THE SELENIUM RECYCLING ENZYME
SELENOCYSTEINE LYASE:
REGULATION AND PHYSIOLOGICAL ROLE IN
GLUCOSE AND LIPID METABOLISM

A DISSERTATION SUBMITTED TO THE GRADUATE DIVISION OF THE
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BY

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To my beloved parents Wilson & Madalena
To my dear love André
ACKNOWLEDGEMENTS

A Ph.D. is a journey in a rough sea. As in all memorable expeditions, there is more to learn while traveling towards the final destination than at the destination itself. In several ways, it is the path and how you face the unexpected obstacles in this path that makes you a Doctor.

The Ph.D. expedition does not allow an easy ride. While you travel through the waves and storms of your topic, figuring out a lot on and about yourself, you also encounter along the way people that make your stressful journey happier, nicer, safer, funnier and – why not – more challenging. They provided the emotional background that is not described in a scientific document.

In my Ph.D. journey, these are the people about whom I’ll talk years ahead to friends, to whom I will silently smile when the nostalgic waves of the Ph.D. path break in the shores of my life. They are the unforgettable people that allowed me to arrive safe and sound in Doctorland.

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Lastly, but far from least, a special thank you to my online and offline friends. During the Ph.D. journey, when the seas were really rough, your relaxing words posted on twitter or facebook walls, in blog comments, or during countless happy hours pointed me to esteemed perspectives required for a safe journey.

None of this work would be possible without the passionate support I get from my family, especially my parents, and from my lovely husband André, who has always been there for me, providing constructive advice, and whose tireless encouragement was a daily and cheerful reminder that the PhD journey, although rough, one day reaches the academic hub from where many other new and exciting journeys may depart.
ABSTRACT

Selenium (Se) is an essential trace element used for biosynthesis of selenoproteins, acquired either through the diet or cellular recycling mechanisms involving the enzyme selenocysteine lyase (Scly). This dissertation characterizes the Se-dependent regulation of Scly gene expression in in vitro and in vivo models. Scly mRNA negatively correlates with Se levels in HepG2 cells, liver and brain tissues, but not in Ht22 cells. Scly is also downregulated in the liver of the Selenoprotein P (Sepp1) knockout (KO) mouse, a model lacking the Se transporter protein with elevated hepatic Se content. Additionally, this dissertation reports the development and characterization of a Scly KO mouse. This mouse model presents diminished Se content and increased expression of selenoprotein mRNAs in the liver compared with wild type mice. Scly KO mice maintain circulating Se levels and most selenoprotein mRNA levels in Se target tissues, such as brain and testis. Disruption of the Scly mechanism also affects hepatic energy metabolism. Scly KO mice raised on a Se adequate diet exhibit hyperinsulinemia and mild hepatic steatosis, with attenuated insulin signaling as measured by Akt phosphorylation and increased oxidative stress, without changes in phosphorylation or protein levels of AMP-activated Protein Kinase alpha, a regulator of cholesterol biosynthesis. On Se restriction, Scly KO mice develop characteristics of metabolic syndrome, such as obesity, fatty liver, hypercholesterolemia and insulin resistance. Moreover, hepatic glutathione peroxidase 1 and circulating Sepp1 levels are also diminished, but mRNA levels of Acetyl-CoA carboxylase are increased. When exposed to a high fat, Se adequate diet, Scly KO mice become more vulnerable to obesity. The findings of this dissertation unveil a strong connection between Se recycling and glucose and lipid metabolism, and demonstrate for the first time in an animal model the unique physiological role of the Se recycling pathway in lipid and glucose metabolism.
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ICP-MS  Inductively Coupled Plasma Mass Spectrometry (p. 47)
IDE  Insulin-degrading Enzyme (p. 121)
IL-1β  Interleukin-1beta (p. 27)
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IR  Insulin Receptor (p. 67)
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kb  kilobase (p. 26)
kDa  kilodalton (p. 8)
KO  Knockout (p. 7)
LDL  Low-density Lipoprotein (p.10)
LXRα  Liver-X Receptor alpha (p. 69)
Lys  Lysine (p. 15)
MCP1  Monocyte Chemotactic Protein 1 (p. 64)
Met  Methionine (p. 4)
MRE  Metal Response Element (p. 28)
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NCBI  National Center for Biotechnology Information (p. 26)
NHANES  National Health and Nutrition Examination Survey (p. 66)
NifS  Nfs1 or Mitochondrial Cysteine Desulfurase (p. 52)
NMD  Nonsense Mediated Decay (p. 11)
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<td>SelR</td>
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<td>SelW</td>
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<tr>
<td>SELECT</td>
<td>Selenium and Vitamin E Cancer Prevention Trial</td>
<td>13</td>
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<tr>
<td>SEM</td>
<td>Standard Error of the Mean</td>
<td>32</td>
</tr>
<tr>
<td>SeMet</td>
<td>Selenomethionine</td>
<td>3</td>
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</tbody>
</table>
Sep15  15-kDa selenoprotein (p. 8)
Sepp1  Selenoprotein P (p. 6)
Ser    Serine (p. 6)
SNP    Single Nucleotide Polymorphism (p. 123)
SOD    Superoxide Dismutase (p. 67)
SPS    Selenophosphate Synthetase (p. 5)
SQS    Squalene Synthase (p. 85)
SREBP  Sterol- regulatory Element Binding Protein (p. 110)
S      Sulfur (p. 1)
T3     Triiodothyronine (p. 65)
T4     Thyroxine (p. 65)
Thr    Threonine (p. 79)
TNFα   Tumor Necrosis Factor alpha (p. 15)
TrxR   Thioredoxin Reductase (p. 5)
UBE2F  Ubiquitin Conjugating Enzyme E2F (p. 26)
UK PRECISE United Kingdom Prevention of Cancer by Intervention with Selenium (p. 66)
USDA  United States Department of Agriculture (p. 3)
WAT    White Adipose Tissue (p. 62)
WT     Wild Type (p. 13)
CHAPTER 1

INTRODUCTION

1.1. History of Selenium and Selenium Research

Selenium (Se) was discovered in 1817 by Swedish chemist John Berzelius. Berzelius isolated the new rare element from reddish mud and named it after the Greek mythological moon goddess Selene. In his groundbreaking report, Berzelius recognized the chemical similarities between Se and sulfur (S) despite the rudimentary technology available at that time [1, 2]. At the time, Se was mainly recognized as a toxic and carcinogenic element for livestock animals and humans, respectively [3]. This perspective didn’t change until 1957, when it was reported that rats deficient in Se and vitamin E developed liver necrosis ([4] reviewed in [5]).

In the seminal report by Schwarz & Foltz mentioned above [4], the sentence: “Se is an essential trace element”, appeared for the first time in scientific history. This statement holds true today, and is corroborated by the vast amount of data published on Se research since 1957. At that time, Se was considered to be essential solely to mammalians, but it was later established to be crucial to all domains of life [6-8].

In the early 70’s, two discoveries impacted the Se research field. First, was the finding that the bovine enzyme, glutathione peroxidase (GPx), contained Se covalently bound in its molecule ([6, 9] reviewed in [5]). Second, it was the observation that Se was also present in two enzymes from bacteria, formate dehydrogenase [10] and glycine reductase [11]. These three were the first selenoproteins to be identified. During the 80’s, studies employing bacterial glycine reductase revealed that Se was present as the amino acid selenocysteine (Sec) [12], a finding that was confirmed two years later with bovine GPx [13]. In 1984, early DNA sequencing technology allowed for the surprising discovery that the Sec residue of bovine GPx was encoded by a TGA stop codon [14, 15], followed once more by the same revelation in the bacterial formate dehydrogenase [16].
presence of a unique tRNA for insertion of Sec was subsequently recognized in bacteria [17] as well as in mammals [18].

The 1990’s brought increased attention to the mechanism behind the recoding of TGA as Sec. In 1990, Zinoni et al. [19] demonstrated in the bacterial formate dehydrogenase gene that a specific sequence downstream of the TGA was required for proper translation into Sec. In the following year, Marla Berry and collaborators identified a stem loop structure that was crucial for the Sec insertion in the 3’ untranslated region of the mRNA for the rat type 1 deiodinase (Dio1) [20]. Named SECIS (Selenocysteine Insertion Sequence), it was recognized as a hallmark of eukaryotic [21, 22] and archaea [23, 24] selenoprotein mRNAs and later used for in silico identification of selenoprotein genes in various DNA databanks [25, 26] and also to generate a selenoprotein database [27].

The molecular mechanism and players of Sec incorporation were characterized in the early 2000’s [28-31]. Since then, the role of Se deficiency and/or supplementation in the pathogenesis of several diseases has been increasingly recognized [32, 33]. Figure 1.1 presents a cartoon depicting the changes in views on the effects of Se on health over time.

Despite the available literature in this field, several mechanistic details of Se metabolism remain to be uncovered. This dissertation attempts to investigate one of these unclear domains of Se metabolism: the role of the cellular Se recycling pathway in physiology.

1.2. Dietary Selenium

1.2.1. Se Intake

Se intake varies widely in the world, ranging from 7 µg to 4990 µg per day, with mean values of 40 µg in Europe and 93 µg (women) to 134 µg (men) in the USA [32, 34]. The world’s most Se deficient areas are found in China and New Zealand, although the latter has overcome the deficiency by importing Se-rich wheat from Australia. Nevertheless, the local values still correlate strongly with the existing amounts in the soil or livestock in the surrounding area. It is worth noting that the
higher mean intake reported for the USA is closely related to the fact that almost 50% of the American population takes dietary supplements that are rich in Se [35].

The World Health Organization [36] recommends a daily intake of 34 µg for adult men and 26 µg for adult women. These values are slightly lower for children (6-21 µg) and higher for pregnant or lactating women (28-42 µg). It should be noted that the calculation for such recommendations is based on values of Se deficiency that are able to epidemiologically cause a significant impairment in health, like in areas of China where Keshan disease is prevalent due to low Se in the soil [37]. Nevertheless, for the proper maintenance of Se metabolism, researchers still struggle to achieve a consensus on a common recommended value. On a study in Keshan disease areas of China, it was found that providing 41 µg of daily Se intake to a very Se-deficient person was sufficient to bring the activity of plasma GPx and plasma Se levels to a satisfactory level [38, 39]. Nevertheless, other researchers will define an adequate intake as the amount to guarantee unrestricted selenoprotein expression; this ranges from 40-55 µg daily [40, 41].

The United States Department of Agriculture National Nutrient Database for Standard Reference (USDA 2005, http://www.nal.usda.gov/fnic/foodcomp) listing of foods with high concentrations of Se includes Brazil nuts, most seafood, barley, wheat and certain meat products (turkey, chicken breast, duck). Nevertheless, one should remember that the Se content of crops varies greatly depending on the surrounding environment in which it grows.

1.2.2. Chemical forms

Dietary Se is found in various chemical forms, and its efficient uptake and metabolism will depend on which form it was ingested. Inorganic Se consists predominantly of selenite and selenate, both water-soluble and strong oxidizers [42]. Organic forms of Se include predominantly the amino acids selenomethionine (SeMet) and Sec [34], although rare forms such as selenoneine, Se-methylselenocysteine and γ-glutamyl-Se-methylselenocysteine may have important biological roles that remain to be unveiled [32]. A comparison between the uptake of inorganic and organic forms of Se demonstrated that approximately 57% of selenite is absorbed by the gastrointestinal tract, while ~97% of SeMet is uptaken by the
same route [43]. Nevertheless, it should be noted that once these forms are absorbed, their metabolic fate is similar; they are converted to selenide in order to act in selenoprotein biosynthesis [43, 44]. Neither Sec nor SeMet accumulate in biological systems, being promptly converted into selenide via recycling of Sec or the transsulfuration pathway [45, 46]. Both routes will be further discussed in this dissertation.

In general, the Se content of plants reflects the Se content of the surrounding soil and its bioavailability. Crops grown in areas with high Se content in the soil, such as the states of Nebraska and Wyoming in the USA, will have high Se concentrations [47]. Livestock Se content will also reflect the Se content of the soil and the forage items they ingest [48]. Besides the dependence on the soil content, plant Se content may also vary according to the presence of ions that can form complexes with Se in the environment [32] and according to the ability of plants to accumulate and metabolize Se [49].

During amino acid synthesis, plants generally employ Se and S nonspecifically in their metabolic processes. Thus, plants form methionine (Met) and SeMet in amounts that reflect the relative S and Se concentrations of the soils in which they are grown. The metabolic processes of SeMet and its downstream metabolites are generally analogous to those of Met in both plants and animals. Nevertheless, once incorporated in animal proteins in place of Met, the Se of SeMet is released when the amino acid is degraded, and from this point on, the metabolic pathways of S and Se take distinct directions [50].

The chemical structure of thio- and seleno-amino acids is shown in Figure 1.2. Although structurally similar, they differ in physiological abundance, reactivity, acid constant dissociation (pKa) and metabolic roles. Met and SeMet are structurally and metabolically analogous, differing in having either S or Se bound to the γ-carbon of their side chains. Nevertheless, Met and SeMet are incorporated into proteins and peptides nonspecifically in amounts that reflect their tissue abundance. SeMet is broken down into methylselenol by the tetrameric enzyme cystathione γ-lyase (Cth), a member of the reverse transsulfuration pathway [51, 52]. This pathway converts mostly homocysteine to cysteine (Cys) which contributes to the generation of sulfide [53]. The methylselenol generated in the Cth-catalyzed reaction of SeMet can be utilized in selenoprotein biosynthesis, including for the synthesis of GPx1 [54]. Due to
this effect on selenoprotein biosynthesis, SeMet is then considered an additional source of biologically essential Se required for de novo synthesis of Sec.

The molecular structures of the amino acids Cys and Sec are also analogues, but Sec is exclusively inserted into genetically unique selenoprotein families, requiring a recoding of the UGA stop codon. The recoding process will be further detailed in section 1.3. Interestingly, Cys can be incorporated in place of Sec in the thioredoxin reductase 1 (TrxR1) and 3 (TrxR3) sequences, utilizing the factors from the Sec synthesis machinery [55]. The Cys incorporation encoded by UGA is upregulated in conditions of low dietary Se, suggesting the presence of a compensatory mechanism. This mechanism would reduce selenoprotein activity and Se as a competitor for Cys insertion into selenoproteins.

1.3. Selenocysteine Incorporation Mechanism and Selenoprotein Biosynthesis

In mammals, Se is acquired through the diet. Once ingested, dietary Se is mostly absorbed and metabolized to produce the unique amino acid Sec, which is cotranslationally incorporated into selenoproteins [56].

The Sec incorporation mechanism involves de novo synthesis of Sec via selenophosphate (Se−P), which is synthesized by Se−P synthetases (SPS) [57]. Two SPS isoforms are found in mammals, SPS1 and SPS2. SPS1 was first isolated from bacteria [58] and subsequently from humans [59]. SPS2 was identified simultaneously in mice, in humans and in the archaeon, Methanococcus jannaschii, by protein alignment analysis [60]. In this study, the possibility of Se-dependent autoregulatory function of selenoprotein translation was suggested since SPS2 is a selenoprotein itself [60].

Although selenoprotein biosynthesis has been shown to not depend on SPS1 in mouse fibroblasts [61], SPS1 was shown to be essential for the process of Dio1 synthesis in a HeLa derivative cell line [59]. The determination of the human SPS1 crystal structure helped to clarify the issue regarding the essentiality of SPS1 for selenoprotein biosynthesis. SPS1 does not contain Sec in its primary sequence, nor the Cys residue at position 17 in the N-terminal glycine-rich loop. This glycine-rich loop is thought to be involved in delivery of Se as perselenide intermediate to the
SPS1, a reaction dependent on potassium ions [57, 62, 63]. The human SPS1 crystal structure revealed that proper catalysis requires consumption of the two high-energy phosphoester bonds present in adenosine triphosphate (ATP). The analysis of this structure also suggested that a mechanism may exist in the cell between the SPS and the Sec synthase (SecS) for the direct transfer of Se-containing molecules such as selenide or Se≈P. This mechanism may play a role in the avoidance of cellular Se toxicity [57].

Contrary to SPS1, it has been successively demonstrated that SPS2 is essential to eukaryotic selenoprotein biosynthesis [30, 31, 61]. Although the crystal structure of SPS2 has not yet been elucidated, the fact that it possesses a Sec residue in its peptide sequence suggested early on that an auto-regulatory mechanism for selenoprotein biosynthesis was in place [60]. In rats, renal SPS2 mRNA has been shown to be significantly upregulated in response to Se deficiency [64]. SPS2 mRNA was also induced in heart, brain and testes of mice with genetic deletion of the Se-transporter protein Selenoprotein P (Sepp1) [65], suggesting that on conditions of low Se availability, SPS2 may facilitate recycling of Se [66].

