metabolize Cys/Sec. Thus, we measured islet mRNA expression of Gclm, Gclc, and Nfs1 via qPCR, but did not observe any significant differences (Figure 6). Additionally, we considered the possibility that sulfur pathways such as the reverse trans-sulfuration pathway and methionine cycle could be affected by the absence of Scly. However, qPCR did not reveal any noticeable differences in Mtr, Cbs, or Cth gene expression (Figure 6).

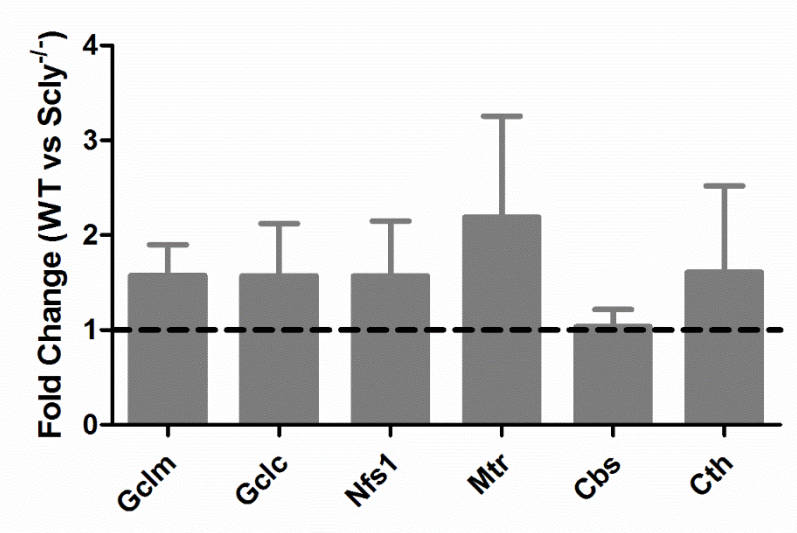


**Figure 6. Gene expression of sulfur pathways in islets of 8-week-old male WT vs. Scly$^{-/-}$ mice.** Fold changes of genes in the glutathione synthesis pathway (Gclm, Gclc), cysteine degradation (Nfs1), methionine cycle (Mtr), and reverse trans-sulfuration pathway (Cbs, Cth). WT gene expression is indicated by the black dotted line. Samples were normalized to the housekeeping gene Cphn2. All samples are represented as means ± S.E.M., n=3 pooled samples per group.

## 2.4 Discussion

We previously demonstrated that Scly$^{-/-}$ mice develop hyperinsulinemia. To the best of our knowledge, the present study is the first to demonstrate that Scly is present in the pancreatic islet. We found that Scly has opposite effects in MIN6 cells and isolated islets, potentially indicating that Scly is physiologically important not only in β-cells but in the other cell types that compose the pancreatic islet. Expression of GPX1, SELENOS, and SEPHS2 were unaffected, indicating Scly is not a critical factor in the biosynthesis of these selenoproteins under Se adequate conditions in the pancreatic islet. Moreover, our qPCR results do not support a compensatory role for sulfur metabolic pathways in the absence of Scly.

Our immunofluorescence studies revealed that Scly was co-localized with DAPI staining (Figure 3A) but Scly could be detected only in the cytosolic fraction when subcellular fractionation was employed (Figure 3B). Selenoprotein synthesis factors such as SBP2 and eEFSec express nuclear localization and export signals and participate in nucleocytoplasmic shuttling, a mechanism which increases Sec incorporation efficiency[119]. It is possible that Scly is involved in nucleocytoplasmic shuttling, but Scly does not appear to have a nuclear export/localization signal[117]. This does not exclude the possibility that Scly is part of a molecular complex that facilitates shuttling. Moreover, we must consider that Scly may be localized near the surface of the nuclear membrane, but not in the nucleus. Further investigation would provide a clearer overview of the biological significance of Scly subcellular localization

Because Scly$^{-/-}$ mice present with fasting hyperinsulinemia, we hypothesized that the insulin secretory pathway is dependent on Scly function. Our results in MIN6 cells indicated that Scly knockdown-enhanced GSIS (Figure 4C), but isolated Scly$^{-/-}$ islets had impaired GSIS when

compared to WT islets (Figure 4E). Since Scly$^{-/-}$ mice have higher fasting serum insulin levels, diminished GSIS could be due to insulin lost during fasting. If this were the case, we would expect to see higher baseline secreted insulin. Nevertheless, it may be useful to measure fasting insulin content. Hyperinsulinemia may also lead to β-cell exhaustion, in which insulin secretion loses efficacy. This could be tested by measuring expression and activity of various components of the insulin secretory pathway such as glucokinase, Pcx, and PDH, among others. MIN6 cells begin to lose their β-cell characteristics and display impaired GSIS at high passage numbers[120]. One limitation to our study is that our cell line was purchased through a manufacturer that could not provide information on how many passages our cells had previously undergone. If these cells were at a high passage number, alterations in key β-cell genes could account for the differences in GSIS response we observed.

One of the advantages of utilizing the MIN6 cell line is that it is a homogeneous population of cells which maintain β-cell like characteristics. While this is undoubtedly useful in understanding signaling pathways in the β-cell, it may not be the most physiologically relevant model in terms of insulin secretion. This is because intra-islet interaction via paracrine signaling also contributes to insulin secretion regulation and β-cell survival. In fact, a study seeking to better understand paracrine signaling loops in the islet found 233 candidates for secreted factors using a bioinformatics database search[121]. Considering Scly expression is not restricted to β-cells (Figure 2), it is conceivable that Scly has direct or indirect consequences on these paracrine factors, influencing the outcome of GSIS in MIN6 versus isolated islets. Future studies evaluating the changes in secreted factors and the expression of their receptors within the islet in Scly$^{-/-}$ mice may clarify the paracrine pathways that may be regulated by Scly.

Our measurements of islet selenoprotein expression mirrored previous findings in the liver that under Se adequate conditions, Scly does not appear to influence selenoprotein biosynthesis, at least for GPX1, SELENOS, and SEPHS2 (Figure 5). This suggests that the pancreatic islet does not have priority over other metabolic tissues such as the liver in the context of seleneoprotein biosynthesis.

Because Sec is toxic, when selenoproteins undergo proteolysis, the released Sec must be further metabolized to avoid damaging effects[122]. As the only identified enzyme with the ability to discriminately degrade Sec, Scly is likely a key player in this pathway. When Scly is deleted, in order to maintain Sec below the threshold for toxicity, Sec decomposition likely occurs through an alternate pathway. Interestingly, enzymes of the sulfur metabolic pathways cannot distinguish between sulfur and its Se analogs, and as a consequence, Se compounds are indiscriminately metabolized through sulfur pathways[123], although the regulatory factors that allow Se metabolism through sulfur pathways are unclear. An important role for Cys lies in its ability to be utilized in glutathione synthesis through the actions of enzymes glutamate cysteine ligase and glutathione synthase (GS). One possibility is that Sec is used to synthesize seleno-glutathione via the glutathione synthesis pathway. Another possibility is that Sec is degraded through cysteine degradation mechanisms. Cysteine desulfurases such as Nfs1 remove the sulfur atom from cysteine, and unlike Scly, can indiscriminately degrade Sec and Cys. In fact, a study in *Trypanosoma brucei* found that the cysteine desulfurase activity containing Nfs protein is able to compensate for the loss of Scly[124].

Potential alterations in the cysteine pathway in response to Scly deletion is particularly intriguing in light of surmounting evidence that an increase in total plasma Cys levels are strongly correlated with increased fat mass, and likely has a causative effect[125,126]. In terms of

islet function, Cys and its biologically active metabolite hydrogen sulfide ($H_2S$) consistently inhibit GSIS in both insulin secreting cell lines and isolated pancreatic islets[127–129]. Recently, it was found that the Cys-dependent reduction of GSIS is due to the direct inhibitory effects of Cys on the activity of pyruvate kinase muscle isoform 2, resulting in a decreased flux of metabolites through the tricarboxylic acid cycle[129]. Moreover, $H_2S$ was found to directly inhibit L-type $Ca^{2+}$ channels that are necessary for insulin vesicle release[128]. Improved understanding of the influence of Scly deletion on glutathione synthesis enzymes and cysteine degradation pathways will provide insight to the fate of Sec and insights to its downstream effects on insulin secretion. Although we failed to establish significance in mRNA expression of sulfur pathway enzymes (Figure 6), it is nevertheless interesting that an increase in fold change in comparing WT vs. $Scly^{-/-}$ islets was observed in each gene that was measured. Moreover, mRNA expression does not always correlate with protein or activity levels. Thus, the possibility that sulfur pathways compensate for the absence of Scly should not be precluded.

Several amino acids act as nutrient signals that can potentiate insulin secretion[130]. Of particular interest, acute alanine stimulation was found to have an insulinotropic effect[131]. It is possible that the alanine produced as a byproduct of Sec decomposition may influence islet function. However, the pool of Sec in relation to other amino acids is small. Thus, the contribution of alanine generated by Sec in insulin secretion is likely minimal.

Our findings elucidate a role for Scly in maintaining pancreatic islet function in a selenoprotein-independent manner. While it is possible that loss of Scly results in alterations in sulfur pathways, further investigation is necessary to determine the metabolites that are affected.

# CHAPTER 3. SEX DIFFERENCES IN SELENOCYSTEINE LYASE REGULATION OF ENERGY METABOLISM

## 3.1 Introduction

In the past, female research subjects have been largely been excluded from biomedical research. It was not until 1993 that the National Institutes of Health (NIH) mandated the inclusion of women in NIH-funded research[132]. Since then, many sex differences in disease pathogenesis have been reported. Relevant to the current study is the increased understanding of the sex-specific pathophysiology of type 2 diabetes[133]. For instance, adipocytes of women are more insulin sensitive than men[133]. One study in mice suggested that this effect could be due to positive effects of estrogen on GLUT1, GLUT4, fatty acid synthase (FAS), and acetyl-CoA carboxylase (ACC), key components of glucose and lipid metabolism[134]. On the other hand, women are at a higher risk of death due to T2D-related cardiovascular issues[135]. Enhancing prevention and treatment strategies for T2D and other diseases would greatly benefit from elucidating mechanistic differences in T2D pathophysiology in males and females.

Sex differences in Se regulation are well documented, but not well understood. In particular, Se appears to be critical to the male reproductive system. Although Se can be found in many tissues, a significant portion of dietary Se is routed to the male reproductive system, and under times of Se deprivation Se uptake is prioritized to the brain and testes[136]. When the Se transport selenoprotein SELENOP is knocked out in mice, the males become infertile while female mice maintain fertility[137]. To further support the sexual dimorphism in Se regulation, male SELENOP$^{-/-}$ mice develop neurological dysfunction to a greater extent than their female counterparts. Because of the Se salvaging capability of Scly and the presence of multiple Sec residues in SELENOP, it has been suggested that their concerted action is necessary for Se

transport and delivery to tissues[117]. Even under Se supplementation, male Scly$^{-/-}$SELENOP$^{-/-}$ mice develop audiogenic seizures and have reduced survival, producing a far worse phenotype than the deletion of either one of these genes[35]. Strikingly, castration is sufficient to rescue Scly$^{-/-}$SELENOP$^{-/-}$ mice, suggesting that the testes competes for Se at the expense of neurological function, further reinforcing the importance of Se to male reproduction.

The hypothalamus is the master regulator of energy homeostasis, sensing nutrient availability and subsequently fine-tuning energy intake and expenditure through its downstream targets[138]. Impairment of selenoprotein synthesis through the deletion of Sec-tRNA$^{[Ser]Sec}$ from the hypothalamus resulted in severe glucose intolerance and insulin resistance in mice[139], indicating that hypothalamic selenoprotein function is necessary to maintain energy metabolism. Nevertheless, we do not know whether Scly is involved in hypothalamic selenoprotein biosynthesis.

Although the association between higher incidence of T2D and elevated Se levels in humans is controversial, it is nevertheless interesting that this association appears to be restricted to men[140]. We previously reported that female Scly$^{-/-}$ mice have a less pronounced metabolic phenotype than their male counterparts[38], suggesting that sex differences in the regulation of Se metabolism may have direct consequences on energy metabolism. In the present study, we hypothesized that the Se demands of the male reproductive system have a detrimental effect on energy metabolism in male Scly$^{-/-}$ mice. We first characterized the metabolic phenotype of female Scly$^{-/-}$ mice. Next, we tested the effects of castration of male mice on body weight, energy expenditure, and measures of insulin secretion and resistance.

## 3.2 Methods

### *3.2.1 Animals*

Protocols are as described in Chapter 2.

### *3.2.2 Castration and Testosterone (TST) replacement*

Five-week-old male mice were anesthetized with isofluorane (2 liters/min) and a 1-2 cm ventral midline incision was made in the scrotum to expose the tunica. The tunica was pierced and the testes pushed out one at a time and excised while all deferential vessels and ducts were replaced. A 0.75 cm incision was made in the neck where either a 60-day controlled release placebo or 1 mg TST pellet (Innovative Research of America, Sarasota, FL) was inserted. For long-term studies, a second pellet was inserted after 60 days. Skin incisions were closed with 6.0 surgical silk. Animals were given ibuprofen and allowed to recover for one week. Sham operations were also performed as a control. Serum TST levels were confirmed using a TST ELISA kit (Crystal Chem, Downers Grove, IL) according to manufacturer's instructions.

### *3.2.3 Measurement of metabolic parameters*

For glucose tolerance test (GTT), mice were fasted overnight and injected intraperitoneally with glucose (1 g/kg of body weight). Tail vein blood was collected at 0, 30, 60, 120 min post-injection and tested for glucose with a glucometer (LifeScan, Milpitas, CA). For fasting insulin, tail blood was collected after a 4-hour fast. Serum insulin was measured using a mouse ultrasensitive insulin ELISA (ALPCO, Salem, NH).