Se≈P serves as the Se donor for Sec biosynthesis [30]. In addition to dietary Se, the Se≈P pool for Sec biosynthesis may include Se derived from selenoprotein degradation. Upon degradation, Sec may be released and subsequently broken down, yielding free Se. Sec biosynthesis occurs on its own tRNA^{[Ser]Sec} [31, 67], as shown in Figure 1.3. Interestingly, the specific tRNA for Sec is first loaded with a serine (Ser), which serves as the backbone for Sec synthesis [45]. Upon phosphorylation of the seryl moiety attached to the tRNA^{[Ser]Sec} by phosphoseryl-tRNA^{[Ser]Sec} kinase (PSTK) and subsequent removal of a hydroxyl group by PSTK, the phosphoserine combined with the Se≈P from SPS becomes the substrate for the enzyme SecS. The SecS-catalyzed reaction substitutes Se≈P for the phosphoryl moiety of Ser. The Sec-loaded tRNA^{[Ser]Sec} can then be methylated at position Um34, a modification required for the synthesis of a subset of selenoproteins [68]. Sec-tRNA^{[Ser]Sec} is thus ready for proper insertion of Sec into the ribosomal A-site for translation [31]. Sec biosynthesis and incorporation into selenoproteins also depend on the protein SECp43 [69, 70], although its specific biochemical function remains unknown.
Synthesis of selenoproteins requires that the UGA stop codon becomes recoded to translate Sec. This energy-consuming mechanism depends on several specific cis and trans elements [56]. The presence of the SECIS in the 3'UTR region of the selenoprotein mRNA represents the cis element [20]. The trans-acting factors are formed by the SECIS-binding protein (SBP2), the tRNA^{Ser}Sec, the ribosomal protein L30 [71], and the Sec elongation factor (EFSec) [72-74]. These four trans-acting factors plus the SECIS element are considered the core factors for Sec incorporation [75]. They act in an orchestrated fashion to allow the SECIS element and the bound complex SBP2/EFSec/tRNA^{Ser}Sec to be able to influence the insertion of the Sec at the UGA codon. The protein-RNA complex would then avert the binding of releasing factors that signal termination [76-78]. Sequence context around the UGA codon has also been shown to exert an effect on termination efficiency and Sec incorporation [75, 77, 79-81].

Attempts to develop whole-body knockout (KO) mouse models for several of the factors involved in Sec production and/or incorporation into selenoproteins, including tRNA^{Ser}Sec, SPS1, SBP2, SecS and SECp43, proved to be unsuccessful, leading mostly to embryonic lethality ([82] and Berry MJ, Hatfield D and Schweizer U, personal communication). Such outcome corroborates the early predictions of Schwarz & Foltz [4], who stated that Se is essential to survival.

1.4. Selenoproteins

Humans have 25 selenoproteins [83] and mice have 24 (summarized in Table 1), all containing the highly reactive Sec in the active site. Sec is usually deprotonated at physiological pH [84, 85], which renders its highly reactive state, and increases the efficiency of selenoprotein enzyme activity. Most selenoproteins participate in reactions involving redox state changes, thus their function is largely to promote antioxidant defense or detoxification [86, 87].

In mice, four selenoprotein isoforms of GPx are responsible for reducing hydroperoxides and lipid and phospholipid hydroperoxides, thus avoiding the propagation of free radicals and reactive oxygen species (ROS) that could ultimately damage cell viability. Glutathione is an essential cofactor in these reactions.
Three selenoprotein isoforms of TrxR mainly control the reduced state of thioredoxin, a class of small redox proteins. TrxR isoforms are involved in regeneration of many intracellular antioxidants that are required to maintain intracellular redox states, reduce nucleotides that are required for DNA synthesis, and have prominent roles in the regulation of gene expression by redox control of transcription factors ([88] reviewed in [86]). The subcellular localization of each isoform differs: TrxR1 is a nuclear and cytosolic protein, TrxR2 is mitochondrial and TrxR3 is found in microsomal fractions [89].

In addition, three isoforms of iodothyronine selenodeiodinases (Dio) control thyroid hormone homeostasis via activation of thyroid hormone globally (Dio1) and locally (Dio2), as well as by inactivating excess circulating thyroid hormone (Dio3). Although these are neither antioxidant nor detoxifying reactions, deiodination of thyroid hormones requires thiol cofactors to regenerate the active enzymes [90]. The presence of Sec at the active sites of these enzymes is required to efficiently perform these reactions.

Selenoprotein R (SelR), also called MsrB1, catalyzes the reduction of methionine-R-sulfoxide residues in proteins. It was discovered through bioinformatics approaches and it was shown to be localized in the cytosol and nucleus [91].

Selenoprotein K (SelK) [92], Selenoprotein M (SelM) [93], Selenoprotein N (SelN) [94], Selenoprotein S (SelS) [95], Selenoprotein T (SelT) and 15-kilodalton (kDa) Selenoprotein (Sep15) [96] reside in the endoplasmic reticulum (ER). With the exception of SelT, they all have been demonstrated to participate in pathways associated with ER redox systems [97], suggesting a role in protein misfolding mechanisms and/or ER-dependent cellular stress regulation. SelS was identified as a part of the ER-associated degradation (ERAD) machinery, binding to Derlin-1 and Derlin-2, proteins that form the ER transmembrane channel for retrotranslocation of misfolded proteins [98-101]. Glycosylated SelK also binds to components of the ERAD complex and the chaperone calnexin, as well as components of the oligosaccharyltransferase complex. The binding partnership with calnexin suggests that SelK may be involved in ERAD of glycosylated misfolded proteins [102]. SelN is a glycoprotein required for proper function of the Ca^{2+}-induced Ca^{2+} release mediated by the ryanodine receptor. SelN possibly serves as a reductase for the several Cys residues of the ryanodine receptor [103]. Sep15 is a binding partner of
UDP-glucose:glycoprotein glucosyltransferase, a chaperone involved in quality control of protein folding in the ER [104]. SelM was implicated in regulation of Ca\(^{2+}\) homeostasis in neurons [105], as was SelT, although the latter was also found to be specifically involved in neuropeptide PACAP release from the pituitary in rats [106].

Selenoprotein H (SelH) is a nuclear protein and may function as a transcription factor [107]. The mRNA levels of Selenoprotein W (SelW) are highly dependent on Se levels [108]. SelW was one of the earliest selenoproteins discovered and is linked to muscle growth and differentiation. SelW was found to contain a thioredoxin-like fold and its possible function may be related to oxidative stress protection [109].

The functions of other eukaryotic selenoproteins such as Selenoprotein O (SelO) and Selenoprotein V (SelV) are still largely unknown.

1.4.1. Serum selenoproteins: GPx3 and Sepp1

Se is transported through the circulatory system bound to plasma proteins such as selenoalbumin, and as Sec residues incorporated into the primary sequence of GPx3 and Sepp1.

GPx3 is mainly produced in the kidneys and secreted into the bloodstream. Although serving as a local pool for Se in the kidneys [110], it is mainly found in circulation. GPx3 plasma levels depend on Se status and individual hypoxic state [111]. Nevertheless, independent of Se status, the plasma GPx3 pool encompasses approximately 20% of the circulating Se ([112, 113] reviewed in [86]). In addition, GPx3 has been shown to regulate the bioavailability of nitric oxide produced by the platelets and vascular cells [114] and to be one of the most highly expressed selenoproteins in the thyroid gland, an organ that maintains high Se content even during Se deficiency [115].

Sepp1 is a unique vertebrate selenoprotein containing multiple Sec residues per protein molecule: 10 in mice and humans; 17 in the fish species Danio rerio [116], Salmo salar, Cyprinus carpio, and Fundulus heteroclitus; 18 in the African clawed toad Xenopus laevis; and 28 in the sea urchin Strongylocentrotus purpuratus [117]. Sepp1 homologs with Cys in place of Sec were found in the nematode Caenorhabditis elegans and in the sea anemone Nematostella vectensis [117].
The Sepp1 primary sequence contains one Sec in the amino terminal region of the protein and a cluster of Sec's in the middle to the carboxyterminal region [113]. Sepp1 mRNA contains two SECIS elements, which were later revealed to function separately during translation [118]. While the second SECIS works primarily to allow incorporation of Sec at the first UGA, the first SECIS is mainly responsible for the coordinated incorporation of the several Sec codons clustered in the C-terminal part of the coding region. The process of incorporating many Sec's in a single translation attempt is the object of several interesting discussions [79, 119-122]. The two SECIS have different processing efficiencies, the second SECIS being much lower than the first one [123]. It was suggested that due to this variation in SECIS processing efficiency, the first UGA of Sepp1 serves as an obstacle to slow down the progress of the ribosome, preventing the stall of several ribosomes in the Sec-rich area of the mRNA [118, 124].

The liver is the primary site of Sepp1 production. In hepatocytes, Sepp1 can potentially bypass the Golgi apparatus and be secreted as an incompletely processed form. This secretory process is dependent on extracellular Ca\(^{2+}\) as well as Ca\(^{2+}\) from the ER-regulated stores [125]. After Sepp1 is released into the bloodstream, it works as the main Se carrier for other tissues in the body possibly via interaction with endothelial cells [126, 127]. Four isoforms of Sepp1 are found mainly in the plasma. The shortest one has a single Sec residue, suggesting that Sepp1 may have an additional unknown function besides serving as a general Se transporter [128]. Sepp1 is thought to preferentially act as a transporter of Se to specific tissues, thus serving as a means of homeostatic redistribution of tissue Se reserves to vital tissues. The specific mechanism of Se transport into the cells is unknown, but there is evidence of interaction between Sepp1 and the low-density lipoprotein (LDL) receptors Apolipoprotein-E receptor 2 (ApoER2) in the brain [129] and testis [130], and interaction between Sepp1 and megalin in the kidneys [131]. Nevertheless, neither ApoER2 nor megalin are found in the liver, and the mechanism of Se uptake carried out in this organ is currently unknown.
1.4.2. Hierarchy of selenoprotein production

Selenoprotein production is regulated on several levels, including transcription, translation and degradation. When Se is limiting, prioritization of the synthesis of some selenoproteins occurs over others. The early observation of such hierarchy involved measuring the activity and mRNA of GPx isoforms in Se deficiency. While GPx1 activity decreased dramatically in Se deficient states, GPx4 only decreased ~40% [132] and the mRNA levels followed the same pattern [133].

The variability of Se pools in different tissues of the body may be associated with the variations in hierarchy. Tissues that uptake more Se, such as liver and kidneys, are likely to possess tighter selenoprotein biosynthesis regulation [43].

Selenoprotein mRNAs vary in turnover and stability levels. The presence of a UGA codon in the sequence of selenoprotein mRNAs makes them potentially susceptible to degradation by the nonsense-mediated decay (NMD) pathway. NMD is a surveillance mechanism that targets mRNAs containing premature stop codons to degradation, ensuring that they do not produce truncated polypeptides. The position of the premature stop codon is important to the fate of the mRNA, and degradation is circumvented if the stop codon is located >55 nucleotides from the last intron splice junction [134, 135]. Se deficiency favors the recognition of the UGA codons in selenoprotein mRNAs as stop codons, preventing Sec incorporation and consequently full-length selenoprotein production [136-138]. Nevertheless, the variability of UGA codon locations and the varying responses to Se deficiency raise the possibility of a mechanism, other than NMD, to act upon selenoprotein mRNAs in a state of Se deficiency [139].

The binding affinity of SBP2 to the different SECIS elements of selenoprotein mRNAs may explain the hierarchy distinction in the mechanism of selenoprotein biosynthesis. Reports from our laboratory determined that SECIS elements from different mRNAs exhibited different abilities to compete for SBP2 [140]. Additionally, SBP2 presents preferential binding to certain mRNAs when exposed to the same Se conditions. This preferential affinity of SBP2 changes according to the cell line analyzed, and it results in the overall hierarchy of selenoprotein synthesis [141].

Whole-body disruption of Sepp1 expression in a mouse model revealed more details of the hierarchical regulation of selenoprotein transcription [65]. A subset of
selenoproteins appears to be more affected by the absence of Se transport than others. The authors of the aforementioned report speculate that the differences in expression in selenoproteins upon disruption of Se transport or Se deficiency are related to increases in NMD, at least in organs that are more affected by the Sepp1 disruption.

The presence of different expression patterns for the same selenoprotein in various tissues of the Sepp1 KO mouse, such as upregulation of GPx4 in the heart and downregulation in brain and testes, added a tissue-specific level of regulation to the selenoprotein hierarchy regulation. Moreover, Sepp1 KO mice on Se supplementation still maintain low levels of Se in the brain [142], corroborating the existence of a tissue-specific mechanism for Se uptake, and thus selenoprotein production.

Another possible molecular explanation for the hierarchy in selenoprotein translation has been recently suggested. As discussed in section 1.3, the tRNA[^Ser]^Sec needs to undergo a methylation step prior to being able to properly insert Sec into the ribosomal site. Interestingly, for the subset of selenoproteins required for survival, the isoform of tRNA[^Ser]^Sec involved in their translation does not contain the methylation site Um34. On the other hand, for stress-related selenoproteins, the Um34 isoform appears to be required for proper Sec incorporation [68]. Thus, the methylation of Um34 seems to be one more level of control for the hierarchy of selenoprotein translation.

1.4.3. Selenoproteins and human health

From the early studies discussed in section 1.1, Se used to be regarded as a toxic element. Se toxicity, or selenosis, is defined by the chronic intake of more than 400 µg daily [143]. Selenosis is characterized in humans by garlic breath, nail and hair loss, disorders of the nervous system, and paralysis [32]. Severe Se deficiency is found in northeastern areas of China, leading to Keshan disease, a reversible congestive cardiomyopathy that may be caused by a Coxsackie virus infection on a background of Se deficiency ([144] reviewed in [145]). Keshan disease was virtually eradicated by supplementation of Se to the affected populations. Nevertheless, Se toxicity and severe Se deficiency are considered rare pathologies. More commonly,
slight changes in Se availability are found in the human population, and these moderate changes are the ones implicated in far more disease states.

For human health, most nutrients are regarded to act in a U-shaped curve profile, and Se is not an exception for this rule [146]. For instance, there is a narrow optimal concentration value where one can weigh the risks and benefits from Se intake. Deviations from this small range will affect several aspects of human health and lead to the problems discussed below. Moreover, due to the action of selenoproteins in most tissues of the body and their crucial participation in anti-inflammatory mechanisms and cell detoxification, this class of proteins is involved in the pathogenesis of several human diseases.

The most researched role of Se in human health is related to cancer prevention [147]. Despite the failure of the Selenium and Vitamin E Cancer Prevention Trial (SELECT) to prove such benefit for prostate cancer [148], rigorous analysis of other clinical trials in comparison to SELECT have demonstrated that the resulting benefits of Se supplementation on cancer prevention depend ultimately on baseline Se levels in participating subjects. Se reduced total cancer incidence when baseline Se levels were lower than 106 µg/L ([149, 150] reviewed in [32]). Furthermore, Se supplementation has been shown to reduce the incidence of liver, esophageal, pancreatic, colon and mammary carcinogenesis [151].

Due to the antioxidant function of several selenoproteins, one could safely predict they would be involved in various aspects of cancer cell physiology. In fact, polymorphisms in GPx2, GPx4, and Sepp1 have been implicated in colorectal cancer, while SelS and Sep15 polymorphisms were implicated in lung and gastric cancer, respectively [33, 147]. Mice whose GPx1 gene has been deleted were more sensitive to oxidative stress than wild type (WT) mice [152] and mice overexpressing this gene had reduced tumorigenicity following injection of cancer cells into nude mice [153]; however, if both GPx1 and GPx2 genes are disrupted, the mice spontaneously develop colon tumors [154]. Sep15 knockdown in colon carcinoma cells from mice and humans inhibited cell and tumor growth [155, 156]. Finally, increased TrxR1 expression occurs in thyroid cancer [157] as well as in oral squamous cell carcinoma [158].

The neurological dysfunctions and male sterility [159] present in Sepp1 KO mice [160, 161] highlighted the crucial importance of maintaining Se homeostasis in
the brain and testes, which appear to be tissues that are hierarchically protected against low circulating Se levels. Dietary Se deficiency affects the Se concentrations in these tissues the least; however, when extreme experimental conditions such as selective sequestration or genetic knockouts are employed to induce unusually severe Se deficiencies, the brain and neuroendocrine tissues are the most affected.

Increases in ROS have been linked to the pathogenesis of several neurodegenerative diseases, including Alzheimer's and Parkinson's Diseases [162]. Not surprisingly, the levels of selenoproteins GPx4 and Sepp1 as well as their gene expression patterns in different brain cell types are altered in individuals with both pathologies [163, 164].

The fact that an entire group of selenoproteins, the deiodinases, are the main regulators of thyroid function exposes the deep relationship between this trace element and endocrine function. Low Se levels have been associated with thyroid disorders such as goiter (reviewed in [165, 166]). In addition, mutations in the SBP2 gene in humans were shown to lead to decreases in Dio2 levels and consequent abnormal thyroid metabolism [167]. Interestingly, mice lacking selenoprotein biosynthesis in the thyroid epithelial cells still maintain normal thyroid function despite the increases in oxidative stress due to a lack of GPx's and TrxR's. The maintenance of normal thyroid hormone levels suggests that either these proteins are not essential to thyrocyte survival or their function is being compensated by an alternative and unknown mechanism [168].

Mice with targeted disruption of Dio2 lack proper adaptive thermoregulation [169] and are more prone to high fat (HF) diet-induced obesity [170]. Overexpression of GPx1 induces hyperinsulinemia and insulin resistance [171, 172] while a lack of Dio3 gene disrupts proper production of insulin in pancreatic β-cells [173]. Moreover, liver-specific deletion of tRNA[^Ser][^Sec] in mice elevates plasma cholesterol levels, suggesting a role of selenoproteins in lipid metabolism [174]. The details of these mouse models and their phenotypical characteristics in relation to energy metabolism will be discussed in subsequent Chapters of this dissertation.

In addition to the roles played by selenoproteins in energy and thyroid metabolism, Se is essential for testosterone biosynthesis and normal development of spermatozoa [175]. In testes, Se is present as part of GPx4, a protein that is crucial for the architecture of the spermatozoan midpiece and a major protector against
oxidative damage of spermatozoan DNA [176]. Subfertile/infertile men displayed improvements in sperm quality upon Se supplementation [177]. Such improvement, however, was dependent on baseline Se levels of the analyzed population.

The data are scarce on the relationship between Se and female fertility. Nevertheless, Se, which is transported through the placenta, is required for proper fetal development. Selenoproteins Sepp1 (Kurokawa S, personal communication), GPx4, GPx1, SelK, TrxR1, SelT, Sep15 (Gilman CL, personal communication), and Dio3 [178, 179] are highly expressed in the placenta, thus suggesting that Se levels are relevant to fetal development at least at a certain stage of pregnancy.

Lastly, immune function also appears to be affected by Se levels. Se deficient mice are more susceptible to viral infection [180]. Se supplementation suppresses HIV replication induced by tumor necrosis factor alpha (TNFα) and protects against coxsackie virus infection and virulence [181]. Deletion of the GPx1 gene rendered mice vulnerable to coxsackie virus-mediated myocarditis as a consequence of increased mutations in the viral genome [182]. Moreover, selenoprotein deficiency caused by T-cell specific deletion of tRNA^{Ser}Sec in mice leads to a dysfunctional adaptive immune response [183].

1.5. Selenocysteine Lyase

Interestingly, the role of Se in human health has mainly been analyzed and measured based on dietary Se [34]. Although the importance of dietary Se is not disputed, this perspective still neglects a source of Se for the cellular pool that may be valuable in conditions of Se limitation. Along with Se delivery, cells have evolved mechanisms to recycle Sec when Se is limiting, in order to maintain essential redox functions. This recycling system utilizes selenocysteine lyase, also called selenocysteine β-lyase (Scly).

Scly was first isolated and characterized from pig livers in 1982 [184], and subsequently identified in bacteria [185, 186]. cDNA for the mouse Scly gene was cloned in 2000 [187], and subsequent BLAST searches have revealed sequence similarity between Scly and several orthologous genes deposited in databases of
species such as humans, platypus, chicken, zebrafish, green spotted pufferfish, sea squirt and nematodes [188].

Scly is a 47.1 kDa enzyme that was shown to function as a homodimer, breaking down Sec into alanine (Ala) and selenide [184, 187, 189] in a reaction that requires pyridoxal 5'-phosphate (PLP) as a cofactor. In anaerobic conditions, the Se product of the Scly enzymatic reaction is elemental Se, while in the presence of thiols, it is selenide [45, 186]. Due to the binding of PLP to a highly conserved lysine (Lys) residue, Scly is considered to be part of the class V PLP-dependent aminotransferase family [190].

The biochemical reaction catalyzed by Scly in physiological conditions is simplified below:

\[
\text{Scly} \quad \text{Sec} \rightleftharpoons \text{Ala} + \text{H}_2\text{Se} \\
\text{PLP}
\]

Scly interacts directly \textit{in vitro} with SPSs [191]. Mouse Scly has been suggested to effectively provide Se from L-Sec to SPS for the production of Se\textsuperscript{~P}, which is the initial step for selenoprotein synthesis [188]. The rat Scly X-ray structure (Figure 1.4A) was demonstrated to be very similar to \textit{E. coli} IscS protein [192] and \textit{Thermotoga maritima} NifS-like protein ([193], Figure 1.4B), both group I Cys desulfurases, a subgroup of the class V aminotransferase family that requires PLP for proper homodimerization. Cys desulfurases are enzymes that break down Cys into Ala and S, a biochemical reaction very similar to that of Scly.

The close structural similarity between Sec and Cys (Figure 1.2) led to the speculation that Scly might utilize both as substrates. In fact, bacterial Cys desulfurases can use Sec and Cys as substrates [187]. Nevertheless, the specificity of the eukaryotic Scly towards Sec was demonstrated in early studies [184] and further explained more recently [194-196]. Briefly, analysis of the rat Scly crystal structure revealed that the critical residue Cys375 directs L-Sec but not L-Cys to the correct position in the active site of Scly, to bind PLP. This analysis indicated that Cys375 was the selenopersulfide-forming residue. In addition, in one of the possible enzyme conformations upon Sec binding, the Se atom further interacts with His133 [194]. A recent study identified Asp146 as a key residue for Sec recognition [195].
The specificity of the active site of Scly for Sec is determined by residue Asp146. Asp146 enables tighter steric distance to Sec than it does to Cys [196]. Therefore, the Scly specificity for Sec is likely to be critical to avoid competition with the larger cellular pool of Cys. In a mutational analysis, substitution of the amino acid residue Asp146 with Lys produced a Scly enzyme able to catalyze Cys desulfuration [195].

Overall, the *in vitro* studies mentioned above suggest a chemical and mechanical sorting control mechanism for the Sec specificity in an environment where Cys is orders of magnitude more available, such as in the cells.

### 1.6. Physiological role of Scly

Although substantial knowledge is available regarding Scly biochemical properties, its physiological role remains unclear.

Early studies showed that most Scly activity was found in the liver, followed by the kidneys [184]. Activity of this enzyme was also detected in rodent tissue homogenates of the pancreas, adrenal gland, heart, lung, testis, brain, thymus, spleen and muscles, but not detected in blood and fat [184]. When the mouse Scly was cloned, the mRNA was found ubiquitously, and the protein was observed at its highest levels in the liver, kidneys and testes [187]. Since the primary metabolism of Se occurs in the liver and kidneys [124], it is possible that Scly activity is connected with Se metabolism.

Scly plays a potentially important role in linking Se to the machinery of selenoprotein synthesis (Figure 1.5). Using knock down approaches in HeLa cells, Scly was recently demonstrated to be required for selenoprotein biosynthesis [197], although its subcellular localization, previously regarded as cytosolic [187], was challenged by the appearance of nuclear staining corresponding to Scly in mouse kidneys and liver [197]. Nevertheless, the physiological role of Scly in cellular Se metabolism and its involvement in whole body Se homeostasis remain largely unknown. In addition, information is also lacking on: a) the regulation of Scly expression and activity; b) the physiological importance of Scly to Se metabolism and to the hierarchy of selenoprotein synthesis; and c) the potential crosstalk between Se recycling and other biochemical pathways.
1.7. Research objectives

The central hypothesis of my dissertation is that Se-regulated Scly plays a unique physiological role in the metabolism of Se in the whole body and/or in tissue-specific Se homeostasis and selenoprotein synthesis.

In the following chapters of the dissertation, I will:

1) Describe the effects of Se treatment on Scly expression. To achieve that, I measured Scly mRNA in response to Se supplementation in different biological models (Chapter 2).

2) Report the characterization of the Scly KO mouse, a model developed as a tool to investigate the role of Scly in whole body Se homeostasis and selenoprotein synthesis. In this study I describe the general phenotype of the mouse model and its Se-dependent characteristics upon an adequate dietary intake of Se (Chapter 3).

3) Provide details revealing a novel physiological role of Scly in energy metabolism. In Chapter 4, I pursued a thorough investigation of the compounding effect of Scly disruption and low Se diet on glucose and lipid homeostasis in mice.

4) Discuss the role of Scly in the aggravation of obesity. In this study, I challenged Scly KO mice with a high fat diet to investigate the importance of Scly on whole-body glucose and lipid homeostasis (Chapter 5).

5) State my concluding remarks in Chapter 6 regarding the scope of this dissertation and summarize the main conclusions drawn from it.
1.8. Figures

*Figure 1.1. Cartoon representation of the different views of Se throughout scientific history. From [198].*
Figure 1.2. Chemical structure of S- and Se-containing amino acids. The pKa value and the protonation state in physiological pH is shown for cysteine and selenocysteine. (From [199], with permission.)
Figure 1.3. Scheme of Sec synthesis on its own tRNA\textsuperscript{[Ser]}\textsuperscript{Sec}. The tRNA\textsuperscript{[Ser]}\textsuperscript{Sec} is first loaded with Ser, a reaction that consumes ATP. Upon action of the enzyme PSTK, the seryl moiety is phosphorylated (PSer). Se~P (or SeP) can then substitute the PSer via action of SecS. After such catalysis, the tRNA is loaded with Sec and ready to be used for selenoprotein biosynthesis [200].
Figure 1.4. Scly crystal structure. A) Ribbon model of rat Scly in a homodimer assembly. B) Comparison with the predicted structure of rat (red), human (orange) Scly and other Cys desulfurases from Thermotoga maritima (blue) and Escherichia coli (green). The stick models in B represent the PLP-binding site and the active site of Scly. Figure from [194].
Figure 1.5. Schematic diagram of Sec incorporation into selenoproteins. The scheme highlights the possible role of Scly (orange circle) in the de novo synthesis of Sec, connecting the degradation of selenoproteins to the incorporation of Sec. Scheme modified from [30].
Table 1. List of Murine Selenoproteins and their Functions.a.

<table>
<thead>
<tr>
<th>Selenoproteins with predominantly antioxidant functions:</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>GPx1</td>
<td>Uses glutathione to detoxify peroxides in aqueous compartment of cytosol</td>
</tr>
<tr>
<td>GPx2</td>
<td>Expressed in cytosol of liver and tissues of digestive system</td>
</tr>
<tr>
<td>GPx3</td>
<td>Antioxidant function in plasma; Se transport to other tissues</td>
</tr>
<tr>
<td>GPx4</td>
<td>Prevents/reverses oxidative damage to lipids in brain, testis and other tissues</td>
</tr>
<tr>
<td>TrxR1</td>
<td>Interacts and/or recycles thioredoxin and other redox regulating pathways</td>
</tr>
<tr>
<td>TrxR2</td>
<td>Located in mitochondria, controls and regulates redox state of local milieu</td>
</tr>
<tr>
<td>TrxR3/TGR</td>
<td>Reduces glutathione disulfide, specific physiological function undefined</td>
</tr>
<tr>
<td>SelH</td>
<td>Transcription factor, regulates transcription of detoxification genes</td>
</tr>
<tr>
<td>SelI</td>
<td>Involved in phospholipids biosynthesis pathway</td>
</tr>
<tr>
<td>SelK</td>
<td>May participate in detoxifying mechanisms in the endoplasmic reticulum</td>
</tr>
<tr>
<td>SelM</td>
<td>Ubiquitous expression, primarily in the brain, involved in calcium handling</td>
</tr>
<tr>
<td>SelN</td>
<td>Mutations associated with several muscle disorders</td>
</tr>
<tr>
<td>SelR/MsrB1</td>
<td>Repairs oxidatively damaged methionine (R-sulfoxides)</td>
</tr>
<tr>
<td>SelS</td>
<td>Helps in detoxification in the endoplasmic reticulum, regulated by glucose</td>
</tr>
<tr>
<td>SelT</td>
<td>Involved in regeneration processes of nervous and endocrine tissues</td>
</tr>
<tr>
<td>SelW</td>
<td>Expressed ubiquitously, may regulate redox state of 14-3-3 proteins</td>
</tr>
<tr>
<td>Sep15</td>
<td>An oxidoreductase that may assist in disulfide formation and protein folding</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Selenoproteins with endocrine functions</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Dio1</td>
<td>Activates thyroid hormone, converts T4 to T3</td>
</tr>
<tr>
<td>Dio2</td>
<td>Regulates local supply of thyroid hormone, by activation and inactivation</td>
</tr>
<tr>
<td>Dio3</td>
<td>Inactivates thyroid hormone in brain, placenta and pregnant uterus</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Selenoproteins with other physiological functions</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>SPS2</td>
<td>Catalyzes formation of Se–P required for synthesis of Sec</td>
</tr>
<tr>
<td>SelP/Sepp1</td>
<td>Primary Se transporter in plasma (10 Sec residues per molecule)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Selenoproteins with unknown function</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>SelO</td>
<td>Identified in silico, redox function?</td>
</tr>
<tr>
<td>SelV</td>
<td>Testis-specific expression</td>
</tr>
</tbody>
</table>

*a Modified from [50]. List compiled from references [83, 88, 90, 92, 95, 96, 105, 107, 201-208]*
CHAPTER 2

REGULATION OF SELENOCYSTEINE LYASE
GENE EXPRESSION

2.1. Introduction

As discussed in the previous Chapter, Se is acquired through the diet and once absorbed by the digestive tract, it is carried mostly to the liver, where it is used for selenoprotein production. All selenoproteins contain the unique amino acid Sec, which is co-translationally incorporated into the peptide chain via the specific tRNA^{Sec} and is usually present as a single residue in the selenoprotein structure.

Because most studies focus on dietary Se, selenoprotein degradation and the subsequent recycling of Sec provides an additional source of Se that is normally neglected when contributions to the Se pool available for cellular homeostasis are measured. The mechanism of selenoprotein degradation is poorly understood. Only the selenoprotein Dio2 was shown to be ubiquitinated and targeted for proteasomal degradation [209]. SelK was demonstrated to bind SelS and Derlin-1 while SelS binds Derlin-1 and Derlin-2 [102], members of the ERAD complex. These interactions suggest that SelK and SelS may play a role in protein degradation mechanisms. The possibility of SelK and SelS participation in such mechanisms, however, still leaves an open question of how these two proteins are themselves degraded.

The biochemical role of Scly in de novo Sec synthesis has been identified to be the main coordinator of Se recycling (see section 1.5). Crystal structure analysis revealed that Scly acts as a homodimer, directing Sec into binding to PLP and to amino acids in the active site that will mostly keep the Se atom always attached to a molecule. Such kinetics is crucial, because unbound Sec can be toxic to the biological systems and cannot be used directly as a substrate for selenoprotein biosynthesis [45, 210]. Unbound Se in its atomic form is also toxic due to its high
reactivity. Cells have evolved mechanisms, such as Se recycling, to balance the reactive properties of Se while preventing the deleterious effects of this element.

Once Sec is released from the peptide chain, the enzyme Scly specifically recognizes the Sec residue [194] and breaks it down into Ala and selenide using PLP as a cofactor for the reaction [189], as detailed in Chapter 1, section 1.5. The Se atom can re-enter the Sec biosynthesis pathway by binding to a phosphate to generate Se–P in a reaction catalyzed by the SPS enzymes in either prokaryotes or eukaryotes [60, 211]. Se–P can in turn be utilized to synthesize Sec on its cognate tRNA\textsuperscript{[Ser]Sec} for incorporation into newly translated selenoproteins as explained in Chapter 1 section 1.3. Therefore, the Se recycling mechanism is a potentially important pathway for allowing Sec derived from the degradation of selenoproteins to be made available for de novo synthesis of Sec and subsequent incorporation into new selenoproteins.

2.1.1. Scly gene structure

The Scly gene is located on chromosome 1 in mouse (gene ID 50880) and chromosome 2 position 2q37.3 in humans (gene ID 51540). The same genes in both genomes flank the Scly gene, upstream by Ubiquitin Conjugating Enzyme E2F (UBE2F), and downstream by Espin-like. In the mouse sequence, the predicted pseudogene Gm7785 is immediately upstream of the Scly open reading frame, intercalating Scly and UBE2F. According to the National Center for Biotechnology Information (NCBI) database, the UBE2F/Scly gene could be read through in humans, potentially working similar to an operon. Nevertheless, data to support such operon mechanism is lacking.

The Scly gene contains 12 exons in both humans and mice. While the human Scly gene is 38.49 kilobases (kb) in length, the mouse gene is 22.74 kb and has decreased intronic space compared with that of the humans.