*3.2.4 Measurement of energy expenditure*

Mice were habituated in the metabolic cages overnight, one week prior to metabolic testing, in order to reduce stress levels which can affect oxygen consumption. Food intake, activity, volume oxygen consumption ($VO_2$), and volume carbon dioxide ($VCO_2$) measurements were obtained over a 24-hour period using the Panlab Oxylet*Pro*™ System (Barcelona, Spain). $VO_2$ measurements were used to calculate the respiratory quotient (RQ), from which preferred substrate utilization was estimated using the Lusk equation. The Lusk equation allows us to calculate fat and carbohydrate oxidation from $VO_2$ consumed and heat produced[141]. Data were analyzed using the Panlab METABOLISM software.

*3.2.5 Immunohistochemistry and islet size determination*

Mice were anesthetized with Avertin and perfused with 4% paraformaldehyde. Pancreases were excised, embedded in paraffin, and sectioned at 5 μm thickness using a microtome (Leica; Wetzlar, Germany). Sections were antigen retrieved by boiling in citrate buffer, as described in chapter 2. M.O.M. basic kit (Vector Labs, Burlingame, CA) was used to stain for insulin according to manufacturer's instructions. Endogenous peroxide activity was blocked using 3% $H_2O_2$ in water for 5 minutes. Vectastain Elite ABC kit (Vector Labs) and DAB substrate kit (Vector Labs) were used for detection according to manufacturer's instructions. Hematoxylin was used as a counterstain to visualize nuclei. Anti-insulin was purchased from Millipore (Billerica, MA). To determine average islet size, images were taken using the 10x objective. Islet size was measured for 6-8 islets per mouse using ImageJ software.

### *3.2.6 Western Blot*

Mouse whole brain was removed and incubated in ice-cold 0.32 M sucrose solution for 5 min before placing in a mouse brain matrix with 1-mm slice intervals, ventral side facing up. Using a razor blade, a 2-mm slice was cut starting at the optic chiasm. The section was placed on a filter paper and the hypothalamus was extracted using a scalpel under a dissecting scope. Tissues were re-suspended in Sigma CelLytic™ MT (St. Louis, MO) with 1% Protease Inhibitor Cocktail III (Calbiochem; San Diego, CA) and disrupted with a sonicator using 3X 10 second pulses over ice. Protein concentration, SDS-PAGE, and Western Blot were performed as described in Chapter 2.

### *3.2.7 Statistical analyses*

For castration studies, Dunnett's multiple comparison was used to compare all groups to WT control following ANOVA. All other statistical analyses were performed as described in Chapter 2.

## 3.3 Results

### *3.3.1 Female Scly$^{-/-}$ mice have increased adiposity but fasting insulin and glucose tolerance remains intact*

It was previously reported that male Scly$^{-/-}$ mice develop a metabolic syndrome-like phenotype that is exacerbated when Se supply is limited[38]. Female Scly$^{-/-}$ mice were not included in the initial study because their phenotype appeared to be milder. In the present study, we fed a low Se diet to female WT and Scly$^{-/-}$ mice and tested the effects of Se deficiency on their metabolic phenotype. At 8 weeks of age, the body weights of female Scly$^{-/-}$ mice were not distinct from their WT counter parts. A time course assessment of body weight revealed a main effect of genotype, time, and interaction. *Post hoc* analysis showed that by 12 weeks of age, female Scly$^{-/-}$ mice were significantly heavier than their WT counterparts, a trend that persisted at least up to 20 weeks of age (Figure 7A,B). This time point was chosen because symptoms of metabolic syndrome in male Scly$^{-/-}$ begin to appear by 20 weeks of age. Female Scly$^{-/-}$ also had a significantly higher percentage of gonadal fat deposition (Figure 7C). We administered an intravenous glucose tolerance test, but found no differences in glucose clearance (Figure 7D,E), further confirming female Scly$^{-/-}$ are insulin sensitive. In contrast to what was previously observed in male Scly$^{-/-}$ mice[38], fasting insulin levels were similar between both genotypes in female mice (Figure 7F). Taken together, our results demonstrate that similar to their male counterparts, a low Se diet in female Scly$^{-/-}$ mice results in increased adiposity, but unlike male mice, female Scly$^{-/-}$ mice do not develop hyperinsulinemia or reduced glucose tolerance.

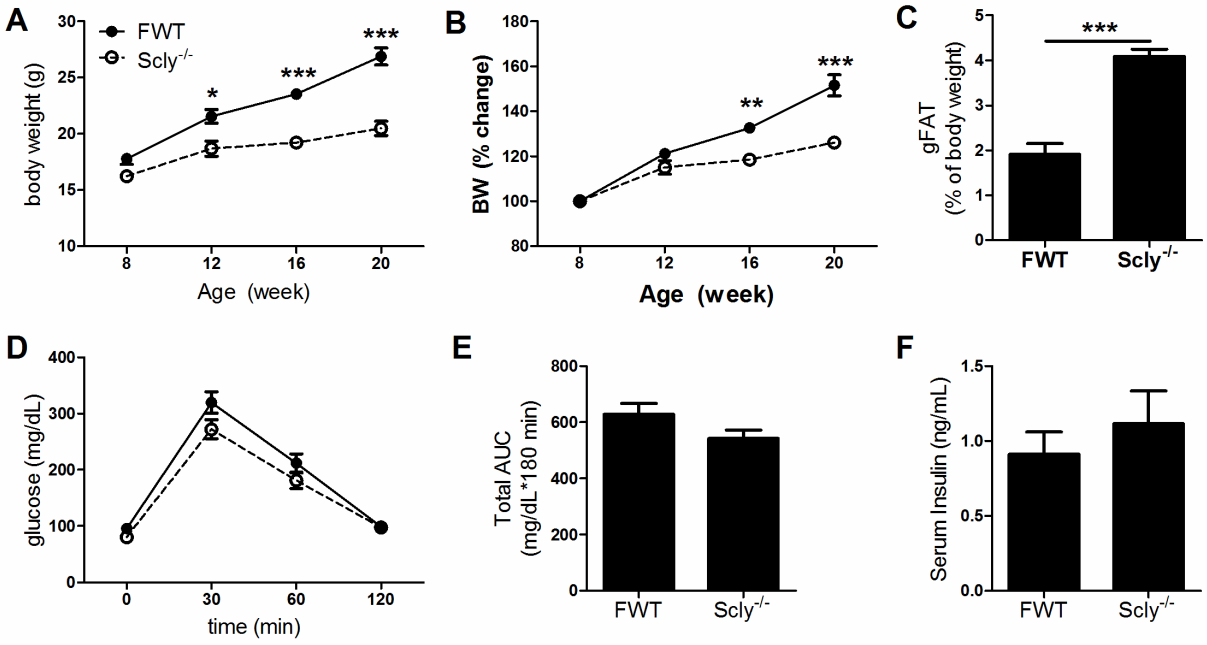


**Figure 7. Metabolic characterization of female Scly-/- mice on a Se deficient diet.** For metabolic characterization, WT: n=4, Scly$^{-/-}$: n=7. Unless otherwise indicated, mice were 22-weeks-old. (A) Mean body weight of female WT and Scly$^{-/-}$ mice at the indicated time points (Two-way ANOVA: time $F_{3,27}$=99.33 $p$<0.0001, genotype $F_{1,27}$=25.38 $p$=0.0007, interaction $F_{3,27}$=13.87 $p$<0.0001), (B) body weight displayed as % change (Two-way ANOVA: time $F_{3,27}$=83.62 $p$<0.0001, genotype $F_{1,27}$=19.88 $p$=0.0016, interaction $F_{3,27}$=9.60 $p$=0.0002), (C) mean % gWAT, (D) mean glucose tolerance test in 20-week-old mice, (E) glucose tolerance area under the curve (AUC), (F) mean fasting serum insulin. All data are represented as means ± S.E.M.

*3.3.2 Castration does not rescue the metabolic phenotype of male Scly$^{-/-}$ mice, but restores fasting insulin levels, while testosterone replacement had no effect*

To determine whether the male reproductive system accounts for the adverse effects on energy metabolism in male Scly$^{-/-}$ mice, we investigated the effects of castration in a long-term study. Scly$^{-/-}$ mice were castrated at 5-weeks-old and were monitored for 4 months. This time point was chosen because we previously reported that Scly$^{-/-}$ mice on a Se adequate diet become significantly obese at this age[38]. If castration rescues energy metabolism dysfunction in Scly$^{-/-}$ mice, there are two possible explanations. The first of these is that removal of the testes results in a diminished demand of Se for reproduction. However, since testes are the main site of TST production, castration results in low TST levels. Thus, to test this possible effect TST, we set up 4 experimental groups consisting of WT sham-operated, Scly$^{-/-}$ sham-operated, Scly$^{-/-}$

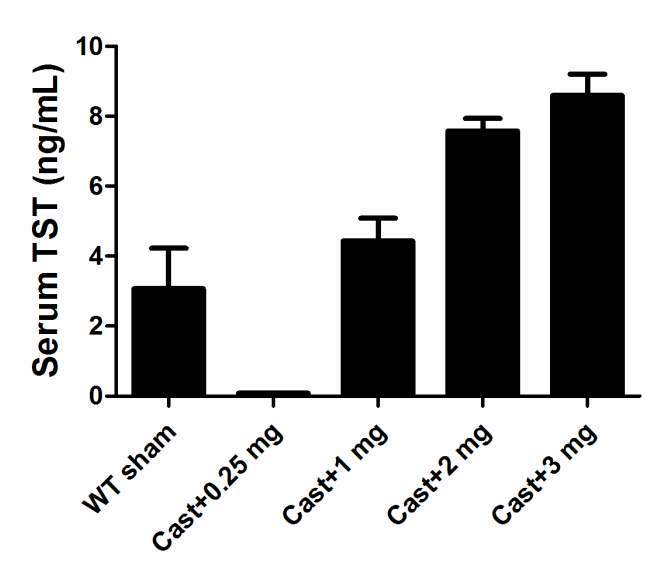


**Figure 8. TST dose-response curve to determine the appropriate dose for TST.**
5-week-old mice were castrated and implanted with the indicated dose of TST pellet. Serum TST was measured one month after implantation. Implantation with 1 mg pellet restored TST to WT levels. For WT sham n=6, for castrated groups n=2-3. All data are represented as means ± S.E.M.

castrated+placebo, and Scly$^{-/-}$ castrated+TST replacement. A dose response curve was used to determine the appropriate TST dose (Figure 8). The 1 mg dose was chosen because it restored TST levels to similar levels as WT mice.

In our first cohort of mice, we observed that in TST implanted mice, hair growth was not restored at the site of TST implantation for the full length of study. In our second cohort, we observed hair growth and less aggressive behavior approximately 30 days after TST implantation, prompting us to test our pellets. We suspected we were sent 30-day release tablets rather than 60-day tablets. Our results show that 15 days after implantation, serum TST levels were approximately 5-6 ng/ml (Table 3). At 30 days, TST levels are halved. By 45 days after implantation, TST levels were indistinguishable from castrated mice. These data are summarized in Table 3. Thus, we proceeded with our study with the following groups: WT sham, Scly$^{-/-}$ sham, and Scly$^{-/-}$ castrated.

**Table 3. TST levels drop to castrated levels 45 days after pellet insertion.** Serum TST (ng/ml) levels at the indicated days post-implantation of TST pellet. Values represent serum TST for a single animal, and values in the third column represent biological replicates.

| | Castrated+placebo | Castrated+1 mg TST | |
|---|---|---|---|
| 15 days | 0.005 | 5.226 | 6.177 |
| 30 days | 0.024 | 3.342 | 3.092 |
| 45 days | 0.038 | 1.469 | 0.069 |

Six weeks post-surgery, Scly$^{-/-}$ sham-operated mice were heavier than WT sham-operated mice, whereas Scly$^{-/-}$ castrated mice weighed less than WT sham-operated mice. This trend continued at 10 weeks, persisting until at least 15 weeks after surgery (Figure 9A). We predicted that if testes played an adverse effect, body weights of castrated mice would be restored to WT. Bonferroni's post-test following ANOVA revealed that castrated Scly$^{-/-}$ mice indeed weighed less than their sham operated counterparts at 10 weeks ($p$<0.001) and 15 weeks ($p$<0.001) post-surgery. However, castrated mice weighed significantly less than WT mice at both 10 weeks ($p$<0.05) and 15 weeks ($p$<0.05) post-surgery. Despite the differences in body weight, both sham-operated and castrated Scly$^{-/-}$ mice had significantly higher inguinal white adipose tissue (WAT) composition when compared to WT sham mice using Dunnett's multiple comparison (Figure 9B). Additionally, food intake was lower than WT in Scly$^{-/-}$ sham mice and Scly$^{-/-}$ castrated mice (Figure 9C).

Indirect calorimetry revealed significant differences in $VO_2$ when comparing WT to Scly$^{-/-}$ sham and a similar trend when comparing WT to castrated mice, although values did not reach significance (Figure 9D,E). Both groups of Scly$^{-/-}$ mice had decreased energy expenditure (Figure 9F,G) and RQ (Figure 9H,I) when compared to WT. WT sham mice preferentially burned carbohydrates compared to Scly$^{-/-}$ sham and castrated mice (Table 4).