2.1.2. Regulation of Scly expression

In terms of direct regulation of gene expression, research on the Scly promoter architecture has been very limited. The Activator Protein-1 (AP-1) binding
site at position -154 was found in the rat Scly promoter [212]. The AP-1 site is regulated by hydrogen peroxide (H$_2$O$_2$) [213] and, in the rat Scly promoter, it is bound specifically to the subunit c-fos of transcription factor AP-1. AP-1 modulates several processes critical for carcinogenesis, such as cell proliferation and invasion [214]. Interestingly, Scly was found in the aforementioned report [212] to be one of the few differentially expressed genes in a comparison of acute glomerulonephritis and chronic glomerulonephritis in rodents. The Scly gene was upregulated in acute glomerulonephritis, and downregulated in chronic states of the disease. A later study identified Major Urinary Proteins 1 and 2 (MUP1 and MUP2) as interactors of Scly via yeast two-hybrid screening [191]. In addition, it was demonstrated that Scly gene expression was increased upon treatment with interleukin-1beta (IL-1β) in kidney mesangial cells, thus suggesting the regulation of Scly could be influenced by the increases in oxidative stress generated by an inflammatory response. It is worth noting that IL-1β is produced by macrophages and monocytes upon acute injury, such as scraping, excessive heat or oxidative injury by H$_2$O$_2$ [215]. It is suggested that Scly participation in renal diseases could be a consequence of an inflammation-mediated oxidative stress injury. The IL-1β effect on Scly gene expression may occur directly on the AP-1 site, induced by changes in the redox tonus of the cell [212].

Besides AP-1 and IL-1β, other gene expression regulators of Scly have not been identified. Nevertheless, the same differential expression approach was used to uncover Scly as the single gene in HepG2 hepatocellular carcinoma cells to respond after infection with Core protein of the hepatitis C virus [216]. Hepatocellular carcinoma is a condition known to decrease cellular availability of Se [217]. In this study, the increased expression of Scly was speculated to protect the cell from oxidative damage, by increasing selenoprotein levels, which in turn would improve antioxidant response. Therefore, an association between Scly expression and the genesis of viral-induced hepatocellular carcinoma remains to be further confirmed.

No other study has reported on the regulation of the Scly gene. Such lack of information on the nature of Scly regulation is surprising, given its potential function in a key step of the selenoprotein biosynthesis pathway [197].
2.1.3. Se regulation of Scly

Se metabolism is closely related to mechanisms that detoxify free radicals in cells. Thus, it is expected that several genes involved in Se metabolism and/or selenoproteins can be regulated by oxidative tonus, including Scly. An in silico approach from our laboratory [218] found putative antioxidant response elements (ARE) and metal response elements (MRE) in most selenoprotein promoters, indicating that at least a subset of selenoproteins is regulated by oxidative status and/or metal availability, respectively. Except for the tRNA[^Ser^Sec] [219-224], relevant binding elements still remain to be identified in the promoter of the genes that encode all other selenoprotein synthesis factors.

Interestingly, Se was promptly ruled out as a regulator of Scly activity [210, 225]. In rats and humans, no correlation was verified between Se levels and Scly activity. In humans, there was no correlation between Scly activity and Se content in the liver, kidney, heart, adrenal and muscle [210]. Nevertheless, this study did not detect Scly activity in the human brain, while in rodents Scly is found to be active in this tissue. Such discrepancy may have resulted from the method employed for tissue collection or from the specific nature of the enzyme in human brains. Rats fed diets with different chemical forms of Se (selenite, SeMet and selenocystine) did not have significant discrepancies for Scly activity in the liver, kidney, testis and muscle, even though those Se diets contained a concentration of Se that was 100-fold lower than that found in the basal torula-yeast diet (0.02 vs 2 ppm) [225].

Despite the negative evidence indicating that Se does not regulate Scly activity, it is possible that Se could modulate Scly mRNA levels in the cell. The possible regulation of Scly gene expression by Se, however, remains to be tested. The concentration of Se (2 ppm) employed previously [225] is now considered to be borderline toxic [226]. Such high concentration of Se, despite not altering Scly activity, could potentially affect protein and mRNA levels of Scly and other Se-related proteins. Moreover, the only recognized function of Scly to date has been its involvement in selenoprotein biosynthesis through Sec catabolism [197]. Nonetheless, the possibility that Scly-dependent pathways other than that of selenoprotein synthesis may exist cannot be discarded.
2.1.4. Scly differential expression in cells and tissues

The existence of a selenoprotein hierarchy, as explained in Chapter 1 section 1.4.2, suggests that different cells and tissues rely differentially on Se. This hierarchy may also indicate variable efficiency of the selenoprotein synthesis pathway. Mouse Scly mRNA was detected in the brain, heart, stomach, liver, kidney, spleen, lung and testis. In addition, Scly protein was shown to be most abundant in the liver, kidneys and testes, followed by spleen, brain and heart [187]. Interestingly, the thyroid, the organ that accumulates more Se per gram of tissue, was not analyzed by these early reports. Nevertheless, the regulation of Scly expression by different concentrations of Se remains to be examined.

As explained in Chapter 1 section 1.4.1, Sepp1 is the plasma Se transporter. It is speculated that with tissue-specific Se deficiency, and specially in those tissues where the requirement for Se is greater, recycling mechanisms should be actively in place to compensate for the limiting Se availability. Sepp1 KO mice was previously described to have lower Se levels in most tissues when fed a Se adequate diet containing ~0.25 ppm Se [142, 160, 227]. On a low Se diet, Sepp1 KO perished after a few weeks of age. On Se supplementation, the Sepp1 KO mouse retained normal Se levels in most tissues, except brain and testis [228]. Interestingly, Sepp1 KO mice had increased hepatic Se content [160]. Therefore, the Sepp1 KO mouse provides a good organismal model to study the effects on Scly expression of a tissue-specific Se deficient environment, as long as the animal is maintained on a Se-adequate diet.

In this Chapter, the regulation of Scly by Se levels will be experimentally addressed by three different approaches:

a) in silico investigation of the Scly gene promoter for response elements relevant to Se regulation;

b) in vitro measurement of the effects of Se treatment on Scly mRNA levels in two different cell lines; and

c) in vivo analysis of Scly mRNA expression in Se-fed mice and in a mouse model of whole-body Se deficiency, the Sepp1 KO mouse. Scly mRNA expression in Sepp1 KO and WT mice will be measured in tissues involved in Se metabolism and/or Se-dependent, such as liver, testis, kidney, brain, lung, heart, spleen, intestines.
2.2. Methodology

2.2.1. Materials

All chemicals used were from Sigma-Aldrich (St. Louis, MO), unless otherwise noted.

2.2.2. Promoter analysis

Transfac 6.0 [229] and Patter-Matrix online softwares were used for promoter analysis. The human Scly gene sequence from NCBI (gene ID 51540) was used as the entry for the Transfac 6.0 online software promoter analysis. A 2,000 base pairs (bp) fragment upstream of the first AUG codon site for translation was specifically used for the analysis, performed with the collaboration of Mr. Mahdi Belcaid, from the Bioinformatics Core Laboratory at the Department of Information and Computer Science, John A. Burns School of Medicine - University of Hawai'i.

2.2.3. Cell culture

Human hepatocellular carcinoma (HepG2) and mouse hippocampal neuronal (Ht22) immortal cell lines from American Type Culture Collection (ATCC, Manassas, VA) were maintained in Dulbecco's Eagle's Modified Medium (Gibco - Invitrogen Life Technologies, Carlsbad, CA) plus 10% fetal bovine serum at 37°C with a 5% CO₂ atmosphere. Each batch of fetal bovine serum was tested for its Se content by mass spectrometry, and the same lot used throughout the study. The chosen lot for this study had ~30 nM Se. Seventy or 170 nM sodium selenite were added to media, reaching final concentrations of 100 nM or 200 nM, respectively. Cells were harvested after 48h of supplementation with Se.

2.2.4. Animals and diets

C57BL/6J wild-type (WT) mice from The Jackson Laboratory (Bar Harbor, ME) were bred, born and raised in the vivarium at the John A. Burns School of
Medicine - University of Hawai‘i. All experiments were conducted in accordance with the principles and procedures approved by the Institutional Animal Care and Use Committee, University of Hawai‘i (protocol number 09-871). Sepp1 heterozygotes were a kind gift of Dr. Raymond Burk from Vanderbilt University (Nashville, TN). Due to fertility issues of Sepp1 KO mice [142], heterozygote mice are used as breeders to obtain homozygote Sepp1 KO for experiments. The development and characterization of Sepp1 KO mice was previously described in detail [142]. Briefly, the Sepp1 KO mouse had the neomycin cassette inserted at exon 2, generating a stop codon downstream in all three possible reading frames and disrupting the transcription of the gene.

Diets formulated for specific Se content (Research Diets, Inc., New Brunswick, NJ) have been previously described [230]. Briefly, pellets contained 12% kcal fat, 68% kcal carbohydrate, plus 0.08 ppm of Se in casein (low), or were supplemented with sodium selenite to contain a total of 0.25 ppm (adequate) or 1 ppm (high) of Se. Mice were maintained in the different Se diets for 3 months. Sepp1 KO mice were maintained in standard mouse chow containing 0.25-0.3 ppm Se.

2.2.5. RNA and Quantitative PCR (qPCR) analysis

Cells were collected and total RNA was extracted using Trizol (Invitrogen Life Technologies, Grand Island, NY) followed by treatment with the RNeasy Clean-Up kit (Qiagen, Valencia, CA). Frozen tissues were pulverized on liquid nitrogen and total RNA was also extracted by the Trizol method. One microgram of total RNA was then reverse-transcribed (High Capacity cDNA Reverse Transcription kit, Life Technologies/Applied Biosystems, Carlsbad, CA) and 10 ng cDNA used in qPCR with SybrGreen (Invitrogen Life Technologies). qPCR results were calculated using the ΔΔ^Ct method, normalized to 18S rRNA or GAPD mRNA as housekeeping genes. PCR primers were used at 100 nM and are listed in Table 2 and Table 3.

2.2.6. Statistical analysis

Results were plotted and analyzed using Prism software (GraphPad Software Inc., La Jolla, CA). Student's t-test or one-way Analysis of variance (ANOVA; 1WA),
were applied according to experimental design. \( P \) values are shown in the figure legends or in graphs. All results are means ± standard error of the mean (SEM).

2.3. Results

2.3.1. *In silico* analysis of Scly gene

*In silico* analysis of the Scly gene promoter employing the Transfac 6.0 and Patter-Matrix softwares revealed the absence of MRE but presence of the putative binding sites for POU domain class 2 homeobox 1 (POU2F1 or Oct1, from Octamer binding Transcription factor 1) and for Nuclear factor-erythroid 2-related factor 2 (Nrf2) with a significant score value (Figure 2.1).

Conservancy analysis using the Scly gene sequence of *Pan troglodytes*, *Canis lupus* and *Bos taurus*, in addition to the human gene, revealed that the Oct1 binding site was conserved in the same position in the Scly promoter. The Nrf2 binding site was also conserved in all four species analyzed.

2.3.2. Effect of Se treatment on Scly expression in vitro

Hepatic HepG2 and neuronal Ht22 cells were treated with three concentrations of Se as sodium selenite, 30 nM, 100 nM or 200 nM. These concentrations in cell lines mimic low, adequate and high Se exposure, respectively. The cell lines HepG2 and Ht22 were selected based on the fact that liver and brain are highly dependent on Se. Treatment with 200 nM Se decreased Scly expression in HepG2 cells compared with 30 or 100 nM of Se (Figure 2.2A). In the mouse hippocampal neuronal Ht22 cell line, Scly gene expression remained unchanged in response to varying concentrations of Se (Figure 2.2B).

2.3.3. Effect of Se treatment on Scly expression in vivo

The effect of three doses of sodium selenite on Scly gene expression was tested *in vivo*. Specific diets containing 0.08 (low), 0.25 (adequate) or 1 ppm (high) of Se [230] were fed to C57BL/6J mice. Scly mRNA levels and Se levels were inversely
correlated in both liver (Figure 2.3A) and brain (Figure 2.3B), tissues that are highly dependent on Se to proper function. As dietary Se increased, Scly mRNA levels decreased.

2.3.4. Scly expression in the Sepp1 KO mouse model

The results from C57BL/6J mice fed different Se diets led to the question of whether the Scly expression would be altered if localized Se deficiency was present in key target tissues. To mimic such condition, Scly gene expression was measured in the Sepp1 KO mouse model.

Despite the low levels of Se in Se-dependent tissues, such as brain and testis, Scly mRNA levels did not change in most tissues of the Sepp1 KO mouse (Figure 2.4). Nevertheless, Scly mRNA levels in the liver decreased significantly in the Sepp1 KO mice when compared with WT littermate mice (Figure 2.4).

2.4. Discussion

In this Chapter, Scly gene expression was investigated in silico, in vitro and in vivo. In silico results described in section 2.3.1 pointed to the presence of Oct-1 and Nrf2 binding sites in the promoter of the Scly gene. Oct-1 is a transcription factor with ubiquitous expression that acts mostly as a repressor and is involved in the development of several tissues, including pancreas. The regulation of carbohydrate-related transcription factors by Oct-1 is attenuated by insulin [231]. In addition, Oct-1 has been shown to act as a sensor molecule for oxidative stress [232, 233]. The presence of an Oct-1 binding element in the promoter of the Scly gene suggests a potential influence of carbohydrate metabolic pathways associated with changes in oxidative stress in Scly regulation.

The analysis also unveiled an Nrf2 binding site at position -927 in the promoter. The Nrf2 transcription factor is a member of the Cap'n'Collar family of bZIP (basic region/leucine zipper) transcription factors and is expressed abundantly in liver, intestine, lung, and kidney [234]. Nrf2 is activated in response to increases in oxidative stress by binding to the ARE in the promoter of genes [234]. The ARE
consensus sequence was described as 5'-TMA\textsubscript{nn}RTGAYnnnGCR\textsubscript{wwww}-3', where 
\( M \) corresponds to nucleotides A or C, \( n \) is any nucleotide, \( R \) corresponds to G or A, \( Y \) 
responds to T or C, and \( W \) corresponds to A or T [235]. Variations of the 
consensus sequence and a minimum of 60% AT content were also found to promote 
ARE-mediated transcription [236, 237]. It should be noted that despite being 
recognized by the software as an Nrf2 binding site, the sequence discovered in the 
Scly gene promoter does not match completely the ARE consensus. This could 
confer different binding affinity for the Nrf2 transcription factor in the Scly promoter, 
influencing levels of transcription. It could also affect interaction with other unknown 
activators or repressors. Nrf2 has been shown to heterodimerize with small Maf or 
Jun proteins in the promoters of genes [238-242], as well as with Parkinson's disease 
associated protein DJ-1 [243]. All these interactors of Nrf2 are thought to enhance 
ARE-mediated gene expression, thus working as activators.

Nrf2 has also been shown to regulate genes involved in mitochondrial 
biogenesis \textit{in vitro} [244]. Recently, a report confirmed that Nrf2 target genes are 
induced under low Se environment [245] or in the low Se hepatocytes of the 
tRNA\textsuperscript{\textsuperscript{[Ser]Sec} liver-specific KO mouse [234]. The presence of an Nrf2 binding site in the 
promoter of the Scly gene indicates that Scly may be regulated by oxidative stress. 
As an Nrf2 target gene, Scly potentially could participate in much broader 
mitochondria-dependent and Se-dependent mechanisms of energy homeostasis 
[169]. Moreover, as seen with the promoter of the Scly, the promoter of the 
antioxidant selenoprotein TrxR1 was previously found to contain both Oct-1 and Nrf2 
binding sites [218], which suggests that Scly and TrxR1 may be regulated by similar 
stimuli. Future investigation of the details of regulation of the Scly gene by Oct1 and 
Nrf2 transcription factors could offer a better understanding of other pathways in 
which Scly might be involved. It is also unknown whether these two transcription 
factors respond to variations in Se levels. Thus analysis of such response could 
translate into a more complete insight towards the major players that regulate Se 
metabolism.

In previous studies examining the regulation of Scly by Se, it was reported 
that Scly activity was neither dependent on Se levels nor on Se chemical form 
(organic or inorganic) [225]. Highest activity of Scly was found in the liver and kidney 
of rats [225]. When the mouse Scly cDNA was first cloned, protein expression was
shown to follow activity levels, with highest expression levels in the liver and kidney as well [187]. It should be noted that the liver is the organ with the second highest accumulation of Se in the body, surpassed only by the kidneys [225]. This correlation indicated a possible relationship between Scly presence and Se metabolism. Interestingly, the brain's supply of Se is low, but very stable, and highly dependent on Se uptake. Se distribution is prioritized to the brain and the organ retains most of the available Se upon Se deficiency [246-248]. Such behavior suggests that a specific mechanism may be in place in the brain to maintain its Se levels. On the other hand, the thyroid, where a significant portion of dietary Se ends, was never analyzed for Scly activity or dependence on Se levels.

Nevertheless, in the present study, Scly expression differed in response to Se according to different cell lines and tissues. In HepG2, a liver-derived stable cell line, Scly mRNA expression decreased following exposure to the highest levels of selenite. A similar response was observed in the liver of mice fed increasing levels of selenite in their diets. Taken together with the previous report on the lack of Scly activity regulation by Se, this result suggests that the regulation of Scly expression by Se levels may also be affected by degradation or post-translational modification mechanisms in this organ. These mechanisms could modulate the Scly levels to maintain the organ with a constant supply of Sec for selenoprotein biosynthesis, thus potentially modulating the selenoprotein levels and hierarchy as well.

The sharp decrease observed in Scly levels in the liver of mice fed a Se-supplemented diet may represent a mechanism to turn off the enzyme in the Se-sufficient liver, maintaining Scly activity levels in equilibrium. The unchanged Scly activity following Se treatment [210, 225] may potentially be a result of a compensatory increase in Scly levels that would maintain proper cellular function in face of a decrease in Se availability.

Interestingly, in mouse hippocampus-derived Ht22 cells, Scly mRNA levels did not change with Se treatment, despite a tendency to increase. In contrast, the brain of WT mice fed increasing levels of Se displayed a sharp decrease in Scly expression. This discrepancy may indicate that in the brain, mechanisms to regulate Se levels are more tightly controlled than in the liver. Activity of Scly was not detected in human brain [210], and was only found in low levels in mouse brain [184]. The influence of Scly on Se levels or chemical forms has not been reported. If
the activity of Scly contributes to the Se pool in the brain despite possible changes in Se levels, the downregulation of Scly mRNA could indicate the participation of Scly in controlling Se availability in the brain. Under low Se conditions that increase Se uptake and storage in the brain, Scly could be actively involved in the maintenance of highly stable Se levels.

The Scly mRNA expression pattern in the Sepp1 KO mice (Figure 2.4) is in accordance with the negative regulation of the Scly gene by Se observed in cell lines (section 2.3.2) and in the tissue analysis of WT mice (section 2.3.3). Nevertheless, Scly expression pattern in Sepp1 KO mice raises the additional question regarding the nature of the interaction and role of these two proteins in selenoprotein production. The unchanged levels of Scly mRNA in most tissues in the Sepp1 KO mice could be explained by the fact that animals were kept on a Se adequate diet, to assure their survival. Nevertheless, hepatic Scly mRNA levels were sharply downregulated when compared with WT controls. This result suggests that instead of compensating for the lack of Sepp1 by activation of recycling mechanisms, the liver could be where these two proteins, Scly and Sepp1, are regulated in a coordinated fashion to maintain Se availability for the other organs of the body. Further studies could help to clarify the relationship between these two proteins in Se metabolism and homeostasis, and shed light into whether the decrease in Scly expression upon high levels of Se is dependent, at least to some extent, on Sepp1.
2.5. Figures

**Figure 2.1. Analysis of human Scly gene promoter.** Human Scly gene promoter presents an Oct-1 (pink triangle) and an Nrf2 (black triangle) binding motifs. The required sequences for each of these motifs are capitalized below the pink and black triangles. Previously found AP-1 binding site is represented in yellow.
Figure 2.2. Scly gene expression *in vitro*. Scly mRNA was measured by qPCR in stable cell lines (A) HepG2 (hepatoma); and (B) Ht22 (hippocampal neurons). Cell cultures were treated with 30, 100 or 200 nM of sodium selenite for 48 hours prior to RNA extraction. The mean result of 100 nM Se was considered the baseline for comparison, and one-way ANOVA (1WA) was performed. Obtained $P$ values are displayed in graphs. **$P<0.01$ and ***$P<0.001$ by Bonferroni’s *post-hoc* test for specific group comparison.
**Figure 2.3. Scly gene expression in vivo.** C57BL/6J wild-type mice were fed diets containing 0.08, 0.25 and 1 ppm of Se as sodium selenite. Graphs indicate mRNA expression of Scly in (A) liver and (B) brain tissues, using the 0.25 ppm levels as baseline for comparison. One-way ANOVA (1WA) was used to compare the means with achieved *P* value indicated in the graphs. Bonferroni’s *post-hoc* analysis was performed and asterisks indicate significance levels between specific groups. *P*<0.05 and **P*<0.01.
**Figure 2.4. Scly gene expression in Sepp1 KO and WT littermates mice.** Mice were maintained on standard mouse chow prior to collection of brain, heart, intestines, kidney, liver, lung and testis. qPCR results were separated by Scly expression amount. *Left graph:* tissues with high Scly copy number; *right graph:* tissues with low copy number of Scly. **P<0.01 by Student’s unpaired t-test with 95% confidence interval.
2.6. Tables

**Table 2.** List of human PCR primer sequences used in Chapter 2.

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<th>Gene</th>
<th>Forward</th>
<th>Reverse</th>
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<td>18S</td>
<td>GCAATTATTCCTCAGCATGAACG</td>
<td>GGGACTTAATCAACGCAAGC</td>
</tr>
<tr>
<td>GAPD</td>
<td>TGACAACTTTGCTGATCCTGAGAAGG</td>
<td>AGGGATGATGTTCTGGAGAGCC</td>
</tr>
<tr>
<td>Scly</td>
<td>GACAGAGAACACCAATGAT</td>
<td>ATTCCTCTCTGACGAAATCAG</td>
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**Table 3.** List of mouse PCR primer sequences used in Chapter 2.

<table>
<thead>
<tr>
<th>Gene</th>
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<tr>
<td>GAPD</td>
<td>TGACATCAAGAAGGTTGGTGA</td>
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<td>Scly</td>
<td>TCCACTCTATGGTAAATGCT</td>
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CHAPTER 3

CHARACTERIZATION OF THE SELENOCYSTEINE
LYASE KNOCKOUT MOUSE

3.1. Introduction

The recycling mechanism of Scly is characterized by the reutilization of Sec, presumably after selenoprotein degradation. It is hypothesized that Scly functions to break down Sec, and deliver the resulting available Se as selenide to the SPS enzymes [57, 194].

As explained in Chapter 1, SPS enzymes can also receive Se directly from dietary sources. Nevertheless, it is still unclear how much of the dietary Se is directly incorporated into the pathway and how much could be provided by the Scly recycling mechanism.

3.1.1. Knockout mouse models of Sec incorporation factors

Attempts to develop whole-body KO mouse models for several of the factors involved in Sec production and/or incorporation into selenoproteins proved to be unsuccessful. Among these attempts are for the tRNA[^Ser][Sec], SPS1, SBP2, SecS and SECp43 factors, most of which led to embryonic lethality ([82] and Berry MJ, Hatfield D and Schweizer U, personal communication). To circumvent the fatal outcome induce by whole-body KOs, some of these factors were knocked out in specific tissues, using Cre-enzyme-mediated recombination. This strategy allowed the animals to survive and develop, providing a better understanding of the roles of these factors in Sec production and/or incorporation into selenoproteins.

Specific KO of the tRNA[^Ser][Sec] gene, Trsp, was developed for several tissues [249]. The gene was conditionally disrupted in liver [174, 250, 251], kidney
podocytes [252], neurons [253], hematopoietic cells [254], macrophages [234], T cells [183], thyrocytes [168], endothelial cells, heart and skeletal muscle [255], bone [256], skin cells expressing keratin 14 [257], and mammary gland [258].

Overall, these tissue-specific KO models lacking selenoprotein expression reinforced the importance of selenoproteins in several steps of development and disease prevention. Endothelial-specific conditional Trsp KO was embryonic lethal at day 14.5 post-conception. This animal presented a poorly developed vascular system and underdeveloped limbs and head [255]. Liver-specific, heart-specific, and neuron-specific conditional KOs all died a few weeks after birth, suffering from hepatocellular degeneration and necrosis, myocardial failure, and cerebellar hypoplasia with severe hippocampus neurodegeneration, respectively [249, 259]. Liver-specific Trsp disruption also provided additional information on the role of selenoproteins in lipid metabolism. Mice with such specific disruption had increased plasma cholesterol levels regardless of gender. Microarray analysis revealed that liver-specific Trsp disruption induced upregulation of genes from the cholesterol biosynthesis pathway, and downregulation of genes involved in cholesterol metabolism and transport [174].

Interestingly, skin-specific Trsp KO was also lethal after birth, but survival lengths were highly variable. These mice had severe alopecia, accumulation of ROS in the hair follicles, and lack of subcutaneous fat, which probably led to hypothermia. Moreover, they had pronounced dysplasia on mucosal and tongue epithelia, which inhibited their suckling capability and led to loss of body weight (BW). Thus, hypothermia and reduced food uptake were suggested to be the compounding effects that led to the skin-specific Trsp KO mice premature death [257].

Surprisingly, kidney podocyte-specific Trsp KO did not present increased oxidative stress nor worsening of diabetic nephropathy. As the diabetic kidney is known for its increased ROS production, there are probably several redundant antioxidant systems, and the ones involving selenoprotein action may be compensated when selenoprotein production is disrupted [252].

Whole-body SBP2 KO proved to be embryonic lethal, but attempts to produce a conditional KO were recently reported [260]. SBP2 is one of the crucial factors involved in Sec incorporation, thus knocking it out implicates on the impairment of overall selenoprotein synthesis. Hepatocyte-specific SBP2 KO reduced expression of
selenoproteins in the liver, but did not impair survival. A neuron-specific SBP2 KO mouse resulted in a milder effect on selenoprotein levels than neuron-specific Trsp KO mouse.

Knockout of the enzyme PSTK, important for tRNA\(^{[\text{Ser}]\text{Sec}}\) loading, has not been reported (Hatfield DL, Berry MJ, personal communication). In fact, such approach would also give a perspective on additional mechanisms that may exist to overcome the lack of Sec, such as the insertion of Cys in place of Sec and the overall consequences of such misstep [55]. Additionally, a KO model has not been attempted for SPS2 enzyme, the selenoprotein that acts in the Sec synthesis pathway [61] and is suggested to auto-regulate the Sec pathway [60].

Knock out of Scly was also not reported prior to the present study and the role of Scly in selenoprotein biosynthesis remains poorly understood. Scly was reported to be actively involved in selenoprotein biosynthesis in HeLa cells [197]. Nevertheless, this study only analyzed GPx1, GPx4 and TrxR1, thus leaving ample room for investigation on the remainder of the selenoproteome. In fact, the development of a whole-body Scly KO mouse model reported herein allows a direct assessment of the role played by Scly on the Se recycling mechanism, Sec synthesis, selenoprotein production, and ultimately in vivo Se metabolism, at the organismal level. Through targeted disruption of the recycling mechanism, one could separate the pathways and/or mechanisms that depend on dietary Se from the ones that depend on Se recycling.

In this Chapter, characterization of the newly developed Scly KO mouse model is described. Gene expression of selenoproteins and Sec synthesis factors as well as gene expression and plasma levels of the redox selenoproteins GPx3 and Sepp1 were measured in order to investigate the physiological role of Scly in Se metabolism. Scly-dependent Se recycling was shown to contribute to selenoprotein synthesis and to specifically coordinate hepatic Se status.
3.2 Methodology

3.2.1. Materials

All reagents employed below were acquired from Sigma-Aldrich (St. Louis, MO), unless otherwise noted.

3.2.2. Scly KO mouse development

C57BL/6J mice with deletion of exon 4 from the Scly gene (Scly KO) were generated by the KnockOut Mouse Project Repository, an initiative supported by the National Center for Research Resources and run by the University of California at Davis. The promoterless β-galactosidase/neomycin reporter cassette flanked by Flp-recombinase (FRT) sites was placed upstream of the genomic site of exon 4 (Figure 3.1A), which contained two critical codons for His and Asp residues, both reported to bind to the Se atom in the enzyme core and to sort Sec from Cys [194, 195]. Efficient splicing to the β-galactosidase reporter cassette results in truncation of the endogenous transcript, a constitutive null mutation in the Scly gene. The targeting vector was inserted into C57BL/6J embryonic stem cells (ESC), antibiotic selected and clones expanded. The recombinant ESC clones were microinjected into C57BL/6J blastocysts to produce chimeras with one wild type and one recombinant Scly allele, which were then mated to generate homozygous Scly KO mice on a C57BL/6J background. Whole-body KO animals were housed and bred in our facility’s vivarium, and genotyped by PCR of digested tail DNA prior to experiments (Figure 3.1B). Primer sequences used for genotyping are listed in Table 4. Mice had their KO status further confirmed by qPCR analysis (Figure 3.1C).

3.2.3. Animals

Age-matched homozygous KO mice and WT homozygous littermates and/or C57BL/6J animals derived from The Jackson Laboratory (Bar Harbor, ME) and bred, born and raised in our mouse facility were used in experiments after weaning in accordance with the Animal Care and Use Committee of University of Hawai'i
(protocol number 09-871). Animals were fed standard mouse chow containing adequate 0.25-0.3 ppm Se. Animals were euthanized by CO₂ asphyxiation or anesthetized with tribromoethanol prior to collection of tissues or perfusion for histological procedures, respectively. All experimental data presented herein are from male mice, except for tissue mRNAs in Figure 3.5 and in Figure 3.6, which included two females, and ovarian and uterine mRNA in the same figure, which were extracted from 3-7 females.

3.2.4. GPx activity assay

Total serum GPx activity was measured by the coupled enzyme procedure. Briefly, 1 µl of serum was incubated at 37°C for 5 minutes in a buffer containing 50 mM potassium phosphate (pH 7.4), 5 mM EDTA, 5 mM reduced glutathione, 5 µg/ml glutathione reductase, 266 µM NADPH, and 400 µM tert-butyl hydroperoxide. The reaction was read at 340 nm in a SpectraMax M3 spectrometer (Molecular Devices, Sunnyvale, CA). Results were calculated with the SoftMax Pro5 software (Molecular Devices).

3.2.5. RNA and qPCR analysis

Total RNA was extracted as described in Chapter 2. Quantitative PCR (qPCR) results were calculated using the ΔΔCt method, normalized to 18S rRNA or GAPD mRNA as housekeeping genes. Mouse selenoprotein PCR primers were described previously [65] and their sequences are listed in Table 4.

3.2.6. Antibodies

Mouse GPx3 antibody (R&D Systems) was diluted according to the manufacturer’s protocols. Rabbit polyclonal Sepp1 antibody against two-thirds of mouse Sepp1 towards the N-terminal was produced by Proteintech Group, Inc., (Chicago, IL) and kindly provided by Dr. Frederick Bellinger. Briefly, the first Sec codon (codon 59) of the N-terminal sequence of Sepp1 cDNA was mutated to a Cys codon using QuikChange II XL Site-Directed Mutagenesis kit (Agilent Technologies,
Santa Clara, CA). Purified protein from transformed bacteria was injected into rabbits and the resulting antisera were affinity-purified with the antigen. Antibody specificity was confirmed by Western Blot using serum from Sepp1 KO and WT mice (Figure 3.2) obtained in our colony and described previously in Chapter 2 and in [65].

3.2.7. Western Blot

Serum samples (1 µl) were separated in 4-20% SDS-PAGE (Bio-Rad, Hercules, CA), transferred in PBS-T buffer with 9% methanol to IPFL membranes (Millipore, Billerica, MA), blocked for 1 hour at room temperature on constant shaking with Odyssey Blocking Buffer (LI-COR Biosciences, Lincoln, NE) and probed for 1.5 hour with specific antibodies. IRDye 800CW or IRDye 680LT (LI-COR Biosciences) was used as the secondary antibody. Membranes were incubated with secondary antibody for 45 minutes. Detection and analysis of Western blots were performed using an Odyssey Infrared Imager (LI-COR Biosciences). Prior to incubation with blocking solution, membranes were stained with Ponceau S for 1 hour at room temperature to allow recording of loading evenness, followed by destaining with at least five washes of distilled water.

3.2.8. Se content

Livers were prepared according to Exova Standard Operating Procedure 7040 for trace metals. The Se content in the livers was measured by Inductively Coupled Plasma Mass Spectrometry (ICP-MS) and quantified by comparison with internal standards at Exova Inc. (Santa Fe Springs, CA).

3.2.9. Statistical analysis

Results were plotted and analyzed using Prism software (GraphPad Software Inc., La Jolla, CA). Statistical tests were applied according to experimental design and are indicated in text or figure legends. P values are also shown in the figure legends or in graphs. All results are means ± SEM.
3.3. Results

3.3.1. General characteristics of Scly KO mice

It has been suggested that Sec recycling is a secondary path to provide Se for selenoprotein production, with diet being the primary path [34]. Based on current knowledge of Scly function [187, 197], we predicted that genetic deletion of Scly would result in phenotypes that are similar to those with low dietary Se intake or disruption of Se transport [142, 175], such as male infertility and/or neuromotor impairments.

Surprisingly, genetic disruption of the Scly gene did not impair embryonic survival, fertility or development, as Scly KO mice gave birth to viable offspring, without weight or size differences from birth to juvenile stage when compared with their WT counterparts. In addition, Scly KO mice fed standard chow containing adequate Se levels had no major neurobehavioral dysfunction [261].

3.3.2. Whole-body Selenium status of Scly KO mice

Se is carried in the bloodstream by several proteins, amongst them selenoalbumin and the selenoproteins Sepp1 and GPx3 [262]. Selenoalbumin has no enzymatic activity, rendering it difficult to measure by conventional methods [263]. Nonetheless, plasma Sepp1 and GPx3 levels are regarded as more effective biomarkers for whole body Se, together with total GPx activity [264, 265].