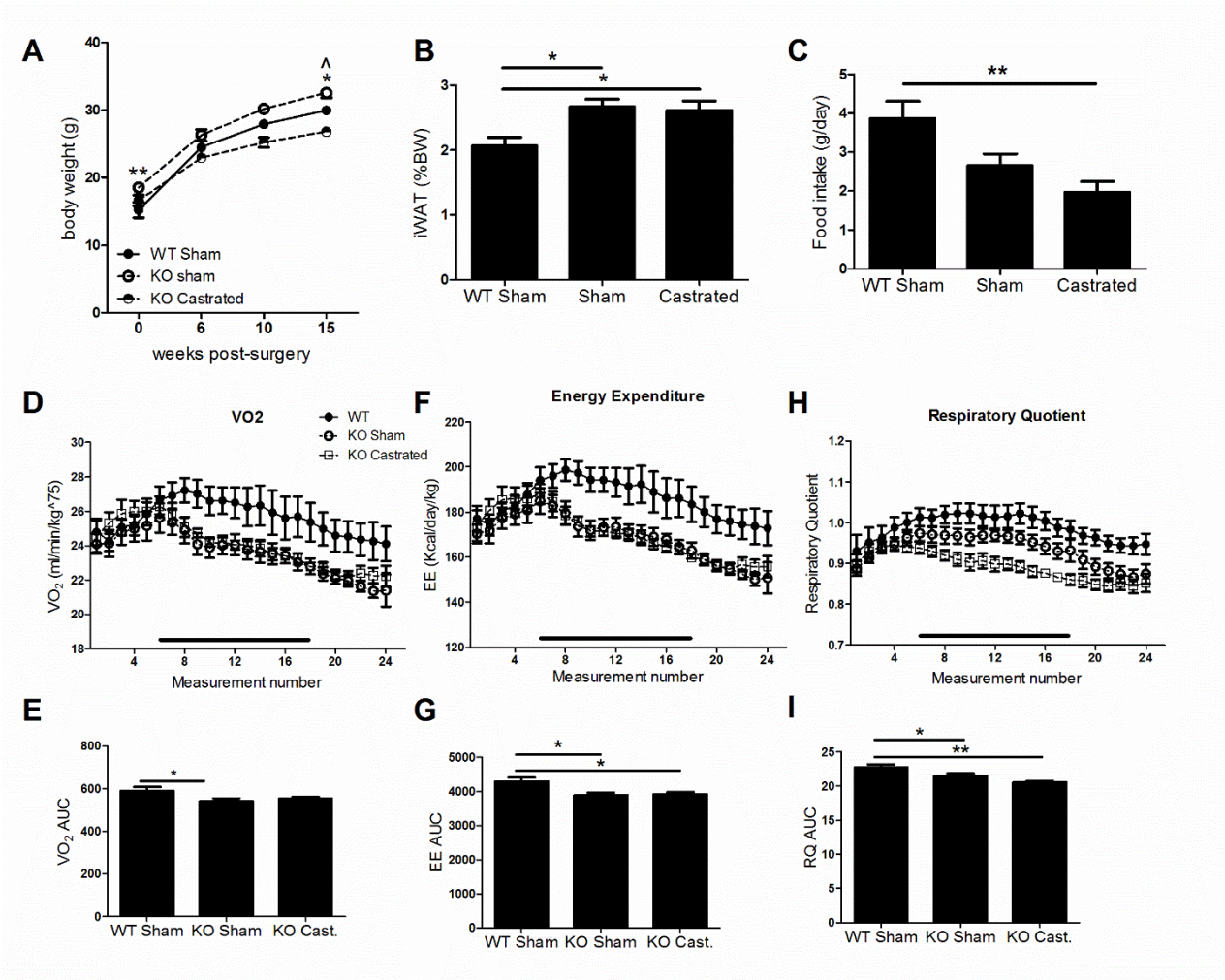


**Figure 9. Bodyweight, adiposity, food intake, and energy expenditure in response to castration.** Unless indicated, all metabolic parameters were measured in 5-month-old mice. For WT sham-operated mice: n=6, Scly$^{-/-}$ sham-operated mice: n=8, Scly$^{-/-}$ castrated mice: n=6. (A) Mean body weight at the indicated time points after surgery. One-way ANOVA was used to test each time point (6 weeks: $F_{2,17}$=5.88, p=0.01; 10 weeks: $F_{2,17}$=14.0, $p$=0.0003; 15 weeks: $F_{2,17}$=18.66, $p$<0.0001) and Dunnett's post-test was used to compare all groups to WT (WT vs. sham: ^$p$<0.05, WT vs. castrated: *$p$<0.05), (B) mean iWAT depots displayed as % of bodyweight (One-way ANOVA: $F_{2,17}$=5.94, $p$=0.01), (C) food intake over 24 hours (One-way ANOVA: $F_{2,15}$=7.29, $p$=0.006), (D) $VO_2$ profile over 24 hours. One measurement was taken every hour, and each point represents an average of n=6 for WT and castrated mice, and n=8 for sham-operated mice. The black bar corresponds to measurements taken during the dark cycle. (E) Area under the curve for $VO_2$ (One-way ANOVA: $F_{2,14}$=3.48, $p$=0.06) (F) energy expenditure over 24 hours, (G) area under the curve for energy expenditure (One-way ANOVA: $F_{2,14}$=5.76, $p$=0.01), (H) RQ over 24 hours, (I) area under the curve for RQ (One-way ANOVA: $F_{2,14}$=8.81, $p$=0.003). For all measurements taken using metabolic cages, Dunnett's post-test was used to compare all columns to WT (*$p$<0.05, **$p$<0.01, ***$p$<0.001). All data are represented as means ± S.E.M.

**Table 4. Preferred substrate utilization of WT sham-operated vs. Scly-/- sham-operated and castrated mice.** Respiratory quotient values, % carbohydrate and fat burned over 24 h. (1-WA: $F_{2,15}$=10.25, $p$=0.0016, Dunnett's post-test comparing all columns to WT: *$p$<0.05, **$p$<0.01).

| Group | RQ (average±S.E.M.) | Carbohydrate % | Fat % |
|---|---|---|---|
| WT sham | 0.98±0.04 | 93.2 | 6.83 |
| $Scly^{-/-}$ sham | 0.93±0.04* | 76.1 | 23.9 |
| $Scly^{-/-}$ castrated | 0.89±0.01** | 62.5 | 37.5 |

In contrast to previous studies showing differences in glucose tolerance between WT and $Scly^{-/-}$mice on an adequate Se diet[38], no differences were observed in this study in glucose tolerance between $Scly^{-/-}$ and WT mice (Figure 10A,B). Nevertheless, fasting serum insulin levels were significantly different among the groups (Figure 10C). Interestingly, Bonferroni's multiple comparison for all conditions revealed significant differences between $Scly^{-/-}$ castrated and $Scly^{-/-}$ sham-operated mice.

To better understand the discrepancies in GTT with previous studies, we measured fasting serum insulin levels over time, comparing $Scly^{-/-}$ to WT mice. This time course study revealed a decrease in serum insulin levels in WT mice between 8 weeks and 12 weeks of age whereas fasting serum insulin levels in $Scly^{-/-}$ mice remains relatively unchanged. This caused significant differences in fasting insulin between WT and $Scly^{-/-}$ mice at 12 weeks of age ($p$<0.05), which persists at 16 weeks of age ($p$<0.001) (Figure 11). However, these differences are abolished by week 20 due to an increase in fasting serum insulin levels in WT mice. Thus, the similar rate of glucose tolerance between WT sham-operated and $Scly^{-/-}$ mice may partially be accounted for by their fasting serum insulin levels.

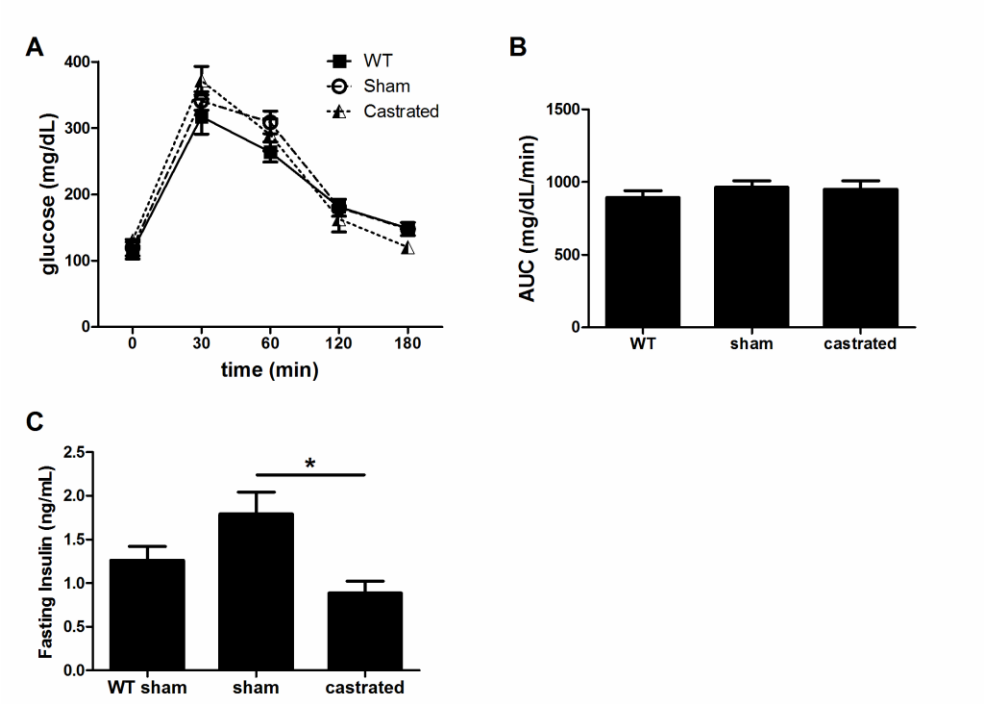


**Figure 10. Effects of castration on parameters of insulin resistance.** All parameters were measured using 5-month-old mice. For WT sham mice: n=6, Scly$^{-/-}$ sham mice: n=8, Scly$^{-/-}$ castrated mice: n=6. (A) GTT, (B) GTT AUC, (C) serum insulin levels after a 4 hour fast (One-way ANOVA: $F_{2,17}$=5.14, $p$=0.01).

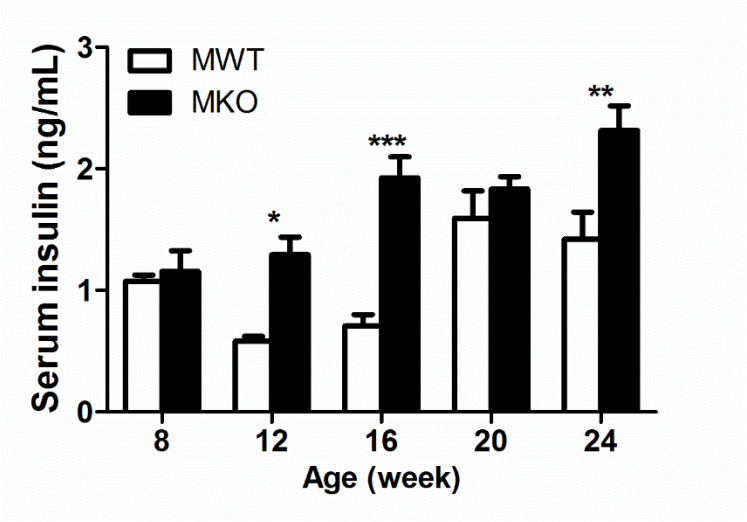


**Figure 11. Time course of fasting insulin secretion in male WT vs. Scly$^{-/-}$.** Insulin secretion after a 4 hour fast at the indicated time points. Using repeated measures two-way ANOVA, there was a main effect for time ($F_{4,28}$=18.45, $p$<0.0001), genotype ($F_{1,28}$=16.03, $p$=0.0052), and interaction ($F_{4,28}$=6.51 $p$=0.0008). Bonferroni's correction revealed significant differences at 12, 16, and 24 weeks (*$p$<0.05, **$p$<0.01, ***$p$<0.001). For WT: n=4, KO: n=6. All data are represented as means ± S.E.M.

*3.3.3 Effects of Castration on islet morphology*

Next, we used immunohistochemistry to stain for insulin-positive cells in order to analyze islet morphology. Scly$^{-/-}$ castrated mice appeared to have smaller islets than WT sham and Scly$^{-/-}$ sham-operated mice (Figure 12 & Figure 13). Islets from castrated Scly$^{-/-}$ mice also appeared to have acinar cell infiltration, which is indicative of β-cell failure[142]. Thus, the decrease in fasting serum insulin was likely due to a reduction in islet mass and density.

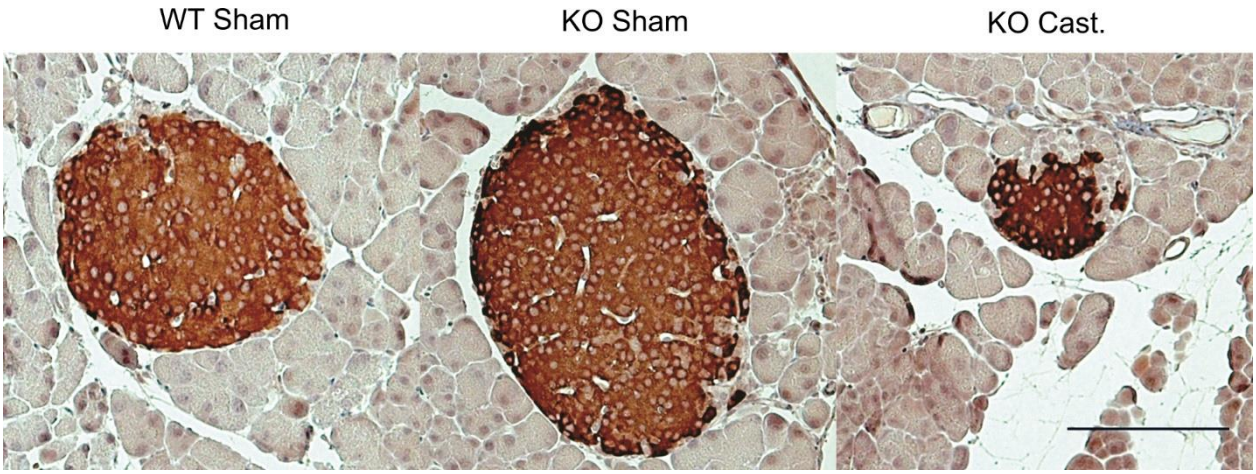


**Figure 12. Pancreatic islets are smaller in Scly-/- castrated than Scly-/- sham-operated or WT sham-operated mice.** Pancreatic sections from 5-month-old WT sham, Scly$^{-/-}$ sham, and Scly$^{-/-}$ castrated mice were labeled with anti-insulin and detected with DAB. Scale bar represents 50 μm. Images are representative of n=1-2.