Investigation was carried out on whether maintenance of Se homeostasis requires the involvement of the putative cellular Se recycling protein Scly. In the serum of KO animals fed a Se adequate diet (0.25-0.3 ppm), Sepp1 protein levels were not statistically different from the serum of WT mice (Figure 3.3A). Total GPx enzyme activity also remained unchanged in Scly KO mice (Figure 3.3B). Likewise, there was no difference in renal GPx3 mRNA expression levels (Figure 3.3C), or GPx3 serum levels (Figure 3.3D) between Scly KO and WT mice. Nevertheless, it should be noted that a non-significant trend towards lower levels of total GPx activity and GPx3 protein levels was observed in Scly KO animals.
3.3.3. Hepatic Se content

The liver is the organ where Scly is most active. Thus, it would likely be the organ where the effects of Scly disruption would be more prominent in terms of Se metabolism. In order to verify whether hepatic Se status was being maintained, whole livers from WT and KO mice fed a Se adequate diet (0.25-0.3 ppm Se) were analyzed by ICP-MS for measurement of their Se content.

The KO mice had diminished Se content in the liver despite being fed the same diet as WT mice (Figure 3.4).

3.3.4. Selenoprotein mRNA expression in Scly KO mice

Because Scly is potentially involved in Se recycling to produce selenoproteins [197], determination of which selenoproteins are more reliant on Se recycling mechanisms for their expression in vivo was carried out on Scly KO mice tissues.

qPCR screening of selenoprotein mRNAs was performed in brown adipose tissue (BAT), brain, heart, intestines, kidney, liver, lung, skeletal muscle, ovaries, pancreas, spleen, testis, uterus and gonadal white adipose tissue (gWAT). Selenoproteins analyzed included Dio1, Dio2, Dio3, GPx1, GPx2, GPx4, SelH, Sel I, SelK, SelM, SelN, Sepp1, SelR, SelS, SelT, SelW, SPS2, TrxR1, TrxR2, TrxR3. All selenoprotein mRNAs listed in Table 1 were measured, except SelO and SelV.

Upon disruption of the Scly gene, mice maintained on a Se adequate diet presented a general upregulation of selenoprotein transcripts in the liver (Figure 3.5). The same response was observed mildly in the gWAT and in the spleen. Interestingly, based on the Sepp1 KO mouse studies [142, 161, 227, 266], tissues that were recognized as Sepp1-dependent for Se transport and distribution, such as brain and testis, had only minimal selenoprotein mRNA changes in the Scly KO mouse model.
3.3.5. Expression of selenoprotein synthesis factors in Scly KO mice

The mRNA levels of the factors involved in selenoprotein synthesis, including the SECIS-binding protein SBP2 [29] and those directly interacting with Scly, such as SPS1 and SPS2 [191] were measured. The mRNAs for these factors were expressed at higher levels in the liver of Scly KO mice, but not in the other tissues (Figure 3.6). Furthermore, in Scly KO mice, elevated expression of the mRNAs for two of the factors, SPS1 and SPS2, was observed in the gWAT, and elevated expression of factors SPS1 and SBP2 was observed in the pancreas.

3.4. Discussion

Given the similar expression profiles of Sepp1 and Scly in the liver and kidneys, their respective roles in selenium transport/storage and recycling, and the requirement of Scly for utilization of Se from Sepp1 in selenoprotein biosynthesis [197], it was hypothesized that Scly KO mice could display a phenotype similar to the Sepp1 KO mice, including neurological and/or reproductive impairment [267]. Surprisingly, neither neurological nor fertility impairment was observed in Scly KO mice on adequate Se intake [261]. Newborn Scly KO mice appeared to have no major observable changes in phenotype compared with WT animals; litters were healthy and had normal size and weight.

Moreover, Se status of Scly KO mice was not disturbed. The measurement of circulating Se levels in mice remains a controversial topic [262]. There is discussion among Se researchers of whether Sepp1, GPx levels/activity or Se content could be the best predictor of Se levels in the plasma [268]. Furthermore, the exact levels required for efficient selenoprotein expression and function probably vary depending on tissue, developmental or metabolic status [65, 226]. Despite the controversy, selected Se parameters measured in the plasma of Scly KO mice indicated that Se levels were kept normal. As described in the results above, renal GPx3 mRNA levels, plasma GPx activity, and GPx3 and Sepp1 levels were unchanged when the animals were fed a diet with adequate amounts of Se (Figure 3.3).
Moreover, Sepp1 circulating levels were maintained. Nevertheless, the amount of Se loaded in the Sepp1 molecule cannot be pinpointed due to the nature of the antigen used for Sepp1 antibody production. The antigen employed in Western blots recognized approximately two-thirds of Sepp1 towards the N-terminal, terminating at the second Sec residue. Ultimately it is unclear whether Sepp1 produced by the Scly KO mice is predominantly full length or if it is truncated at a Sec residue, where the UGA would be circumventing the Sec incorporation machinery and acting as a standard stop codon. If such scenario is established, this would handicap Sepp1 of its function in Se transport and storage, placing a stronger reliance on dietary and Se recycling mechanisms for physiological Se homeostasis. Moreover, Sepp1 is known for its glycosylated residues [269], which could contribute to proper folding for specific site recognition. It remains to be further investigated if circulating Sepp1 in Scly KO mice is fully glycosylated and/or properly folded.

Compared with the results with regard to selenoproteins discussed in section 3.3.2, the KO mice exhibited organ-specific changes in Se. Moreover, the decrease in hepatic Se levels does not carry over to the bloodstream if enough Se is available through the diet. Due to the decreases in Se content observed in the liver, selenoprotein production was predicted to be impaired in the organ, thus causing an overall decrease in selenoprotein levels. Nevertheless, the increase in oxidative stress caused by such a decrease might result in compensatory upregulation in selenoprotein transcripts.

The disruption of Scly revealed an interesting pattern for the selenoprotein transcriptome, as observed in Figure 3.5. Disruption of Sec recycling was hypothesized to cause a decrease in Se availability directed towards selenoprotein production. Nevertheless, the screening of several tissues of Scly KO mice revealed that most tissues presented unchanged selenoprotein mRNA expression patterns compared with WT, but up- or downregulation of specific messages was also observed (Figure 3.5). Thus, most of the selenoprotein transcriptome was not severely affected by disruption of Scly in most tissues, including brain and testis, that rely/depend more on Se for proper function. This discrepancy suggests that the Se recycling pathway is a secondary pathway for Se acquisition in the highly Se-dependent tissues, with diet likely being the main source of Se for selenoprotein production in these tissues. The maintenance of proper selenoprotein antioxidant
function probably relies more on dietary Se than on the secondary recycling mechanism.

Nevertheless, the liver was significantly affected by disruption of Scly, which is consistent with the fact that the liver is the primary site of Scly activity (Chapter 1, section 1.6). Most selenoprotein mRNAs were upregulated in the liver of Scly KO mice (Figure 3.5), as well as factors involved in selenoprotein synthesis (Figure 3.6), including SBP2 [29] and SPS enzymes [191]. Such outcome warranted further research, which will be addressed in Chapter 4.

Ultimately, most dietary chemical forms of Se are converted to Sec and utilized for selenoprotein production. It is unknown how much Sec is produced from the Se recycling pathway, but results from Chapter 2 suggest that when dietary Se is limited, Scly may become more abundant and contribute, at least partially, to Sec synthesis. It should be noted that expression of some selenoproteins is very responsive to dietary changes in Se, while expression of others is more resistant to such changes. The existence of a hierarchy in selenoprotein production [88, 270] may in part be a result of the intricate mechanisms of NMD [65, 271], discussed in Chapter 1, section 1.4.2. Additionally, the physiological role of each selenoprotein in specific tissues may also dictate their dependence on Scly.

Although Scly is very specific in distinguishing the Se of Sec from the sulfur of Cys [194], there are other lyases involved in the degradation of these two amino acids. One is the Nickel-Iron-Sulfur (NiFS) cysteine desulfurase, whose primary function is to recycle Cys, but has also been shown to have Scly activity in bacteria [272]. Mammalian NiFS is ubiquitously expressed in tissues and found mostly in mitochondria [273], where it is primarily involved in the biogenesis and regulation of iron/sulfur-containing proteins [274]. Another possibility is the prioritized activation of the transsulfuration pathway, via the enzyme Cth, which breaks down SeMet into methylselenol [51, 52]. Usually, Cth converts homocysteine into Cys to further contribute to the generation of sulfide [53]. Nevertheless, the methylselenol generated in the Cth-catalyzed reaction of SeMet can be utilized in selenoprotein biosynthesis, specifically of GPx1 [54], which renders SeMet as an additional source of biologically essential Se required for de novo synthesis of Sec. Cth could potentially be more active towards SeMet to compensate for the lack of Scly in the mouse model. It remains to be investigated whether the Scly KO mouse adapts, at
least partially, to the lack of the enzyme by triggering a compensatory mechanism utilizing proteins that may have Sec lyase activity such as NiFS [272], and/or helping supplement Se to the selenoprotein synthesis pathway coming from a different chemical form, such as that derived from the action of Cth. The structural similarity between NiFS and Scly was illustrated in Figure 1.4 of Chapter 1.

Examination of the rate of Se absorption by the digestive tract and the renal Se excretion levels in the Scly KO mice could also provide useful insights into Se metabolism. Both the intestines and kidneys play major roles in Se homeostasis. Intestines are the primary site for Se absorption. In human intestinal Caco-2 cell monolayers, it has been reported that SeMet treatment increases intracellular Se content more effectively than selenite [275-277]; this effect is further enhanced when Met is present, possibly because Met and SeMet may compete for the same transporter [277]. Nevertheless, it should be noted that selenite has a lower apparent permeability coefficient. Thus, the contrast with SeMet absorption may be of limited physiological relevance. *In vivo* studies are required to clarify how the Se uptake occurs in the intestines. Nevertheless, the kidneys are where excess Se must be triaged to excretion. This organ monitors whole-body Se status, avoiding disposal of needed Se, and promoting the retention of Se under conditions of low Se status. Such retention would possibly require Se recycling activity, where Scly could be involved. Thus, further analysis of these organs could potentially reveal the adaptations that the Scly KO model may have developed to compensate the lack of Se recycling mechanisms.
3.5. Figures

**Figure 3.1. Generation of Scly KO mouse and genotyping.** A) Schematic representation of the construct cassette used to generate Scly KO mice. Numbers in yellow boxes represent exons of the Scly gene. B) Typical genotype result with PCR band size differentiation in 1.5% agarose gel. A 100 bp DNA standard ladder was run in the first lane. C) Scly qPCR in liver, lung, testis and spleen of WT and Scly KO mice. In A, *FRT* = Flp recombinase; *neo* = neomycin; *loxP* = genomic sites of targeted recombination driven by Cyclic Recombinase (Cre).
**Figure 3.2. Confirmation of Sepp1 antibody specificity.** Western Blot was used to assess the antibody specificity to Sepp1, using 1 µl of sera from WT and Sepp1 KO mice. The Sepp1 KO mice lacked a band in the size where Sepp1 is usually observed, at approximately 50 kDa. Molecular weight markers are indicated in the figure. The bottom figure shows the even protein loading of the Western Blot membrane after staining with Ponceau S.
Figure 3.3. Whole body Se status of Scly KO mice. A) Sepp1 levels in the serum measured by Western Blot and its respective graph quantification expressed in arbitrary units. B) Total serum GPx activity. C) GPx3 mRNA expression in kidneys, the primary site of serum GPx3 production. D) GPx3 protein expression in the serum and its respective graph quantification. Ponceau-stained membranes were used as loading controls for (A) and (D). Sample size and $P$ values are displayed in graphs, except in (D), where $n=3$. Student's unpaired t-test was performed to compare WT and KO means in all panels.
Figure 3.4. Hepatic Se content in WT and Scly KO mice fed a Se adequate diet.

Results were normalized by liver weight and the $P$ value was obtained by Student's unpaired $t$-test, applied to compare the means between both groups. Each square represents one individual mouse. It should be noted that the Y axis is broken to accentuate the difference.
### Figure 3.5. Gene expression map of selenoproteins in tissues of Scly KO versus WT mice.

Age-matched animals were fed standard chow and had their total RNA extracted for qPCR analysis. Selenoprotein genes are displayed in the columns in descending order of expression in WT. Yellow, blue, gray and white represent genes that are upregulated, downregulated, unchanged or not detected in Scly KO mice, respectively. Results were normalized relative to 18S rRNA (BAT, brain, liver and skeletal muscle) or GAPD mRNA (heart, intestines, kidney, lung, ovaries, pancreas, spleen, testis, uterus and gWAT). Asterisks represent significance by Student's unpaired *t*-test with 95% confidence interval. *P<0.05, **P<0.01 and ***P<0.001, n=4-11, depending on tissue.
Figure 3.6. Gene expression map of selenoprotein factors in several tissues of Scly KO mice. Age-matched animals were fed standard chow and had their total RNA extracted for qPCR analysis. Yellow, blue and gray represent tissues where the specified gene is upregulated, downregulated or unchanged in the Scly KO mice, respectively. Results of gene expression of SPS1, SPS2 or SBP2 were normalized relative to 18S rRNA (BAT, brain, liver and skeletal muscle) or GAPD mRNA (heart, intestines, kidney, lung, ovaries, pancreas, spleen, testis, uterus and gWAT). Asterisks represent significance by Student's unpaired t-test. *P<0.05, **P<0.01 and ***P<0.001, n=4-11, depending on tissue.

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59
### Table 4. List of mouse PCR primer sequences used in Chapter 3.

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CHAPTER 4

DISRUPTION OF THE SELENIUM-RECYCLING ENZYME
SELENOCYSTEINE LYASE
INDUCES METABOLIC SYNDROME IN MICE

4.1. Introduction

*In vitro* and *in vivo* hepatic Scly downregulation in the presence of high levels of selenite, as seen in Chapter 2, is evidence that recycling mechanisms are probably attenuated when Se is abundant. This suggests that Scly would be required when Se is limiting. Data in Chapter 3 suggest that mice with disrupted Se recycling still maintain normal Se status in many tissues, as evidenced by unchanged plasma GPx3 activity and protein levels, as well as unchanged Sepp1 levels. In addition, the unchanged production of selenoprotein mRNAs in most tissues other than liver suggests that dietary Se is sufficient for Se homeostasis in most tissues.

Nevertheless, as stated in Chapter 3, most studies of Se metabolism focus on dietary Se and neglect the Se recycling mechanism. Se content was decreased in the liver of Scly KO mice, concomitant to an overall selenoprotein transcript upregulation in the same tissue. Taken together, these two results suggested that Scly disruption mostly affected Se recycling in the liver. A major function of the liver is detoxification, and it has a regulatory role on overall energy metabolism in vertebrates. The liver is also responsible for synthesizing several plasma proteins, including Sepp1, the Se transporter, and it is also an endocrine organ. Therefore, the liver may be the site where Se and glucose metabolism possibly interconnect. This relationship will be analyzed in light of metabolic diseases and discussed in this Chapter.
Type 2 diabetes is a global epidemic that affects more than 25 million people in the United States alone, according to the American Diabetes Association (ADA, http://diabetes.org). It is a chronic lifestyle disease characterized by insulin resistance and hyperglycemia. Insulin resistance is interpreted as the failure of insulin to exert the suppression of hepatic glucose output and/or the efficient glucose uptake in target tissues. The progression of type 2 diabetes follows a pattern that varies individually, but whose outcomes are similarly characterized by hyperglycemia and loss of insulin synthesis and secretion due to pancreatic β-cell failure, as summarized in Figure 4.1. As a consequence of a chronic hyperglycemic state, the risks for developing neuropathy, retinopathy, cardiovascular diseases and kidney failure increases.

The leading risk for development of type 2 diabetes is the presence of pre-diabetes. It is estimated that 79 million Americans are pre-diabetics. Pre-diabetes is defined by the ADA as a condition in which "blood glucose levels are higher than normal but not yet high enough to be diagnosed as diabetes". Clinically, one is considered pre-diabetic if he/she presents glucose intolerance, measured by glucose tolerance test (GTT; normal is <140 mg/dL, pre-diabetic between 140-200 mg/dL); hemoglobin A1C levels between 5.7-6.5% (normal is <5.7%); or fasting plasma glucose between 100-126 mg/dL (normal is <100 mg/dL).