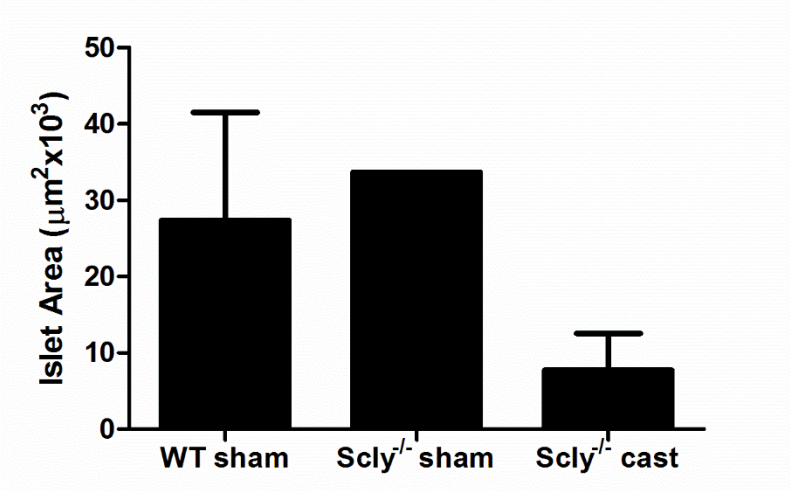


**Figure 13. Quantification of mean islet area in response to castration.** Islet size was measured and averaged for 6-8 islets per mouse, n=1-2.

*3.3.4 Hypothalamic selenoprotein expression decreases in both male and female Scly$^{-/-}$ mice, even under Se adequate conditions*

Recently, it was reported that loss of selenoproteins in the hypothalamus through a conditional knockout of Sec-tRNA$^{[Ser]Sec}$ resulted in T2D in mice[139]. Thus, we measured selenoprotein levels via Western Blotting in the hypothalamus of male and female Scly$^{-/-}$ mice under Se adequate conditions. In males, Scly$^{-/-}$ GPX1 levels were reduced to 29%±0.03 of the levels found in WT hypothalamus, SELENOM was reduced to 46%±0.05, and SELENOS was reduced to 71%±0.18 (**Error! Reference source not found.**A). In female mice, GPX1 expression was at 44%±0.04, SELENOM levels at 24%±0.07, and SELENOS at 72%±0.07 of WT hypothalamus levels. On the other hand, expression of housekeeping selenoproteins SEPHS2 and TXNRD1 were unchanged (**Error! Reference source not found.**B).

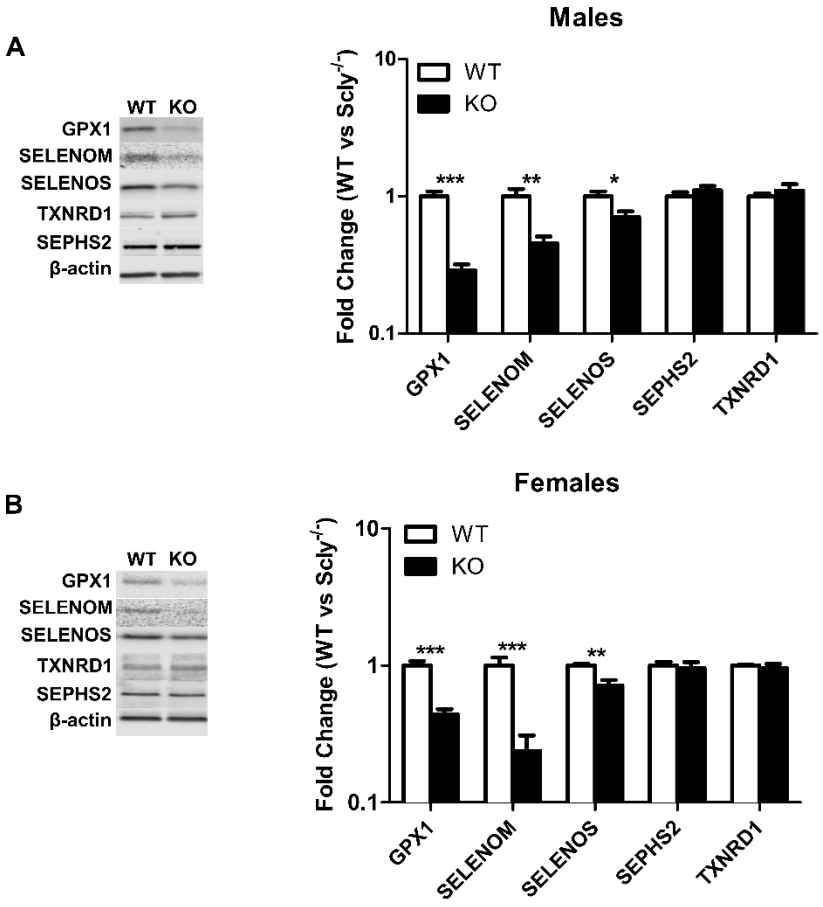


**Figure 14. Hypothalamic selenoprotein expression in WT vs. Scly$^{-/-}$ mice.**
(A) Hypothalamic selenoprotein expression in male WT vs. male Scly$^{-/-}$ mice, n=6 and (B) female WT vs. female Scly$^{-/-}$ mice, n=5. B-actin was used as a loading control. WT protein expression was normalized to 1. Unpaired, two-tailed t-test was used to compare WT vs. Scly$^{-/-}$ for each protein (*$p$<0.05, **$p$<0.01, ***$p$<0.001). All data are represented as means ± S.E.M.

### 3.4 Discussion

Sex differences in Se and incidence of T2D in humans have been previously noted in a number of studies in which increased Se intake was correlated with susceptibility to T2D in men but not women[104]. However, the mechanism behind the sex differences in the relationship between Se and energy metabolism is unclear. We previously observed that female Scly$^{-/-}$ mice do not develop a metabolic phenotype to the same extent as their male counterparts. Thus, we wondered whether the sexually dimorphic outcome of Se in T2D could be attributed to sex-specific regulation of Scly. In order to answer this question, we first characterized female mice. Our results revealed that in accordance with our previous observations, unlike their male counterparts, female Scly$^{-/-}$ mice have normal glucose tolerance, insulin signaling, and fasting insulin levels (Figure 7D-G). To our surprise, female Scly$^{-/-}$ mice developed higher body fat composition than WT mice (Figure 7C). The protective effects of estrogen against insulin resistance have been well documented[143]. For instance, pre-menopausal women are protected from the impacts of a high fat diet on insulin resistance when compared to age-matched men, but these effects are abolished in menopausal women[144]. Additionally, estrogen replacement has been shown to directly improve insulin sensitivity[145]. Thus, we suspect that the presence of estrogen may have masked the effects of increased adiposity on insulin resistance in female Scly$^{-/-}$ mice.

Scly was previously found to be expressed in spermatocytes and the Leydig cells of the testes[117]. In addition, castration of Scly$^{-/-}$SELENOP$^{-/-}$ mice attenuated their severe neurological phenotype, suggesting Se distribution to the testes is prioritized[146]. Together, these results reinforce the importance of Se delivery and recycling to testes function and male fertility. Thus, the possibility that the Se demands of the testes produces adverse effects on the function of

metabolic tissues in Scly$^{-/-}$ mice could not be excluded. We therefore castrated Scly$^{-/-}$ mice to evaluate whether testes removal could rescue the metabolic dysfunction in Scly$^{-/-}$ mice. Without the testes, perhaps circulating Se would be more readily available for distribution to metabolic tissues. An unanticipated result of the castration was that the body weights of castrated Scly$^{-/-}$ mice were lower than WT mice (Figure 9A). TST deficiency in men leads to loss of lean body mass[147], and a similar phenomenon in mice may have been a confounding factor in our studies.

Body fat composition was higher in Scly$^{-/-}$ mice than in WT, and indistinguishable between sham-operated and castrated Scly$^{-/-}$ mice, despite the lower body weight of the castrated mice (Figure 9B). Interestingly, food intake was lower in both Scly$^{-/-}$ groups compared to WT mice (Figure 9C). As an adipokine, leptin levels rise with increasing fat accumulation, regulating hunger in response to fat stores. We previously reported that Scly$^{-/-}$ mice indeed have increased circulating leptin[38], and thus we speculate that the decreased food intake is a direct result of increased leptin in Scly$^{-/-}$ mice.

To our surprise, we did not observe changes in GTT between WT sham-operated and Scly$^{-/-}$ sham-operated mice (Figure 10A,B), as previously reported[38]. One disadvantage of the GTT test at a single time point is that we are only able to obtain a snapshot of glucose tolerance. As age is a known causative factor in obesity and insulin resistance development[148], it is reasonable to consider that by 20 weeks of age, WT mice develop age-dependent insulin resistance. To address this, we conducted a time course study, revealing that at 20 weeks of age, fasting serum insulin in WT and Scly$^{-/-}$ mice are not significantly different (Figure 11). Although the insulin levels may suggest similar levels of insulin resistance between WT and Scly$^{-/-}$ sham mice, insulin resistance can only be confirmed using glucose clamps. Nevertheless, the insulin levels at 20-weeks-old may partially account for the lack of difference observed in

GTT. One interesting finding was that although male WT and Scly$^{-/-}$ mice had similar fasting insulin levels at 8-weeks-old, there was an inexplicable drop in serum fasting insulin levels at 12-weeks-old in WT mice while Scly$^{-/-}$ insulin levels were maintained (Figure 11). This raises the question of whether Scly$^{-/-}$ mice develop hyperinsulinemia or fail to experience a drop in insulin levels. Further investigation is necessary to determine whether a significant decrease in fasting insulin levels after 8 weeks of age is a typical event in WT mice.

We found castration to significantly reduce fasting serum insulin levels (Figure 10C). Assessment of islet morphology revealed islets from Scly$^{-/-}$ castrated mice to be smaller than from Scly$^{-/-}$ sham-operated and WT sham-operated mice (Figure 12 & Figure 13). The smaller β-cell mass in Scly$^{-/-}$ castrated mice likely accounts for the lower fasting serum insulin levels, which may be the outcome of either of two possible explanations. On one hand, it is possible that castration rescued insulin sensitivity and β-cell function, eliminating the need for hyperplasia as a compensatory mechanism, resulting in what appears to be smaller β-cell mass. If this were the case, we would likely see improved body fat composition, energy expenditure, glucose tolerance, and fasting blood glucose levels, in Scly$^{-/-}$ castrated mice; however, none of these were observed. Thus, it is more likely that altered insulin secretion in castrated Scly$^{-/-}$ mice is due to β-cell decompensation, which is characterized by a reduction of β-cell mass[149].
If this is the case, we should see a reduction in markers of β-cell identity such as Pdx1 and musculoaponeurotic fibrosarcoma oncogene family A (MafA). Androgen deficiency in men has been known to have detrimental effects on obesity and insulin sensitivity[150]. More recently, it was found that TST has direct effects in maintaining β-cell function in male mice[151]. Hence, Scly$^{-/-}$ castrated mice are likely experiencing a poorer outcome in terms of β-cell health as a consequence of TST deficiency.

Our finding that body fat composition is higher than WT in Scly$^{-/-}$ mice regardless of sex (Figure 7C & Figure 9B), points to a likely involvement of Scly in lipogenesis and/or lipolysis. However, we previously reported Scly was expressed minimally in the WAT when compared to the liver, while another study which measured Scly activity in rat tissues failed to detect Scly activity in the WAT[31,38], indicating that the regulatory action of fat stores through Scly is likely indirect. We demonstrate that even on a Se adequate diet, expression of selenoproteins was diminished in the hypothalamus when Scly$^{-/-}$ was absent (Figure 14). This is in contrast to a previous study from our laboratory which found selenoprotein mRNA from whole brain to be unchanged in Scly$^{-/-}$ mice[152], suggesting that Scly may play a more prominent role in selenoprotein biosynthesis in certain brain regions than others.

Crosstalk between the hypothalamus and WAT is well documented, and mostly attributed to the adipokine leptin[153]. Recently, it was found that direct sympathetic efferent contact with the WAT is critical to the action of leptin on lipolysis via the hypothalamus[154]. Thus, it is tempting to speculate that Scly influences WAT function by regulating selenoprotein expression in the hypothalamus. In support of this, a recent study found that deletion of Sec-tRNA$^{[Ser]Sec}$ from the hypothalamus results in increased adiposity and insulin resistance[139]. The effects were found to be a direct result of increased oxidative stress due to the absence of Sec-tRNA$^{[Ser]Sec}$.

We previously showed that SELENOM knockout mice develop obesity and decreased leptin signaling[79]. Interestingly, SELENOM was one of the selenoproteins which we found to be downregulated in the hypothalamus of Scly$^{-/-}$ mice. The reduction in hypothalamic selenoprotein expression was observed at 10-weeks-old of age, prior to significant changes in energy metabolism. Thus, diminished selenoprotein expression is likely a contributing factor to

alterations in energy metabolism, rather than a consequence. This invites speculation that Scly may protect against alterations in lipid metabolism, through the regulation of select selenoproteins.

Taken together, our results suggest a novel role for Scly in regulating energy metabolism through the hypothalamus. Scly appears to be critical for efficient hypothalamic selenoprotein biosynthesis, particularly for the Se-responsive selenoproteins. An interesting avenue for further investigation would be to determine whether hypothalamic Scly protects against high fat diet and whole-body insulin resistance.

# CHAPTER 4. CONCLUSION

## 4.1 Final Remarks

Until the generation of Scly$^{-/-}$ mice, much of the pioneering work involving Scly function was accomplished through *in vitro* methods[31]. Scly is known to be involved in selenoprotein biosynthesis and Se recycling[117], but its physiological role has only recently begun to be appreciated. It is proposed that Scly coordinates with SELENOP to supply Se to tissues such as the brain and testes[117]. Yet, we found that in contrast with SELENOP$^{-/-}$ mice, Scly$^{-/-}$ mice fail to develop a neurological phenotype, nor do they exhibit impairments in fertility[152]. Under a Se deficient diet, Scly$^{-/-}$ mice develop mild deficits in spatial learning accompanied by a reduction in brain GPX activity as well as GPX1 and GPX4 expression, indicating a role for Scly in regulating brain selenoprotein expression when exogenous Se supply is low, and it is likely due to its Se recycling ability. In a follow up study, we found that Scly$^{-/-}$ mice develop a metabolic syndrome-like phenotype in a sex-dependent fashion, uncovering a relationship between Scly and pathways involving energy metabolism[38].

The current project advances our understanding of Scly in energy metabolism. One of the most striking features of the Scly$^{-/-}$ mice, which paved the way for the current project, is its development of severe hyperinsulinemia[38]. This is particularly interesting to us because of previous reports which suggest that selenoproteins are critical in regulating pancreatic islet function[60,86,111]. Therefore, we pursued the investigation of Scly function in the pancreatic islet. While changes were not observed in GSIS, our results are nonetheless exciting, as to the best of our knowledge, they were the first to describe Scly expression in the pancreatic islet. Because we did not find islet selenoprotein expression to be influenced by Scly on a Se sufficient diet, we considered the possibility that Scly may be regulating insulin secretion and/or

islet function in an enzyme-activity independent manner. For instance, the ability of sulfur pathways to compensate for Sec accumulation may influence insulin secretion as an off-target effect. Our characterization of female mice revealed that female Scly$^{-/-}$ mice also develop higher body fat composition, but were likely protected from insulin resistance due to the presence of estrogen. Impaired hypothalamic selenoprotein biosynthesis in both male and female Scly$^{-/-}$ mice paves the way for a previously unexplored role for Scly in the brain-fat axis.

Finally, an important topic for discussion is the translational relevance of Scly$^{-/-}$ mice to human studies. Presently, literature citing Scly in human metabolic diseases is scarce, with only one study identifying an association between human Scly polymorphisms and alterations in lipid pathways[41]. Nevertheless, further investigation of Scly in T2D and metabolic syndrome should be pursued to better understand human health. An interesting consideration is the relationship between supra-nutritional Se intake, Scly, and T2D. Human clinical trials have uncovered a positive correlation between Se intake and increased incidence of T2D when baseline Se levels are already at the upper tolerable limit[51]. Given that Scly is inversely regulated by increasing Se levels *in vitro* (Figure 4A), it is conceivable that human Scly activity may also be impacted by fluctuating serum Se concentrations. As modern technology has improved food import and export, human health concerns due to Se deficiency have mostly been eradicated. Despite receiving an adequate supply, at least 18-19% of adults reported taking Se supplements[155]. The potential threat of T2D with supra-nutritional Se intake is alarming, and further understanding of Se metabolism is necessary in preventing unwanted effects on energy metabolism.

### 4.2 Future Directions

An intriguing finding in our immunofluorescence images of pancreatic islets was an apparent association of Scly with the nucleus in contrast to it being found solely in the cytosolic fraction when nuclear and cytosolic fractions were separated. As the majority of Scly substrates are in the cytosol, a predominant cytosolic localization would have been expected. However, as other components of selenoprotein biosynthesis have been found to participate in nucleocytoplasmic shuttling[119,156], it is possible that Scly is also involved in this process. Scly is not known to contain a nuclear localization or nuclear export signal[117], and therefore nuclear import would likely involve association with a transport protein. Alternatively, Scly is associated with the cytoplasmic side of the nuclear membrane. Future studies will attempt high-resolution separation of immunostaining within the nucleus as opposed to the nuclear membrane. If targeted to the nuclei, future experiments could focus on the identification of potential interactor protein(s) that allows Scly to enter the nucleus. In the context of metabolic diseases, it would be valuable to determine if conditions such as glucotoxicity or lipotoxicity influence the subcellular localization of Scly.

While selenoprotein biosynthesis is well-studied, much less is known about Sec degradation. Scly is unique in that it has evolved to specifically accept Sec as a substrate in vertebrates, despite the ability of sulfur pathway enzymes to participate in Se metabolism[32]. This raises speculation that efficient Sec decomposition is beneficial, but more in-depth studies involving Scly are necessary to fully understand the advantage of a system which recognizes Sec with such high specificity. While we assessed gene expression of sulfur pathway enzymes in the present study, measuring protein and activity levels would provide a more accurate representation of how the sulfur pathways are affected by Scly deletion and how they might

compensate to handle an expected increase in Sec level in the absence of Scly.

Thus far, the physiological function of Scly has been attributed to its ability to generate selenide. Nevertheless, we observe metabolic dysfunction in $Scly^{-/-}$ mice despite selenoprotein biosynthesis remaining intact in most tissues. An important consideration is that Scly regulation of energy metabolism may occur independent of its enzymatic activity. One way to test this would be to inactivate the Sec decomposing capacity of Scly by introducing a mutation in its active site. If Scly enzymatic activity is critical in its regulation of energy metabolism, we expect a similar outcome to Scly deletion. In contrast, if Scly enzymatic activity is negligible, mutation of the active site will likely not impact energy metabolism.

We have also provided insight to the potential role of Scly in regulating energy metabolism through the hypothalamus, suggesting Scly may regulate the brain-fat axis. Possibly, in the absence of Scly, inefficient selenoprotein biosynthesis in the hypothalamus drives WAT accumulation. One explanation is that decreased selenoprotein expression could lead to increased oxidative stress and/or neurodegeneration in the hypothalamus, leading to detrimental consequences in lipolysis and/or lipogenesis in the periphery. Conditional knockout studies involving Scly deletion from the hypothalamus would clarify the role of Scly in the brain-fat axis. Moreover, it would be interesting to restore Scly expression in the hypothalamus to determine if WAT lipogenic/lipolytic pathways are rescued, which could be determined by measuring body fat composition and lipid pathway enzymes such as hormone sensitive lipase, ACC1, lipoprotein lipase, and peroxisome proliferator-activated receptor. Overall, these studies will begin to uncover a mechanistic role for Scly in the hypothalamic regulation of energy metabolism.

REFERENCES


1. Herrera, B. M. & Lindgren, C. M. The Genetics of Obesity. *Curr. Diab. Rep.* **10,** 498–505 (2010).
2. Bergman, R. N., Phillips, L. S. & Cobelli, C. Physiologic evaluation of factors controlling glucose tolerance in man: measurement of insulin sensitivity and beta-cell glucose sensitivity from the response to intravenous glucose. *J. Clin. Invest.* **68,** 1456–1467 (1981).
3. Muscogiuri, G., Salmon, A. B., Aguayo-Mazzucato, C., Li, M., Balas, B., Guardado-Mendoza, R., Giaccari, A., Reddick, R. L., Reyna, S. M., Weir, G., DeFronzo, R. A., Remmen, H. V. & Musi, N. Genetic Disruption of SOD1 Gene Causes Glucose Intolerance and Impairs β-Cell Function. *Diabetes* **62,** 4201–4207 (2013).
4. Chan, J. Y., Luzuriaga, J., Maxwell, E. L., West, P. K., Bensellam, M. & Laybutt, D. R. The balance between adaptive and apoptotic unfolded protein responses regulates β-cell death under ER stress conditions through XBP1, CHOP and JNK. *Mol. Cell. Endocrinol.* **413,** 189–201 (2015).
5. Donath, M. Y. & Shoelson, S. E. Type 2 diabetes as an inflammatory disease. *Nat. Rev. Immunol.* **11,** 98–107 (2011).
6. Poungvarin, N., Lee, J. K., Yechoor, V. K., Li, M. V., Assavapokee, T., Suksaranjit, P., Thepsongwajja, J. J., Saha, P. K., Oka, K. & Chan, L. Carbohydrate response element-binding protein (ChREBP) plays a pivotal role in beta cell glucotoxicity. *Diabetologia* **55,** 1783–1796 (2012).
7. Vasu, S., McClenaghan, N. H., McCluskey, J. T. & Flatt, P. R. Effects of lipotoxicity on a novel insulin-secreting human pancreatic β-cell line, 1.1B4. *Biol. Chem.* **394,** 909–918 (2013).
8. Marban, S. & Roth, J. in *Lessons Anim. Diabetes* **VI,** 201–224 (Birkhauser, 1996).
9. Kim, B., McLean, L. L., Philip, S. S. & Feldman, E. L. Hyperinsulinemia induces insulin resistance in dorsal root ganglion neurons. *Endocrinology* **152,** 3638–3647 (2011).
10. Baum, J., Simons, B. E., Unger, R. H. & Madison, L. L. Localization of glucagon in the alpha cells in the pancreatic islet by immunofluorescent technics. *Diabetes* **11,** 371–374 (1962).
11. Unger, R. H., Eisentraut, A. M., McCall, M. S. & Madison, L. L. Measurements of endogenous glucagon in plasma and the influence of blood glucose concentration upon its secretion. *J. Clin. Invest.* **41,** 682–9 (1962).
12. Ravnskjaer, K., Hogan, M. F., Lackey, D., Tora, L., Dent, S. Y. R., Olefsky, J. & Montminy, M. Glucagon regulates gluconeogenesis through KAT2B- and WDR5-mediated epigenetic effects. *J. Clin. Invest.* **123,** 4318–4328 (2013).
13. Hauge-Evans, A. C., King, A. J., Carmignac, D., Richardson, C. C., Robinson, I. C. A. F., Low, M. J., Christie, M. R., Persaud, S. J. & Jones, P. M. Somatostatin secreted by islet delta-cells fulfills multiple roles as a paracrine regulator of islet function. *Diabetes* **58,** 403–411 (2009).
14. Dezaki, K., Hosoda, H., Kakei, M., Hashiguchi, S., Watanabe, M., Kangawa, K. & Yada, T. Endogenous ghrelin in pancreatic islets restricts insulin release by attenuating Ca2+ signaling in beta-cells: implication in the glycemic control in rodents. *Diabetes* **53,** 3142–3151 (2004).
15. Ashcroft, F. M., Harrison, D. E. & Ashcroft, S. J. Glucose induces closure of single potassium channels in isolated rat pancreatic beta-cells. *Nature* **312,** 446–448 (1984).
16. Straub, S. G. & Sharp, G. W. G. Hypothesis: one rate-limiting step controls the magnitude of both phases of glucose-stimulated insulin secretion. *Am. J. Physiol. Cell Physiol.* **287,** C565-571 (2004).