In the early stages of pre-diabetes, the body tries to compensate the excess glucose by producing more insulin (reviewed in [278]). Insulin is a peptide hormone produced by the β-cells in the islets of Langerhans of the endocrine pancreas. The rise in circulating levels of insulin is called hyperinsulinemia, and it is established initially as a consequence of an increase in the number and activity of pancreatic β-cells. Hyperinsulinemia leads to constant activation of insulin signaling phosphorylation cascades in target tissues, such as white adipose tissue (WAT) and skeletal muscle, which will ultimately advance to a state of insulin resistance, which is characterized by a fatigue of the insulin signaling cascade. Insulin resistance is a hallmark of type 2 diabetes. After chronic stimulation to increase production of insulin, the pancreatic β-cells start to weaken, leading to pancreatic failure.
Nevertheless, it should be pointed out that these two conditions, pre-diabetes and type 2 diabetes, are more complicated to be discretely separated than it is suggested by the numbers of inflicted people above. This lack in distinction occurs because not all symptoms are present in patients at the same time, and the degrees in which the symptoms will interfere with one's metabolism vary greatly.

Insulin resistance is considered as one of the most relevant risk factors for the establishment of metabolic syndrome by the NCBI (http://www.ncbi.nlm.nih.gov/pubmedhealth/PMH0004546/). Metabolic syndrome is a combination of medical complications including obesity, hypertension, dyslipidemia and glucose intolerance that will lead to one's increased risk to develop cardiovascular diseases and type 2 diabetes [279]. There is still debate on whether this syndrome is caused by a single factor or several factors. Moreover, up to 40% of obese individuals never develop cardiovascular risk nor progress to type 2 diabetes, which is intriguing [279-281]. Nevertheless, it is important to highlight that the major risk factor for metabolic syndrome is obesity.

Obesity is a preventable lifestyle disease and a global public health problem that affects approximately a third of the United States population according to the 2007-2008 data compilation from the Centers of Disease Control and Prevention (http://www.cdc.gov/obesity/data/trends.html). Obesity develops mostly due to hypercaloric food intake and/or lack of physical activity, and it is an independent factor for the development of metabolic syndrome.

Human obesity is characterized by body mass index (BMI) over 30 and exaggerated BW due to lipid accumulation in most WAT depots, particularly in the waist region and upper body ("central obesity"). Consequences of obesity include disturbances in lipid metabolism in the liver and in the circulatory system, elevated plasma cholesterol and triglycerides, high blood pressure and increased risk for cardiovascular diseases and type 2 diabetes, as insulin resistance also becomes present. One of the main treatments of obesity involves changes in lifestyle towards a more balanced, hypocaloric diet, concomitant with the elimination of sedentary behavior.

Insulin resistance in the WAT results in lipolysis, with subsequent release of free fatty acids (FFA) into the bloodstream. Elevated circulating FFAs lead to excessive hepatic triglyceride synthesis, which will result in non-alcoholic fatty liver
disease (**NAFLD**), also called hepatic steatosis [282, 283]. NAFLD is another pathology commonly associated with type 2 diabetes, obesity and hypertriglyceridemia, therefore potentially present in metabolic syndrome patients [284]. If insulin resistance is not prevented, NAFLD may progress into non-alcoholic steatohepatitis (**NASH**) and further to cirrhosis.

### 4.1.2. Endocrine control of metabolic syndrome and obesity

Obesity is a risk factor for the development of metabolic syndrome that can lead to type 2 diabetes. Dysregulation of pancreatic insulin production and/or secretion is the primary cause of type 2 diabetes, leading to metabolic overload, and impacting WAT depots. The body interprets such metabolic overload as a chronic injury, thus a chronic and peculiar inflammatory state is presented, called metaflammation or metabolic inflammation [285]. Metaflammation is not accompanied by either infection, autoimmunity or severe tissue injury. It is a low-grade inflammation, coordinated mainly by the affected WAT via the action of the adipocytes behaving as immune cells and producing ROS, cytokines and adipokines [286].

On an endocrine level, several adipokines are dysregulated in obesity, such as leptin and adiponectin [287-289]. Leptin is a 16 kDa protein that inhibits appetite through activation of hypothalamic receptors. Defects in the leptin receptor and/or in its signaling cascade in the hypothalamus have been associated with obesity. Obese individuals present high levels of circulating leptin, leading to leptin resistance [290]. Adiponectin is an anti-inflammatory adipocytokine with insulin-sensitizing properties, acting by suppression of hepatic gluconeogenesis, and enhancement of insulin signaling in target tissues [291, 292]. Physiologically, the role of adiponectin in the pathogenesis of metabolic syndrome is well established [293]. Circulating levels of leptin correlate with fat mass [294], while adiponectin is downregulated in obese states [295].

Adipocytes can also produce some proinflammatory cytokines, such as TNFα [296] and Monocyte Chemotactic Protein 1 (**MCP1**). Both are positively correlated to obesity. TNFα has been shown to decrease adiponectin transcription via phosphorylation of Peroxisome Proliferator-Activated Receptor gamma (**PPARγ**), a
transcription factor involved in several adipogenic pathways. MCP1 mediates the insertion of macrophages into the obese WAT, establishing an important proinflammatory state that may lead to or compound insulin resistance [297, 298].

Tissue-resident and newly recruited macrophages inserted into the adipose tissue on obese states also release proinflammatory cytokines, such as Interleukin-6 (IL-6), TNFα and resistin. IL-6 has been reported to induce insulin resistance in cells, and its circulating levels are elevated in type 2 diabetics [299]. Resistin is known to be elevated in obesity, but its specific role remains unclear ([300, 301] reviewed in [302]).

Thyroid hormones exert strong effects on obesity. They are produced by the thyrocytes in the thyroid gland, and contain iodine atoms in their molecules. The primary thyroid hormone in the bloodstream is L-3, 3', 5, 5' tetraiodothyronine or thyroxine (T4), a pro-hormone with four iodines and long half-life. To become biologically active, T4 requires removal of one iodine, producing L-3, 3', 5' triiodothyronine (T3). This deiodination step is catalyzed by the Dio selenoenzymes, which have Sec in their catalytic site, as explained in Chapter 1 section 1.4. Deiodination allows tight and local regulation of the levels of thyroid hormones, with Dio1 and Dio2 mostly converting T4 into T3, and Dio3 converting T4 into inactive reverse T3, or T3 into T2 [303].

One of the classical physiological roles of thyroid hormones is the regulation of metabolism and basal metabolic rate via stimulation of energy expenditure ([304] reviewed in [305]). Dio2, specially, controls adaptive thermogenesis induced by cold [169, 306] and by diet [307] in the BAT, and it has been connected to insulin resistance upon a high fat diet challenge [170]. The role of the deiodinases in insulin-regulated glucose metabolism will be discussed in section 4.1.5 of this Chapter.

4.1.3. Effects of Se in glucose and lipid metabolism

The effects of Se on glucose metabolism are an unresolved issue among researchers. Se has been shown to adversely affect glucose homeostasis, despite being considered a safe supplement with potential to prevent several human diseases, including cancer ([35, 308, 309] reviewed in [147]).
The Third National Health and Nutrition Examination Survey (NHANES III) found a positive association between serum Se and prevalence of type 2 diabetes in American adults [310, 311]. Three additional case-control studies found reduced incidence of type 2 diabetes upon high Se status ([312-314] reviewed in [32]).

In the SELECT [315] and in the Nutritional Prevention of Cancer (NPC) trials [316], subjects were administered Se supplements for assessment of effects on the incidence of prostate and skin cancers, respectively. After 7 years, both trials were prematurely terminated. In the SELECT, it was revealed that those subjects who received high doses of supplemental Se presented a trend, although not significant ($P=0.16$), towards increased risk of glucose intolerance. Nevertheless, a 10-year follow-up analysis of SELECT participants revealed both a lack of influence of Se supplementation in the outcome of type 2 diabetes prevalence [148] and no effect on prostate cancer protection [317]. In contrast, a post-hoc analysis of the NPC trial showed a significant increase in risk of type 2 diabetes in those supplemented with 200 µg of Se [32].

One possible reason for such discrepancy is the value of baseline serum Se among trial participants [150]. The baseline Se status of men on SELECT trial was 136 µg/L, while in the NPC trial, the baseline Se was 114 µg/L. The higher baseline of SELECT may have caused selenoprotein expression to plateau or surpass a threshold of risk, as is observed in rats at equivalent concentrations [318]. Trial participants in the SELECT study probably could not benefit from Se supplementation because their Se system was already saturated.

The randomized United Kingdom Prevention of Cancer by Intervention with Selenium (UK PRECISE) pilot study unveiled the effects of Se on lipid profile. Early studies have suggested that low serum Se could increase cardiovascular morbidity and mortality [319, 320], and the association of those effects with lipid content was investigated by the UK PRECISE. The pilot study revealed that Se supplementation up to 300 µg/day was only modestly beneficial to plasma cholesterol. The baseline serum Se of this study, however, was 88.8 µg/L, considerably lower than in the SELECT or NPC trials. In terms of benefits of Se supplementation for glucose and lipid profile, it reinforces the importance of the U-shaped association, where most benefits fall in a midrange concentration, while harm is prevalent in the extreme sides of the curve [32]. Combined, these clinical controversies underline our limited
understanding of the effects of Se supplementation, specifically regarding its influence on glucose and lipid metabolism and on the metabolic syndrome and type 2 diabetes outcomes.

4.1.4. Molecular crosstalk of Se with glucose and lipid metabolism

The production of insulin, the hormone that coordinates glucose metabolism in vertebrates, was reported to be dependent on the activity of the antioxidant enzymes superoxide dismutase (SOD) and selenoprotein GPx1, both responsible for the detoxification of peroxides and superoxides in cells [172]. Insulin secretion occurs upon feeding, and once in the bloodstream, insulin acts in the cells of target tissues involved in carbohydrate and lipid metabolism, such as liver, WAT and skeletal muscle. Binding of insulin to its specific receptor trigger a tyrosine phosphorylation cascade that ultimately will activate downstream kinases, such as Phosphoinositide 3-Kinase (PI3K) and Protein Kinase B/Akt (PKB/Akt or simply Akt), which will ultimately signal to glucose transporter (GLUT)-containing vesicles to translocate GLUT from the vesicles to the plasma membrane, allowing glucose to enter the cell and be used metabolically.

The specific mechanism connecting Se and glucose pathways is still unclear. Several studies, in vitro and in vivo, have suggested that independent selenoproteins are responsible for linking these metabolic pathways, such as GPx1 [171], Sepp1 [267], Dio2 [170, 321], Dio3 [173], SelS [95, 322] and SelN [323]. Paradoxically, while Se itself can act as an insulin-mimetic, some selenoproteins such as GPx1 could work as insulin antagonists [324].

The mode of action of Se as an insulin sensitizer is believed to occur through the inactivation of critical tyrosine phosphatases [325], such as protein tyrosine phosphatase 1B (PTP1B) and phosphatase and tensin homolog (PTEN) [326-328]. These proteins are known inhibitors of insulin signaling, PTP1B dephosphorylating the insulin receptor (IR) and insulin receptor substrate (IRS), and PTEN dephosphorylating downstream effectors PI3K and Akt. On the other hand, the selenoprotein-induced insulin antagonism is suggested to occur through a burst of superoxide that is released when insulin binds to its receptor in the cell membrane of target tissues [329]. The superoxide anion is immediately converted to peroxide by
the membrane-bound NAD(P)H oxidase homolog NOX4 [330]. The membrane-bound peroxide anion reaches the cytosol where it is then quickly converted to hydrogen peroxide (H$_2$O$_2$) by the enzyme SOD. The H$_2$O$_2$ is broken down by GPx1 into water + oxygen, and this mechanism increases the probability of reducing the cysteine residue present in the active site of PTP1B, leading to the activation of this enzyme [331]. Active PTP1B then dephosphorylates the IR and IRS, turning off the insulin signaling cascade [332]. Nevertheless, both actions of Se in insulin signaling involve the negative regulator PTP1B. This protein, therefore, may be an important factor in the interconnection between Se and glucose metabolic pathways.

Interestingly, Se deficiency has also been demonstrated to affect lipid concentration and fatty acid composition in the liver [333]. The responsible mechanism encompasses the action of GPx, via downregulation of lipid peroxidation.

4.1.5. Effects of selenoproteins in glucose and lipid metabolism

Various selenoproteins have been established to be closely involved in carbohydrate [95, 170, 172, 267, 323] and lipid [174] metabolism. A description of the role that these selenoproteins play on energy metabolism is presented below.

The liver-specific Trsp KO mouse lacked the gene for tRNA$^{\text{Ser}Sec}$, thus lacking overall selenoprotein expression even on Se adequate diet. This model presented drastic decreases in total liver Se, inducing severe liver and WAT necrosis that ultimately led to death of the mouse by hepatic failure [174]. In addition, the study found an increase in ApoE levels in the plasma of transgenic mice, with concomitant elevated plasma cholesterol levels and increased expression of genes from the cholesterol biosynthesis pathway. On the other hand, the transgenic mouse model of tRNA$^{\text{Ser}Sec}$ that does not express stress-related selenoproteins was found to ameliorate the lipid metabolic profile compared with the liver-specific Trsp KO mice.

Interestingly, increased hepatic Se levels were also found in the Sepp1 KO mice model [266]. The animal did not have increases in plasma cholesterol; instead, non-esterified fatty acids were decreased. Moreover, Sepp1 KO mice displayed reduced post-prandial plasma insulin and improved glucose tolerance. After challenge with a high fat diet, Sepp1 KO mice presented increased food intake
without changes in BW, a probable consequence of its reported increase in basal energy expenditure [267]. The suggested mechanism for such effect involves inactivation of Adenosine Monophosphate-Kinase alpha (AMPKα), a major coordinator of hepatic carbohydrate and lipid metabolism. AMPKα is inhibited by high glucose level in the pancreas [334, 335], thus functioning as a sensor to allow insulin secretion. In the liver, AMPKα is regulated by several factors, including the adipocytes hormones leptin and adiponectin, and by insulin via Akt signaling ([336] reviewed in [337]). Phosphorylated AMPKα inactivates the enzyme Acetyl-CoA carboxylase 1 (ACC1) by phosphorylating the Ser79 residue of the molecule [338].

Hepatic steatosis, hyperinsulinemia and hypercholesterolemia have been linked at the molecular level to increased transcription of hepatic ACC1 [339-341], which is a critical enzyme for de novo lipogenesis. Once phosphorylated, ACC1 is unable to catalyze malonyl-CoA production, a crucial step for fatty acid biosynthesis in the liver [341]. High levels of malonyl-CoA favor esterification of FFA to produce triglycerides, sparing the FFAs from oxidation [342].

Interestingly, most of the hepatic genes involved in de novo glucose synthesis and/or cholesterol biosynthesis share to some extent their expression control. Nuclear transcription factors such as the PPAR family (mainly PPARα and PPARγ), the PPARγ coactivator 1 alpha (PGC1α), and the liver X receptor alpha (LXRα), have been shown to participate in the direct activation of genes involved in lipogenesis or from the insulin signaling pathway and/or GLUT translocation to the membrane [343]. Sepp1 gene expression in HepG2 cells is enhanced by PGC1α interacting with two other transcription factors, the forkhead box protein class O1a (FoxO1a) and the hepatocyte nuclear factor 4 alpha (HNF-4α) [344]. Moreover, Sepp1 was demonstrated to be under transcriptional control similar to the gluconeogenic enzyme glucose-6-phosphatase, suggesting that the existing orchestrated mechanistic regulation towards increasing gluconeogenesis on insulin resistance might involve Sepp1.

Levels of plasma Sepp1 have been shown to be elevated in human patients with type 2 diabetes [267, 345], a pathology closely related to metabolic syndrome [346]. Moreover, the same report described a negative correlation between hepatic Sepp1 mRNA and metabolic clearance rate of glucose, and a positive correlation between serum Sepp1 and fasting plasma glucose.
Another selenoprotein recognized to participate in glucose metabolic regulation is GPx1. GPx1 KO mice displayed enhanced protection against high-fat-induced insulin resistance [329]. Such effect was attributed to increased ROS generation induced by insulin, which led to oxidation of inhibitory phosphatases, and a consequent increase in Akt phosphorylation and glucose uptake in target tissues. Phosphatase PTP1B is affected by Se supplementation via alterations in its redox regulation, promoted by GPx1 activity; once PTP1B is more active, hepatic lipogenesis is induced [327].

Interestingly, Labunskyy et al. [347] proposed that either overexpression or deficiency of selenoproteins such as Sepp1 or GPx1 could lead to a type 2 diabetes phenotype. Using the transgenic mouse containing the mutant tRNA\(^{[\text{Ser]}\text{Sec}}\) that impairs translation of only a subset of selenoproteins [348], the authors concluded that the animal developed a mild type 2 diabetes phenotype despite lower GPx1 levels in the liver and kidneys. This is paradoxical because overexpression of GPx1 mRNA and/or increases in GPx1 activity have been linked to hyperglycemia, hyperinsulinemia and obesity [349] and GPx1 KO to increased insulin sensitivity [329]. It is important to note, however, that the mutant tRNA\(^{[\text{Ser]}\text{Sec}}\) mouse was reported to have a 48% decrease in plasma Se levels, but a ~67% and 40% decrease in hepatic Se content and Sepp1 gene expression, respectively [348]. On the other hand, Sepp1 KO mice fed a Se adequate diet had lower Se levels in most tissues except liver [142] and instead presented increased insulin sensitivity even when fed a high fat diet [267]. Thus, the question remains as to whether the mild type 2 diabetes phenotype seen by Labunskyy et al. [347] is independent of Sepp1 levels or if it is affecting another factor related to Se and glucose metabolism, such as Scly.