17. Jitrapakdee, S., Vidal-Puig, A. & Wallace, J. C. Anaplerotic roles of pyruvate carboxylase in mammalian tissues. *Cell. Mol. Life Sci. CMLS* **63,** 843–854 (2006).
18. Turrens, J. F. Mitochondrial formation of reactive oxygen species. *J. Physiol.* **552,** 335–344 (2003).
19. Tiedge, M., Lortz, S., Drinkgern, J. & Lenzen, S. Relation between antioxidant enzyme gene expression and antioxidative defense status of insulin-producing cells. *Diabetes* **46,** 1733–1742 (1997).
20. Bindokas, V. P., Kuznetsov, A., Sreenan, S., Polonsky, K. S., Roe, M. W. & Philipson, L. H. Visualizing superoxide production in normal and diabetic rat islets of Langerhans. *J. Biol. Chem.* **278,** 9796–9801 (2003).
21. Leloup, C., Tourrel-Cuzin, C., Magnan, C., Karaca, M., Castel, J., Carneiro, L., Colombani, A.-L., Ktorza, A., Casteilla, L. & Pénicaud, L. Mitochondrial reactive oxygen species are obligatory signals for glucose-induced insulin secretion. *Diabetes* **58,** 673–681 (2009).
22. Nolan, C. J., Madiraju, M. S. R., Delghingaro-Augusto, V., Peyot, M.-L. & Prentki, M. Fatty Acid Signaling in the -Cell and Insulin Secretion. *Diabetes* **55,** S16–S23 (2006).
23. Newsholme, P., Cruzat, V., Arfuso, F. & Keane, K. Nutrient regulation of insulin secretion and action. *J. Endocrinol.* **221,** R105–R120 (2014).
24. Kryukov, G. V., Castellano, S., Novoselov, S. V., Lobanov, A. V., Zehtab, O., Guigó, R. & Gladyshev, V. N. Characterization of Mammalian Selenoproteomes. *Science* **300,** 1439–1443 (2003).
25. Lee, B. J., Worland, P. J., Davis, J. N., Stadtman, T. C. & Hatfield, D. L. Identification of a selenocysteyl-tRNA(Ser) in mammalian cells that recognizes the nonsense codon, UGA. *J. Biol. Chem.* **264,** 9724–9727 (1989).
26. Berry, M. J., Banu, L., Chen, Y. Y., Mandel, S. J., Kieffer, J. D., Harney, J. W. & Larsen, P. R. Recognition of UGA as a selenocysteine codon in type I deiodinase requires sequences in the 3' untranslated region. *Nature* **353,** 273–276 (1991).
27. Howard, M. T., Carlson, B. A., Anderson, C. B. & Hatfield, D. L. Translational redefinition of UGA codons is regulated by selenium availability. *J. Biol. Chem.* **288,** 19401–19413 (2013).
28. Seyedali, A. & Berry, M. J. Nonsense-mediated decay factors are involved in the regulation of selenoprotein mRNA levels during selenium deficiency. *RNA N. Y. N* **20,** 1248–1256 (2014).
29. Yant, L. J., Ran, Q., Rao, L., Van Remmen, H., Shibatani, T., Belter, J. G., Motta, L., Richardson, A. & Prolla, T. A. The selenoprotein GPX4 is essential for mouse development and protects from radiation and oxidative damage insults. *Free Radic. Biol. Med.* **34,** 496–502 (2003).
30. Jakupoglu, C., Przemeck, G. K. H., Schneider, M., Moreno, S. G., Mayr, N., Hatzopoulos, A. K., de Angelis, M. H., Wurst, W., Bornkamm, G. W., Brielmeier, M. & Conrad, M. Cytoplasmic thioredoxin reductase is essential for embryogenesis but dispensable for cardiac development. *Mol. Cell. Biol.* **25,** 1980–1988 (2005).
31. Esaki, N., Nakamura, T., Tanaka, H. & Soda, K. Selenocysteine lyase, a novel enzyme that specifically acts on selenocysteine. Mammalian distribution and purification and properties of pig liver enzyme. *J. Biol. Chem.* **257,** 4386–4391 (1982).
32. Omi, R., Kurokawa, S., Mihara, H., Hayashi, H., Goto, M., Miyahara, I., Kurihara, T., Hirotsu, K. & Esaki, N. Reaction Mechanism and Molecular Basis for Selenium/Sulfur Discrimination of Selenocysteine Lyase. *J. Biol. Chem.* **285,** 12133–12139 (2010).
33. Tobe, R., Mihara, H., Kurihara, T. & Esaki, N. Identification of proteins interacting with selenocysteine lyase. *Biosci. Biotechnol. Biochem.* **73,** 1230–1232 (2009).

34. Burk, R. F. & Hill, K. E. Regulation of Selenium Metabolism and Transport. *Annu. Rev. Nutr.* **35,** null (2015).
35. Byrns, C. N., Pitts, M. W., Gilman, C. A., Hashimoto, A. C. & Berry, M. J. Mice Lacking Selenoprotein P and Selenocysteine Lyase Exhibit Severe Neurological Dysfunction, Neurodegeneration, and Audiogenic Seizures. *J. Biol. Chem.* **289,** 9662–9674 (2014).
36. Dimkovikj, A., Fisher, B., Hutchison, K. & Van Hoewyk, D. Stuck between a ROS and a hard place: Analysis of the ubiquitin proteasome pathway in selenocysteine treated Brassica napus reveals different toxicities during selenium assimilation. *J. Plant Physiol.* **181,** 50–54 (2015).
37. Pilon, M., Owen, J. D., Garifullina, G. F., Kurihara, T., Mihara, H., Esaki, N. & Pilon-Smits, E. A. H. Enhanced selenium tolerance and accumulation in transgenic Arabidopsis expressing a mouse selenocysteine lyase. *Plant Physiol.* **131,** 1250–1257 (2003).
38. Seale, L. A., Hashimoto, A. C., Kurokawa, S., Gilman, C. L., Seyedali, A., Bellinger, F. P., Raman, A. V. & Berry, M. J. Disruption of the Selenocysteine Lyase-Mediated Selenium Recycling Pathway Leads to Metabolic Syndrome in Mice. *Mol. Cell. Biol.* **32,** 4141–4154 (2012).
39. Sors, T. G., Ellis, D. R. & Salt, D. E. Selenium uptake, translocation, assimilation and metabolic fate in plants. *Photosynth. Res.* **86,** 373–389 (2005).
40. Seale, L. A., Gilman, C. L., Hashimoto, A. C., Ogawa-Wong, A. N. & Berry, M. J. Diet-Induced Obesity in the Selenocysteine Lyase Knockout Mouse. *Antioxid. Redox Signal.* **23,** 761–774 (2015).
41. Gao, C., Hsu, F.-C., Dimitrov, L. M., Okut, H., Chen, Y.-D. I., Taylor, K. D., Rotter, J. I., Langefeld, C. D., Bowden, D. W. & Palmer, N. D. A genome-wide linkage and association analysis of imputed insertions and deletions with cardiometabolic phenotypes in Mexican Americans: The Insulin Resistance Atherosclerosis Family Study. *Genet. Epidemiol.* **41,** 353–362 (2017).
42. Ezaki, O. The insulin-like effects of selenate in rat adipocytes. *J. Biol. Chem.* **265,** 1124–1128 (1990).
43. Akbaraly, T. N., Arnaud, J., Rayman, M. P., Hininger-Favier, I., Roussel, A.-M., Berr, C. & Fontbonne, A. Plasma selenium and risk of dysglycemia in an elderly French population: results from the prospective Epidemiology of Vascular Ageing Study. *Nutr. Metab.* **7,** 21 (2010).
44. Navarro-Alarcón, M., López-G de la Serrana, H., Pérez-Valero, V. & López-Martínez, C. Serum and urine selenium concentrations as indicators of body status in patients with diabetes mellitus. *Sci. Total Environ.* **228,** 79–85 (1999).
45. Park, K., Rimm, E. B., Siscovick, D. S., Spiegelman, D., Manson, J. E., Morris, J. S., Hu, F. B. & Mozaffarian, D. Toenail selenium and incidence of type 2 diabetes in U.S. men and women. *Diabetes Care* **35,** 1544–1551 (2012).
46. Bleys, J., Navas-Acien, A. & Guallar, E. Serum Selenium and Diabetes in U.S. Adults. *Diabetes Care* **30,** 829–834 (2007).
47. Laclaustra, M., Navas-Acien, A., Stranges, S., Ordovas, J. M. & Guallar, E. Serum selenium concentrations and diabetes in U.S. adults: National Health and Nutrition Examination Survey (NHANES) 2003-2004. *Environ. Health Perspect.* **117,** 1409–1413 (2009).
48. Stranges, S., Marshall, J. R., Natarajan, R., Donahue, R. P., Trevisan, M., Combs, G. F., Cappuccio, F. P., Ceriello, A. & Reid, M. E. Effects of Long-Term Selenium Supplementation on the Incidence of Type 2 DiabetesA Randomized Trial. *Ann. Intern. Med.* **147,** 217–223 (2007).

49. Lippman, S. M., Klein, E. A., Goodman, P. J., Lucia, M. S., Thompson, I. M., Ford, L. G., Parnes, H. L., Minasian, L. M., Gaziano, J. M., Hartline, J. A., Parsons, J. K., Bearden, J. D., Crawford, E. D., Goodman, G. E., Claudio, J., Winquist, E., Cook, E. D., Karp, D. D., Walther, P., Lieber, M. M., Kristal, A. R., Darke, A. K., Arnold, K. B., Ganz, P. A., Santella, R. M., Albanes, D., Taylor, P. R., Probstfield, J. L., Jagpal, T. J., Crowley, J. J., Meyskens, F. L., Baker, L. H. & Coltman, C. A. Effect of Selenium and Vitamin E on Risk of Prostate Cancer and Other Cancers: The Selenium and Vitamin E Cancer Prevention Trial (SELECT). *JAMA* **301,** 39 (2009).
50. Klein, E. A., Thompson, I. M., Tangen, C. M., Crowley, J. J., Lucia, M. S., Goodman, P. J., Minasian, L. M., Ford, L. G., Parnes, H. L., Gaziano, J. M., Karp, D. D., Lieber, M. M., Walther, P. J., Klotz, L., Parsons, J. K., Chin, J. L., Darke, A. K., Lippman, S. M., Goodman, G. E., Meyskens, F. L. & Baker, L. H. Vitamin E and the risk of prostate cancer: the Selenium and Vitamin E Cancer Prevention Trial (SELECT). *JAMA* **306,** 1549–1556 (2011).
51. Rayman, M. P. & Stranges, S. Epidemiology of selenium and type 2 diabetes: can we make sense of it? *Free Radic. Biol. Med.* **65,** 1557–1564 (2013).
52. Rayman, M. P. Selenium and human health. *Lancet Lond. Engl.* **379,** 1256–1268 (2012).
53. Mao, J., Bath, S. C., Vanderlelie, J. J., Perkins, A. V., Redman, C. W. G. & Rayman, M. P. No effect of modest selenium supplementation on insulin resistance in UK pregnant women, as assessed by plasma adiponectin concentration. *Br. J. Nutr.* **115,** 32–38 (2016).
54. Labunskyy, V. M., Lee, B. C., Handy, D. E., Loscalzo, J., Hatfield, D. L. & Gladyshev, V. N. Both Maximal Expression of Selenoproteins and Selenoprotein Deficiency Can Promote Development of Type 2 Diabetes-Like Phenotype in Mice. *Antioxid. Redox Signal.* **14,** 2327–2336 (2011).
55. Wang, X., Zhang, W., Chen, H., Liao, N., Wang, Z., Zhang, X. & Hai, C. High selenium impairs hepatic insulin sensitivity through opposite regulation of ROS. *Toxicol. Lett.* **224,** 16–23 (2014).
56. Pinto, A., Juniper, D. T., Sanil, M., Morgan, L., Clark, L., Sies, H., Rayman, M. P. & Steinbrenner, H. Supranutritional selenium induces alterations in molecular targets related to energy metabolism in skeletal muscle and visceral adipose tissue of pigs. *J. Inorg. Biochem.* **114,** 47–54 (2012).
57. Campbell, S. C., Aldibbiat, A., Marriott, C. E., Landy, C., Ali, T., Ferris, W. F., Butler, C. S., Shaw, J. A. & Macfarlane, W. M. Selenium stimulates pancreatic beta-cell gene expression and enhances islet function. *FEBS Lett.* **582,** 2333–2337 (2008).
58. Steinbrenner, H., Hotze, A.-L., Speckmann, B., Pinto, A., Sies, H., Schott, M., Ehlers, M., Scherbaum, W. A. & Schinner, S. Localization and regulation of pancreatic selenoprotein P. *J. Mol. Endocrinol.* **50,** 31–42 (2013).
59. McClung, J. P., Roneker, C. A., Mu, W., Lisk, D. J., Langlais, P., Liu, F. & Lei, X. G. Development of insulin resistance and obesity in mice overexpressing cellular glutathione peroxidase. *Proc. Natl. Acad. Sci. U. S. A.* **101,** 8852–8857 (2004).
60. Wang, X. D., Vatamaniuk, M. Z., Wang, S. K., Roneker, C. A., Simmons, R. A. & Lei, X. G. Molecular mechanisms for hyperinsulinaemia induced by overproduction of selenium-dependent glutathione peroxidase-1 in mice. *Diabetologia* **51,** 1515–1524 (2008).
61. Loh, K., Deng, H., Fukushima, A., Cai, X., Boivin, B., Galic, S., Bruce, C., Shields, B. J., Skiba, B., Ooms, L. M., Stepto, N., Wu, B., Mitchell, C. A., Tonks, N. K., Watt, M. J., Febbraio, M. A., Crack, P. J., Andrikopoulos, S. & Tiganis, T. Reactive oxygen species enhance insulin sensitivity. *Cell Metab.* **10,** 260–272 (2009).

62. Tanaka, Y., Tran, P. O. T., Harmon, J. & Robertson, R. P. A role for glutathione peroxidase in protecting pancreatic beta cells against oxidative stress in a model of glucose toxicity. *Proc. Natl. Acad. Sci. U. S. A.* **99,** 12363–12368 (2002).
63. Merry, T. L., Tran, M., Stathopoulos, M., Wiede, F., Fam, B. C., Dodd, G. T., Clarke, I., Watt, M. J., Andrikopoulos, S. & Tiganis, T. High-fat-fed obese glutathione peroxidase 1-deficient mice exhibit defective insulin secretion but protection from hepatic steatosis and liver damage. *Antioxid. Redox Signal.* **20,** 2114–2129 (2014).
64. Gurzov, E. N., Tran, M., Fernandez-Rojo, M. A., Merry, T. L., Zhang, X., Xu, Y., Fukushima, A., Waters, M. J., Watt, M. J., Andrikopoulos, S., Neel, B. G. & Tiganis, T. Hepatic oxidative stress promotes insulin-STAT-5 signaling and obesity by inactivating protein tyrosine phosphatase N2. *Cell Metab.* **20,** 85–102 (2014).
65. Wang, X., Yun, J.-W. & Lei, X. G. Glutathione peroxidase mimic ebselen improves glucose-stimulated insulin secretion in murine islets. *Antioxid. Redox Signal.* **20,** 191–203 (2014).
66. Brigelius-Flohé, R., Müller, M., Lippmann, D. & Kipp, A. P. The yin and yang of nrf2-regulated selenoproteins in carcinogenesis. *Int. J. Cell Biol.* **2012,** 486147 (2012).
67. Tersey, S. A., Maier, B., Nishiki, Y., Maganti, A. V., Nadler, J. L. & Mirmira, R. G. 12-lipoxygenase promotes obesity-induced oxidative stress in pancreatic islets. *Mol. Cell. Biol.* **34,** 3735–3745 (2014).
68. Kuzuya, M., Ando, F., Iguchi, A. & Shimokata, H. Glutathione peroxidase 1 Pro198Leu variant contributes to the metabolic syndrome in men in a large Japanese cohort. *Am. J. Clin. Nutr.* **87,** 1939–1944 (2008).
69. Misu, H., Takamura, T., Takayama, H., Hayashi, H., Matsuzawa-Nagata, N., Kurita, S., Ishikura, K., Ando, H., Takeshita, Y., Ota, T., Sakurai, M., Yamashita, T., Mizukoshi, E., Yamashita, T., Honda, M., Miyamoto, K., Kubota, T., Kubota, N., Kadowaki, T., Kim, H.-J., Lee, I., Minokoshi, Y., Saito, Y., Takahashi, K., Yamada, Y., Takakura, N. & Kaneko, S. A Liver-Derived Secretory Protein, Selenoprotein P, Causes Insulin Resistance. *Cell Metab.* **12,** 483–495 (2010).
70. Hurst, R., Armah, C. N., Dainty, J. R., Hart, D. J., Teucher, B., Goldson, A. J., Broadley, M. R., Motley, A. K. & Fairweather-Tait, S. J. Establishing optimal selenium status: results of a randomized, double-blind, placebo-controlled trial. *Am. J. Clin. Nutr.* **91,** 923–931 (2010).
71. Caselli, C. Role of adiponectin system in insulin resistance. *Mol. Genet. Metab.* **113,** 155–160 (2014).
72. Misu, H., Ishikura, K., Kurita, S., Takeshita, Y., Ota, T., Saito, Y., Takahashi, K., Kaneko, S. & Takamura, T. Inverse correlation between serum levels of selenoprotein P and adiponectin in patients with type 2 diabetes. *PloS One* **7,** e34952 (2012).
73. Yang, S. J., Hwang, S. Y., Choi, H. Y., Yoo, H. J., Seo, J. A., Kim, S. G., Kim, N. H., Baik, S. H., Choi, D. S. & Choi, K. M. Serum selenoprotein P levels in patients with type 2 diabetes and prediabetes: implications for insulin resistance, inflammation, and atherosclerosis. *J. Clin. Endocrinol. Metab.* **96,** E1325-1329 (2011).
74. Hellwege, J. N., Palmer, N. D., Ziegler, J. T., Langefeld, C. D., Lorenzo, C., Norris, J. M., Takamura, T. & Bowden, D. W. Genetic variants in selenoprotein P plasma 1 gene (SEPP1) are associated with fasting insulin and first phase insulin response in Hispanics. *Gene* **534,** 33–39 (2014).
75. Speckmann, B., Walter, P. L., Alili, L., Reinehr, R., Sies, H., Klotz, L.-O. & Steinbrenner, H. Selenoprotein P expression is controlled through interaction of the coactivator PGC-1alpha with FoxO1a and hepatocyte nuclear factor 4alpha transcription factors. *Hepatol. Baltim. Md* **48,** 1998–2006 (2008).

76. Speckmann, B., Sies, H. & Steinbrenner, H. Attenuation of hepatic expression and secretion of selenoprotein P by metformin. *Biochem. Biophys. Res. Commun.* **387,** 158–163 (2009).
77. Ferguson, A. D., Labunskyy, V. M., Fomenko, D. E., Araç, D., Chelliah, Y., Amezcua, C. A., Rizo, J., Gladyshev, V. N. & Deisenhofer, J. NMR Structures of the Selenoproteins Sep15 and SelM Reveal Redox Activity of a New Thioredoxin-like Family. *J. Biol. Chem.* **281,** 3536–3543 (2006).
78. Reeves, M. A., Bellinger, F. P. & Berry, M. J. The Neuroprotective Functions of Selenoprotein M and its Role in Cytosolic Calcium Regulation. *Antioxid. Redox Signal.* **12,** 809–818 (2010).
79. Pitts, M. W., Reeves, M. A., Hashimoto, A. C., Ogawa, A., Kremer, P., Seale, L. A. & Berry, M. J. Deletion of Selenoprotein M Leads to Obesity without Cognitive Deficits. *J. Biol. Chem.* **288,** 26121–26134 (2013).
80. Ozcan, L., Ergin, A. S., Lu, A., Chung, J., Sarkar, S., Nie, D., Myers Jr., M. G. & Ozcan, U. Endoplasmic Reticulum Stress Plays a Central Role in Development of Leptin Resistance. *Cell Metab.* **9,** 35–51 (2009).
81. McAninch, E. A. & Bianco, A. C. Thyroid hormone signaling in energy homeostasis and energy metabolism. *Ann. N. Y. Acad. Sci.* **1311,** 77–87 (2014).
82. Leiria, L. B., Dora, J. M., Wajner, S. M., Estivalet, A. A. F., Crispim, D. & Maia, A. L. The rs225017 polymorphism in the 3'UTR of the human DIO2 gene is associated with increased insulin resistance. *PloS One* **9,** e103960 (2014).
83. Vernia, S., Cavanagh-Kyros, J., Barrett, T., Jung, D. Y., Kim, J. K. & Davis, R. J. Diet-induced obesity mediated by the JNK/DIO2 signal transduction pathway. *Genes Dev.* **27,** 2345–2355 (2013).
84. Marsili, A., Aguayo-Mazzucato, C., Chen, T., Kumar, A., Chung, M., Lunsford, E. P., Harney, J. W., Van-Tran, T., Gianetti, E., Ramadan, W., Chou, C., Bonner-Weir, S., Larsen, P. R., Silva, J. E. & Zavacki, A. M. Mice with a Targeted Deletion of the Type 2 Deiodinase Are Insulin Resistant and Susceptible to Diet Induced Obesity. *PLoS ONE* **6,** e20832 (2011).
85. Christoffolete, M. A., Doleschall, M., Egri, P., Liposits, Z., Zavacki, A. M., Bianco, A. C. & Gereben, B. Regulation of thyroid hormone activation via the liver X-receptor/retinoid X-receptor pathway. *J. Endocrinol.* **205,** 179–186 (2010).
86. Medina, M. C., Molina, J., Gadea, Y., Fachado, A., Murillo, M., Simovic, G., Pileggi, A., Hernández, A., Edlund, H. & Bianco, A. C. The thyroid hormone-inactivating type III deiodinase is expressed in mouse and human beta-cells and its targeted inactivation impairs insulin secretion. *Endocrinology* **152,** 3717–3727 (2011).
87. Medina, M. C., Fonesca, T. L., Molina, J., Fachado, A., Castillo, M., Dong, L., Soares, R., Hernández, A., Caicedo, A. & Bianco, A. C. Maternal Inheritance of an Inactive Type III Deiodinase Gene Allele Affects Mouse Pancreatic β-Cells and Disrupts Glucose Homeostasis. *Endocrinology* **155,** 3160 (2014).
88. Akiyama, S., Ogiwara, T., Aoki, T., Tsunekawa, K., Araki, O. & Murakami, M. Glucagon-like peptide-1 stimulates type 3 iodothyronine deiodinase expression in a mouse insulinoma cell line. *Life Sci.* **115,** 22–28 (2014).
89. Kryukov, G. V., Kryukov, V. M. & Gladyshev, V. N. New mammalian selenocysteine-containing proteins identified with an algorithm that searches for selenocysteine insertion sequence elements. *J. Biol. Chem.* **274,** 33888–33897 (1999).
90. Dikiy, A., Novoselov, S. V., Fomenko, D. E., Sengupta, A., Carlson, B. A., Cerny, R. L., Ginalski, K., Grishin, N. V., Hatfield, D. L. & Gladyshev, V. N. SelT, SelW, SelH, and

Rdx12: genomics and molecular insights into the functions of selenoproteins of a novel thioredoxin-like family. *Biochemistry (Mosc.)* **46,** 6871–6882 (2007).
91. Moustafa, M. E. & Antar, H. A. A bioinformatics approach to characterize mammalian selenoprotein T. *Biochem. Genet.* **50,** 736–747 (2012).
92. Tanguy, Y., Falluel-Morel, A., Arthaud, S., Boukhzar, L., Manecka, D.-L., Chagraoui, A., Prevost, G., Elias, S., Dorval-Coiffec, I., Lesage, J., Vieau, D., Lihrmann, I., Jégou, B. & Anouar, Y. The PACAP-Regulated Gene Selenoprotein T Is Highly Induced in Nervous, Endocrine, and Metabolic Tissues during Ontogenetic and Regenerative Processes. *Endocrinology* **152,** 4322–4335 (2011).
93. Grumolato, L., Ghzili, H., Montero-Hadjadje, M., Gasman, S., Lesage, J., Tanguy, Y., Galas, L., Ait-Ali, D., Leprince, J., Guérineau, N. C., Elkahloun, A. G., Fournier, A., Vieau, D., Vaudry, H. & Anouar, Y. Selenoprotein T is a PACAP-regulated gene involved in intracellular Ca2+ mobilization and neuroendocrine secretion. *FASEB J.* **22,** 1756–1768 (2008).
94. Dhillon, S. S., McFadden, S. A., Chalmers, J. A., Centeno, M.-L., Kim, G. L. & Belsham, D. D. Cellular leptin resistance impairs the leptin-mediated suppression of neuropeptide Y secretion in hypothalamic neurons. *Endocrinology* **152,** 4138–4147 (2011).
95. Chalmers, J. A., Jang, J. J. & Belsham, D. D. Glucose sensing mechanisms in hypothalamic cell models: glucose inhibition of AgRP synthesis and secretion. *Mol. Cell. Endocrinol.* **382,** 262–270 (2014).
96. Nazarians-Armavil, A., Chalmers, J. A., Lee, C. B., Ye, W. & Belsham, D. D. Cellular insulin resistance disrupts hypothalamic mHypoA-POMC/GFP neuronal signaling pathways. *J. Endocrinol.* **220,** 13–24 (2014).
97. Ye, Y., Shibata, Y., Yun, C., Ron, D. & Rapoport, T. A. A membrane protein complex mediates retro-translocation from the ER lumen into the cytosol. *Nature* **429,** 841–847 (2004).
98. Gao, Y., Hannan, N. R. F., Wanyonyi, S., Konstantopolous, N., Pagnon, J., Feng, H. C., Jowett, J. B. M., Kim, K.-H., Walder, K. & Collier, G. R. Activation of the selenoprotein SEPS1 gene expression by pro-inflammatory cytokines in HepG2 cells. *Cytokine* **33,** 246–251 (2006).
99. Turanov, A. A., Shchedrina, V. A., Everley, R. A., Lobanov, A. V., Yim, S. H., Marino, S. M., Gygi, S. P., Hatfield, D. L. & Gladyshev, V. N. Selenoprotein S is involved in maintenance and transport of multiprotein complexes. *Biochem. J.* **462,** 555–565 (2014).
100. Walder, K., Kantham, L., McMillan, J. S., Trevaskis, J., Kerr, L., De Silva, A., Sunderland, T., Godde, N., Gao, Y., Bishara, N., Windmill, K., Tenne-Brown, J., Augert, G., Zimmet, P. Z. & Collier, G. R. Tanis: a link between type 2 diabetes and inflammation? *Diabetes* **51,** 1859–1866 (2002).
101. Karlsson, H. K. R., Tsuchida, H., Lake, S., Koistinen, H. A. & Krook, A. Relationship between serum amyloid A level and Tanis/SelS mRNA expression in skeletal muscle and adipose tissue from healthy and type 2 diabetic subjects. *Diabetes* **53,** 1424–1428 (2004).
102. Olsson, M., Olsson, B., Jacobson, P., Thelle, D. S., Björkegren, J., Walley, A., Froguel, P., Carlsson, L. M. S. & Sjöholm, K. Expression of the selenoprotein S (SELS) gene in subcutaneous adipose tissue and SELS genotype are associated with metabolic risk factors. *Metabolism.* **60,** 114–120 (2011).
103. Gao, Y., Feng, H. C., Walder, K., Bolton, K., Sunderland, T., Bishara, N., Quick, M., Kantham, L. & Collier, G. R. Regulation of the selenoprotein SelS by glucose deprivation and endoplasmic reticulum stress - SelS is a novel glucose-regulated protein. *FEBS Lett.* **563,** 185–190 (2004).

104. Schomburg, L. & Schweizer, U. Hierarchical regulation of selenoprotein expression and sex-specific effects of selenium. *Biochim. Biophys. Acta BBA - Gen. Subj.* **1790,** 1453–1462 (2009).
105. Stoedter, M., Renko, K., Hög, A. & Schomburg, L. Selenium controls the sex-specific immune response and selenoprotein expression during the acute-phase response in mice. *Biochem. J.* **429,** 43–51 (2010).
106. Burk, R. F., Hill, K. E., Olson, G. E., Weeber, E. J., Motley, A. K., Winfrey, V. P. & Austin, L. M. Deletion of Apolipoprotein E Receptor-2 in Mice Lowers Brain Selenium and Causes Severe Neurological Dysfunction and Death When a Low-Selenium Diet Is Fed. *J. Neurosci.* **27,** 6207–6211 (2007).
107. Olson, G. E., Winfrey, V. P., NagDas, S. K., Hill, K. E. & Burk, R. F. Apolipoprotein E Receptor-2 (ApoER2) Mediates Selenium Uptake from Selenoprotein P by the Mouse Testis. *J. Biol. Chem.* **282,** 12290–12297 (2007).
108. Davis, C. D., Tsuji, P. A. & Milner, J. A. Selenoproteins and cancer prevention. *Annu. Rev. Nutr.* **32,** 73–95 (2012).
109. Behne, D., Weiler, H. & Kyriakopoulos, A. Effects of selenium deficiency on testicular morphology and function in rats. *J. Reprod. Fertil.* **106,** 291–297 (1996).
110. Hill, K. E., Zhou, J., McMahan, W. J., Motley, A. K. & Burk, R. F. Neurological Dysfunction Occurs in Mice with Targeted Deletion of the Selenoprotein P Gene. *J. Nutr.* **134,** 157–161 (2004).
111. Prevost, G., Arabo, A., Jian, L., Quelennec, E., Cartier, D., Hassan, S., Falluel-Morel, A., Tanguy, Y., Gargani, S., Lihrmann, I., Kerr-Conte, J., Lefebvre, H., Pattou, F. & Anouar, Y. The PACAP-regulated gene selenoprotein T is abundantly expressed in mouse and human β-cells and its targeted inactivation impairs glucose tolerance. *Endocrinology* **154,** 3796–3806 (2013).
112. Jitrapakdee, S., Wutthisathapornchai, A., Wallace, J. C. & MacDonald, M. J. Regulation of insulin secretion: role of mitochondrial signalling. *Diabetologia* **53,** 1019–1032 (2010).
113. Ishihara, H., Asano, T., Tsukuda, K., Katagiri, H., Inukai, K., Anai, M., Kikuchi, M., Yazaki, Y., Miyazaki, J. I. & Oka, Y. Pancreatic beta cell line MIN6 exhibits characteristics of glucose metabolism and glucose-stimulated insulin secretion similar to those of normal islets. *Diabetologia* **36,** 1139–1145 (1993).
114. Dimauro, I., Pearson, T., Caporossi, D. & Jackson, M. J. A simple protocol for the subcellular fractionation of skeletal muscle cells and tissue. *BMC Res. Notes* **5,** 513 (2012).
115. Gotoh, M., Maki, T., Satomi, S., Porter, J., Bonner-Weir, S., O'Hara, C. J. & Monaco, A. P. Reproducible high yield of rat islets by stationary in vitro digestion following pancreatic ductal or portal venous collagenase injection. *Transplantation* **43,** 725–730 (1987).
116. Schuppin, G. T., Bonner-Weir, S., Montana, E., Kaiser, N. & Weir, G. C. Replication of Adult Pancreatic-Beta Cells Cultured on Bovine Corneal Endothelial Cell Extracellular Matrix. *In Vitro Cell. Dev. Biol. Anim.* **29A,** 339–344 (1993).
117. Kurokawa, S., Takehashi, M., Tanaka, H., Mihara, H., Kurihara, T., Tanaka, S., Hill, K., Burk, R. & Esaki, N. Mammalian Selenocysteine Lyase Is Involved in Selenoprotein Biosynthesis. *J. Nutr. Sci. Vitaminol. (Tokyo)* **57,** 298–305 (2011).
118. Schulze, T., Morsi, M., Brüning, D., Schumacher, K. & Rustenbeck, I. Different responses of mouse islets and MIN6 pseudo-islets to metabolic stimulation: a note of caution. *Endocrine* **51,** 440–447 (2016).
119. de Jesus, L. A., Hoffmann, P. R., Michaud, T., Forry, E. P., Small-Howard, A., Stillwell, R. J., Morozova, N., Harney, J. W. & Berry, M. J. Nuclear assembly of UGA decoding

complexes on selenoprotein mRNAs: a mechanism for eluding nonsense-mediated decay? *Mol. Cell. Biol.* **26,** 1795–1805 (2006).
120. Cheng, K., Delghingaro-Augusto, V., Nolan, C. J., Turner, N., Hallahan, N., Andrikopoulos, S. & Gunton, J. E. High passage MIN6 cells have impaired insulin secretion with impaired glucose and lipid oxidation. *PloS One* **7,** e40868 (2012).
121. Yang, Y. H. C., Szabat, M., Bragagnini, C., Kott, K., Helgason, C. D., Hoffman, B. G. & Johnson, J. D. Paracrine signalling loops in adult human and mouse pancreatic islets: netrins modulate beta cell apoptosis signalling via dependence receptors. *Diabetologia* **54,** 828–842 (2011).
122. Burk, R. F. Molecular biology of selenium with implications for its metabolism. *FASEB J. Off. Publ. Fed. Am. Soc. Exp. Biol.* **5,** 2274–2279 (1991).
123. Daniels, L. A. Selenium metabolism and bioavailability. *Biol. Trace Elem. Res.* **54,** 185–199 (1996).
124. Poliak, P., Hoewyk, D. V., Oborník, M., Zíková, A., Stuart, K. D., Tachezy, J., Pilon, M. & Lukeš, J. Functions and cellular localization of cysteine desulfurase and selenocysteine lyase in Trypanosoma brucei. *FEBS J.* **277,** 383 (2010).
125. Elshorbagy, A. K., Refsum, H., Smith, A. D. & Graham, I. M. The association of plasma cysteine and gamma-glutamyltransferase with BMI and obesity. *Obes. Silver Spring Md* **17,** 1435–1440 (2009).
126. Carter, R. N. & Morton, N. M. Cysteine and hydrogen sulphide in the regulation of metabolism: insights from genetics and pharmacology. *J. Pathol.* **238,** 321–332 (2016).
127. Kaneko, Y., Kimura, Y., Kimura, H. & Niki, I. L-cysteine inhibits insulin release from the pancreatic beta-cell: possible involvement of metabolic production of hydrogen sulfide, a novel gasotransmitter. *Diabetes* **55,** 1391–1397 (2006).
128. Tang, G., Zhang, L., Yang, G., Wu, L. & Wang, R. Hydrogen sulfide-induced inhibition of L-type Ca2+ channels and insulin secretion in mouse pancreatic beta cells. *Diabetologia* **56,** 533–541 (2013).
129. Nakatsu, D., Horiuchi, Y., Kano, F., Noguchi, Y., Sugawara, T., Takamoto, I., Kubota, N., Kadowaki, T. & Murata, M. L-cysteine reversibly inhibits glucose-induced biphasic insulin secretion and ATP production by inactivating PKM2. *Proc. Natl. Acad. Sci. U. S. A.* **112,** E1067-1076 (2015).
130. McClenaghan, N. H., Barnett, C. R. & Flatt, P. R. Na+ cotransport by metabolizable and nonmetabolizable amino acids stimulates a glucose-regulated insulin-secretory response. *Biochem. Biophys. Res. Commun.* **249,** 299–303 (1998).
131. Brennan, L., Shine, A., Hewage, C., Malthouse, J. P. G., Brindle, K. M., McClenaghan, N., Flatt, P. R. & Newsholme, P. A nuclear magnetic resonance-based demonstration of substantial oxidative L-alanine metabolism and L-alanine-enhanced glucose metabolism in a clonal pancreatic beta-cell line: metabolism of L-alanine is important to the regulation of insulin secretion. *Diabetes* **51,** 1714–1721 (2002).
132. Clayton, J. A. & Collins, F. S. Policy: NIH to balance sex in cell and animal studies. *Nature* **509,** 282–283 (2014).
133. Lovejoy, J. C., Sainsbury, A. & the Stock Conference 2008 Working Group. Sex differences in obesity and the regulation of energy homeostasis. *Obes. Rev.* **10,** 154–167 (2009).
134. Macotela, Y., Boucher, J., Tran, T. T. & Kahn, C. R. Sex and Depot Differences in Adipocyte Insulin Sensitivity and Glucose Metabolism. *Diabetes* **58,** 803–812 (2009).
135. Regensteiner, J. G., Golden, S., Huebschmann, A. G., Barrett-Connor, E., Chang, A. Y., Chyun, D., Fox, C. S., Kim, C., Mehta, N., Reckelhoff, J. F., Reusch, J. E. B., Rexrode, K. M., Sumner, A. E., Welty, F. K., Wenger, N. K., Anton, B. & American Heart Association

Diabetes Committee of the Council on Lifestyle and Cardiometabolic Health, Council on Epidemiology and Prevention, Council on Functional Genomics and Translational Biology, and Council on Hypertension. Sex Differences in the Cardiovascular Consequences of Diabetes Mellitus: A Scientific Statement From the American Heart Association. *Circulation* **132,** 2424–2447 (2015).
136. Brown, D. G. & Burk, R. F. Selenium retention in tissues and sperm of rats fed a Torula yeast diet. *J. Nutr.* **103,** 102–108 (1973).
137. Olson, G. E., Winfrey, V. P., Nagdas, S. K., Hill, K. E. & Burk, R. F. Selenoprotein P is required for mouse sperm development. *Biol. Reprod.* **73,** 201–211 (2005).
138. Jr, M. G. M. & Olson, D. P. Central nervous system control of metabolism. *Nature* **491,** 357–363 (2012).
139. Yagishita, Y., Uruno, A., Fukutomi, T., Saito, R., Saigusa, D., Pi, J., Fukamizu, A., Sugiyama, F., Takahashi, S. & Yamamoto, M. Nrf2 Improves Leptin and Insulin Resistance Provoked by Hypothalamic Oxidative Stress. *Cell Rep.* **18,** 2030–2044 (2017).
140. Ogawa-Wong, A. N., Berry, M. J. & Seale, L. A. Selenium and Metabolic Disorders: An Emphasis on Type 2 Diabetes Risk. *Nutrients* **8,** 80 (2016).
141. Lusk, G. Animal Calorimetry: Analysis of the oxidation of mixtures of carbohydrate and fat. *J. Biol. Chem.* **59,** 41–42 (1924).
142. Dalbøge, L. S., Almholt, D. L. C., Neerup, T. S. R., Vassiliadis, E., Vrang, N., Pedersen, L., Fosgerau, K. & Jelsing, J. Characterisation of age-dependent beta cell dynamics in the male db/db mice. *PloS One* **8,** e82813 (2013).
143. Zhu, L., Martinez, M. N., Emfinger, C. H., Palmisano, B. T. & Stafford, J. M. Estrogen signaling prevents diet-induced hepatic insulin resistance in male mice with obesity. *Am. J. Physiol. - Endocrinol. Metab.* **306,** E1188–E1197 (2014).
144. Carr, M. C. The emergence of the metabolic syndrome with menopause. *J. Clin. Endocrinol. Metab.* **88,** 2404–2411 (2003).
145. Mauvais-Jarvis, F., Clegg, D. J. & Hevener, A. L. The Role of Estrogens in Control of Energy Balance and Glucose Homeostasis. *Endocr. Rev.* **34,** 309–338 (2013).
146. Pitts, M. W., Kremer, P. M., Hashimoto, A. C., Torres, D. J., Byrns, C. N., Williams, C. S. & Berry, M. J. Competition between the Brain and Testes under Selenium-Compromised Conditions: Insight into Sex Differences in Selenium Metabolism and Risk of Neurodevelopmental Disease. *J. Neurosci.* **35,** 15326–15338 (2015).
147. Vermeulen, A., Goemaere, S. & Kaufman, J. M. Testosterone, body composition and aging. *J. Endocrinol. Invest.* **22,** 110–116 (1999).
148. Kim, G.-Y., Lee, Y. M., Cho, J.-H., Pan, C.-J., Jun, H. S., Springer, D. A., Mansfield, B. C. & Chou, J. Y. Mice expressing reduced levels of hepatic glucose-6-phosphatase-α activity do not develop age-related insulin resistance or obesity. *Hum. Mol. Genet.* **24,** 5115–5125 (2015).
149. Weir, G. C. & Bonner-Weir, S. Five stages of evolving beta-cell dysfunction during progression to diabetes. *Diabetes* **53 Suppl 3,** S16-21 (2004).
150. Yu, I.-C., Lin, H.-Y., Sparks, J. D., Yeh, S. & Chang, C. Androgen Receptor Roles in Insulin Resistance and Obesity in Males: The Linkage of Androgen-Deprivation Therapy to Metabolic Syndrome. *Diabetes* **63,** 3180–3188 (2014).
151. Navarro, G., Xu, W., Jacobson, D. A., Wicksteed, B., Allard, C., Zhang, G., De Gendt, K., Kim, S. H., Wu, H., Zhang, H., Verhoeven, G., Katzenellenbogen, J. A. & Mauvais-Jarvis, F. Extranuclear Actions of the Androgen Receptor Enhance Glucose-Stimulated Insulin Secretion in the Male. *Cell Metab.* **23,** 837–851 (2016).

152. Raman, A. V., Pitts, M. W., Seyedali, A., Hashimoto, A. C., Seale, L. A., Bellinger, F. P. & Berry, M. J. Absence of selenoprotein P but not selenocysteine lyase results in severe neurological dysfunction. *Genes Brain Behav.* **11,** 601–613 (2012).
153. Jung, C. H. & Kim, M.-S. Molecular mechanisms of central leptin resistance in obesity. *Arch. Pharm. Res.* **36,** 201–207 (2013).
154. Zeng, W., Pirzgalska, R. M., Pereira, M. M. A., Kubasova, N., Barateiro, A., Seixas, E., Lu, Y.-H., Kozlova, A., Voss, H., Martins, G. G., Friedman, J. M. & Domingos, A. I. Sympathetic neuro-adipose connections mediate leptin-driven lipolysis. *Cell* **163,** 84–94 (2015).
155. Bailey, R. L., Gahche, J. J., Lentino, C. V., Dwyer, J. T., Engel, J. S., Thomas, P. R., Betz, J. M., Sempos, C. T. & Picciano, M. F. Dietary supplement use in the United States, 2003-2006. *J. Nutr.* **141,** 261–266 (2011).
156. Small-Howard, A., Morozova, N., Stoytcheva, Z., Forry, E. P., Mansell, J. B., Harney, J. W., Carlson, B. A., Xu, X., Hatfield, D. L. & Berry, M. J. Supramolecular Complexes Mediate Selenocysteine Incorporation In Vivo. *Mol. Cell. Biol.* **26,** 2337–2346 (2006).