Obese people express less Dio2 and Dio3 in the liver [350]. Interestingly, Dio2 KO mice were also susceptible to high-fat-induced obesity [170], besides failing to properly thermoregulate upon cold exposure [169]. These two outcomes directly connect local production of thyroid hormone T3 to energy metabolic regulation. In addition, Dio2 KO mice are insulin resistant even on a normal chow diet, and accumulate triglycerides in the liver, despite not yet displaying weight gain [170].

Inability to properly activate thyroid hormone via Dio2 was linked to glucose intolerance through hepatic insulin resistance. Intriguingly, Dio3-dependent thyroid
hormone inactivation was observed to also lead to glucose intolerance, but through a different physiological route [173]. Dio3 is highly expressed in the β-cells of the human and mouse pancreas, and Dio3 KO mice had impaired expression of β-cells genes, including insulin. Moreover, insulin secretion is also impaired. Thus, the glucose intolerance in Dio3 KO mice is a consequence of intrinsic inability to increase circulating insulin levels upon glucose challenge. In addition, it should be noted that the human Dio2 gene expression is inhibited by the heterodimerization of LXR with the retinoid X-receptor (RXR) [351]. The Dio2 regulation by a classic lipogenic transcription factor was observed in LXRα and LXRβ double KO mice, which ectopically express Dio2 in the liver [352], and suggests a strong role of Dio2 inhibition in the pathogenesis of obesity and its hepatic consequences, such as NAFLD.

SelS mRNA was higher in omental adipose tissue of a Chinese population with type 2 diabetes [353], and it is found in the human plasma [95]. Hepatic SelS expression increases after activation with TNFα [354] and the gene was suggested to be a receptor for serum amyloid A [355] which is an apolipoprotein associated with HDL. Serum amyloid A is secreted by liver and adipose tissue during inflammatory processes, thus it is possible that upon chronic inflammation as in type 2 diabetes, this protein plays a role in lipid metabolism [356]. Despite its localization in the ER membrane, SelS can also be secreted by hepatoma cells [95]. Moreover, it was found to be increased in fasting liver, skeletal muscle and WAT of diabetic mice [356], suggesting it may play a role in glucose metabolism.

Mutations in the SelN gene were correlated to several muscular disorders. A fraction of patients with a G -> A mutation leading to congenital hypotrophy of slow-twitch muscle fibers [323] also presented abnormal oral GTT, suggesting a potential implication of SelN in insulin resistance. This notion requires further investigation.

Se availability from diet and/or Se recycling mechanisms in order to maintain proper selenoprotein production is important to glucose and lipid homeostasis in vivo. Both may also contribute to maintaining proper energy function. Impaired Se recycling in the whole-body of mice provides an excellent opportunity to connect these two pathways, Se and energy metabolism, and to separate the roles of each one. As previously described, the disruption of several selenoproteins leads to impaired energy metabolism.
In Chapter 3, it was revealed that Scly KO mice upregulate hepatic selenoprotein mRNA production. In this Chapter, this result is further studied taking into account the obesity developed by Scly KO mice. This unexpected effect on energy metabolism led to a detailed investigation of a possible connection between Se recycling and energetic pathways. To further challenge the Se recycling pathway, Scly KO mice were also fed a diet with low Se levels. Se deficiency accentuated the Se recycling-dependent characteristics of the Scly KO mice. The results connecting Se and energy metabolism are further discussed in this Chapter.

4.2 Methodology

4.2.1. Materials

All reagents used in this study were purchased from Sigma-Aldrich (St. Louis, MO), unless otherwise noted.

4.2.2. Animals

Experimental mice were used as detailed in Chapter 3. Animals were euthanized by CO₂ asphyxiation or anesthetized with tribromoethanol prior to collection of tissues or perfusion for histological procedures, respectively. Body weight (BW) was measured prior to sacrifice, and liver and adipose tissue (inguinal, epididymal and brown fat) weights were measured prior to freezing in liquid nitrogen. Due to milder phenotype presented by the females (Figure 4.2), they were excluded from the analysis and only male mice are reported in this Chapter, except in figure 4.11A, where two females were included in mRNA analysis.

4.2.3. Diets

Unless on dietary Se regimen, animals were maintained on standard mouse chow containing 0.25-0.3 ppm Se. Diets formulated for specific Se content were as previously described [230] and the same used in Chapter 2. These diets contained
12% kcal fat, 68% kcal carbohydrate, plus 0.08 ppm of Se in casein (low), or were supplemented with sodium selenite to contain a total of 0.25 ppm of Se (adequate). Mice remained on Se diets for at least 3 months.

4.2.4. Food consumption

Food consumption was measured twice weekly for two months by weighing the leftovers of 100g of low Se diet supplied weekly into cages. Each cage had 2-3 mice and the consumption was averaged and analyzed per cage. Diet pellets had 3.90 kcal/g and kcal of food intake was calculated by multiplying this factor by the total amount of food consumed per week.

4.2.5. Histology

Liver tissues were paraffin-embedded. Samples were deparaffinized for staining with hematoxylin & eosin (H&E).

4.2.6. Glucose tolerance test (GTT) and insulin tolerance test (ITT)

For GTT, animals were fasted for 4 hours prior to testing and injected with glucose at 1 mg/g BW. For ITT, mice were injected intraperitoneally with 0.75 mU/g BW of human insulin (Humulin R, Eli Lilly). We used strips/glucometer (OneTouch Ultra, LifeScan, Milpitas, CA) for GTT and ITT measurements at described time points. Area under the curve (AUC) was calculated for individual mice and averaged.

4.2.7. Serum hormones

Serum insulin (Alpco Immunoassays, Salem, NH) and serum adiponectin (Otsuka Pharmaceutical Co. Ltd., Tokyo, Japan), were measured by ELISAs following the manufacturer’s protocol. Serum T4 levels were measured by radioimmunoassay as described previously [357] in collaboration with Dr. Ann-Marie Zavacki, Dr. Alessandro Marsili and Dr. P. Reed Larsen, at the Thyroid Lab, Brigham and Women’s Hospital and Harvard Medical School, Boston, MA.
4.2.8. Cholesterol assay

Serum cholesterol was assayed by using commercial kits (Cayman Chemical Company, Ann Harbor, MI, and Wako Pure Chemical Industries, Osaka, Japan) according to the manufacturer's protocol. Both kits showed comparable results in their controls.

4.2.9. Flow cytometry

Cytokines TNFα, IL-6 and MCP1 concentrations were measured using the mouse Th1/Th2 cytometric bead array kit (BD Biosciences, San Jose, CA) following the manufacturer's protocol. Data was collected in a BD FACSCaliber flow cytometer.

4.2.10. GPx activity assay

GPx activity in liver lysates was measured by the coupled enzyme procedure, as explained in Chapter 3. Assay was performed with 40 ng of total protein.

4.2.11. RNA and qPCR analysis

RNA and qPCR analysis was performed as described in Chapter 3. Mouse selenoprotein PCR primers were described previously [65] and their sequences are listed in Table 4.

4.2.12. Western Blot

Liver tissues were pulverized and resuspended in CelLytic MT, sonicated and centrifuged for 10 minutes at 12,000 g and supernatant collected. The protein concentration was determined by Bio-Rad Protein Assay solution (Bio-Rad, Hercules, CA). Protein samples consisting of 5 µg (Sepp1 detection) or 20 µg (detection of all other proteins) or 1 µl of serum were separated in 4-20% SDS-PAGE.
(Bio-Rad), transferred to IPFL membranes (Millipore, Billerica, MA) and the protocol was carried out as described in Chapter 3 section 3.2.7.

4.2.13. Antibodies

All commercial mouse antibodies used in this Chapter were diluted according to the manufacturer’s protocols and included pAkt Ser473, pAkt Thr308, Akt, AMPKα, pAMPKα Thr172 (Cell Signaling, Beverly, MA), GPx3, GPx1 (R&D Systems), and mouse β-actin antibodies (Sigma-Aldrich). It should be noted that pAMPKα Thr172 antibody was specific for human, but it crossreacted with the correspondent phosphorylated Thr183 from the mouse AMPKα sequence. The rabbit polyclonal Sepp1 antibody was custom-produced by Proteintech Group Inc. (Chicago, IL) as described in Chapter 3, and it was used diluted 1:1,000 for liver samples and 1:2,000 for serum samples.

4.2.14. Insulin challenge

Phosphorylated proteins were detected by Western Blot after insulin challenge, as described previously [267]. Briefly, animals fed 0.25-0.3 ppm Se chow were fasted for 4 hours prior to injection with a sub-lethal dose of 10 mU/g BW of human insulin (Humulin R, Eli Lilly). After twenty minutes, mice were sacrificed by CO2 asphyxiation and their livers were collected. The fresh liver was homogenized in a Polytron homogenizer at 15,000 rpm in buffer containing 20 mM Tris, 5 mM EDTA, 10 mM Na4P2O7, 1% NP-40, 1% protease inhibitor cocktail and 1% phosphatase inhibitor cocktail (Pierce-Thermo Scientific, Rockford, IL). Tissue lysates were solubilized by continuous stirring for 1 hour at 4°C and centrifuged for 60 minutes at 14,000x g. Supernatants (20 µg) were electrophoresed as described above.

4.2.15. Oxidative stress assay

Oxidative stress was determined by measuring lipid peroxidation end products using the OxiSelect HNE-His Adduct ELISA kit (Cell Biolabs, Inc., San Diego, CA) and following the manufacturer’s protocol.
4.2.16. **Statistical analysis**

Results were plotted and analyzed using Prism software (GraphPad Software Inc., La Jolla, CA). Applied statistical tests varied depending on experimental design and are indicated in figure legends. *P* values and sample size (*n*) are indicated in figure legends or graphs. All results are expressed as means ± SEM.

4.3. **Results**

4.3.1. **Effects of Se intake on Scly KO mice**

4.3.1.1. **Body and fat weights**

As presented in Chapter 3, Scly KO mice had changes in the liver, the main site of Scly expression and function in the body [225] and the major site for regulation of glucose and lipid metabolism. In order to verify how much the pathways involved in energy metabolism rely on dietary Se upon disruption of Scly, mice were fed diets with 0.08 ppm or 0.25 ppm Se for at least 3 months.

When Scly KO mice fed a low Se diet reached ~3 months of age, differences in fat accumulation became apparent (Figure 4.2). Interestingly, males were more affected than females, which presented only mild fat accumulation. The male Scly KO mice were heavier (Figure 4.3A), had larger inguinal (ingWAT; Figure 4.3B) and epididymal (eWAT; Figure 4.3C) white fat depots, and increased interescapular brown fat (iBAT; Figure 4.3D) on an adequate Se diet than WT mice in the same diet. The overall fat accumulation became even more prominent when the animals were Se-restricted (Figure 4.3). Interestingly, the ingWAT of WT mice also became slightly heavier after Se restriction (Figure 4.3B).
4.3.1.2. Liver size and histology

Detailed investigation of the Scly KO mouse liver was carried out. Livers of Scly KO mice were found to be visually normal when animals were fed an adequate Se diet. Nevertheless, livers of male mice were larger (Figure 4.4A) and heavier (Figure 4.4B) upon low Se dietary intake.

Histological analysis of Scly KO liver revealed mild accumulation of lipid droplets in hepatocytes of mice on an adequate Se diet. This histological result became more pronounced in the liver of Scly KO mice fed a low Se diet (Figure 4.5).

4.3.1.3. Hepatic cytokines

It is known that a steatotic liver will have increased infiltration of immune cells, which will lead to increased levels and/or production of some inflammation markers, known as cytokines. The levels of IL-6, TNFα and MCP1 in the liver of WT and Scly KO mice were measured. Hepatic IL-6 was reduced in the Scly KO mice, an effect that was independent of Se levels (Figure 4.6A). IL-6 reduction in Scly KO mice was mostly an effect of genotype ($P_{\text{genotype}}=0.0431$). Nevertheless, insulin resistance increases IL-6 levels, which suggests the infiltrated macrophages of Scly KO mice are producing IL-6 indendent of metabolic status. TNFα levels were decreased in low Se diet in Scly KO mice (Figure 4.6B), also a result contrary to what is expected from a steatotic liver, but that indicated a possible direct involvement of Scly in cytokine production. The effect on TNFα production was only dependent on genotype ($P_{\text{genotype}}=0.0184$). Despite hepatic steatosis, MCP1 levels were maintained in the liver of Scly KO mice regardless of diet or genotype (Figure 4.6C).

4.3.1.4. Serum parameters

Increased fat weight together with hepatic steatosis are the classic characteristics of imbalanced lipid metabolism [358], and are often associated with the early stages of metabolic syndrome, which is additionally characterized by hyperinsulinemia. Therefore, this investigation was carried out to assess whether
insulin function was disturbed in the Scly KO mice. Fasting serum insulin levels were almost doubled in the KO animals fed an adequate Se diet (Figure 4.7A). On the low Se diet, insulin levels of the Scly KO mice were increased even further.

Adiponectin levels were unchanged in the Scly KO mice, despite the increased accumulation of fat in the WAT depots (Figure 4.7B). In addition, Se deficiency increased circulating adiponectin levels in the WT mice compared with a Se adequate diet (Figure 4.7B).

Circulating T4 levels were increased in the Scly KO mice on a low Se diet and decreased in an adequate Se diet compared with WT mice. Interestingly, T4 levels in Scly KO mice were maintained at the same levels on both diets, while the WT decreased as expected in low Se dietary intake (Figure 4.7C).

Fasting serum cholesterol levels were unchanged in the mice fed a Se adequate diet. Nevertheless, on low Se, the Scly KO mice increased their serum cholesterol compared with WT mice, which maintained their cholesterol levels (Figure 4.7D).

4.3.1.5. Glucose tolerance

The rise in serum insulin is likely to be a result of compensatory increase in its production in pancreatic β-cells, responding to peripheral insulin resistance. Whole-body glucose sensitivity, as measured by GTT, showed a trend towards intolerance in normal diet (Figure 4.8A). It is important to note that the response after 30 minutes of glucose overload was the same in WT and Scly KO mice, an indication that glucose tolerance was maintained.

Nevertheless, when Scly KO mice were Se deficient, they became glucose intolerant compared with WT (Figure 4.8B). In addition, their response to insulin stress, as measured by ITT, was also impaired (Figure 4.8C).

Taken together, these results suggest that Scly KO mice fed an adequate Se diet is in the early stages of metabolic syndrome, with hyperinsulinemia and hepatic steatosis. Upon a low Se diet, the same mice developed obesity, hypercholesterolemia and insulin resistance, aggravating the metabolic syndrome phenotype.
4.3.2. Effect of insulin challenge on the Scly KO mice liver

4.3.2.1. Oxidative stress

Several proteins are activated in the liver when serum insulin levels rise, and these factors trigger increased demand for the insulin-coupled phosphorylation cascade to function. An overstimulated insulin cascade may reach a threshold, when the phosphorylation is no longer transduced and the final outcome, the translocation of GLUT to the membrane, becomes halted, leading to insulin resistance. To verify whether Scly KO mice were developing hepatic insulin resistance, animals fed standard mouse chow were challenged with a sub-lethal dose of insulin. Oxidative stress levels were monitored by measuring the HNE-his adduct and GPx activity measurement. Phosphorylation levels of Akt and AMPKα were also assessed.

Results from HNE assay indicated increased oxidative stress levels in the liver of Scly KO mice compared with the WT mice (Figure 4.9A). GPx activity was decreased by approximately 40% in the Scly KO mice liver after insulin challenge (Figure 4.9B).

4.3.2.2. Phosphorylation levels

Insulin-challenged Scly KO mice had decreased levels of Akt phosphorylation on two sites, the Ser 473 (pAkt Ser473) and threonine (Thr) 308 (pAkt Thr308) in comparison with WT mice (Figure 4.9C). Phosphorylation of Ser473 and Thr308 are crucial for activation of the kinase activity of Akt regulated by insulin [359]. The result indicated that the Scly disruption leads to attenuation of the hepatic insulin response.

Strikingly, there was no difference in phosphorylation levels of AMPKα (pAMPKα Thr172) between Scly KO and WT mice subjected to insulin challenge (Figure 4.9D).

4.3.3. Effect of Scly disruption on gene expression of glucose and lipid related genes

With an inefficient response to insulin and the consequent lack of internal glucose, cells change their milieu and several compensatory mechanisms become
activated, inducing the expression of several genes. Transcription factors PPARα, PPARγ, PGC1α and LXRα were upregulated in the liver of Scly KO mice on an adequate Se (Figure 4.10A). Interestingly, mRNA levels of GLUT2, the hepatic glucose receptor isoform, were not changed in Scly KO mice compared with those of WT mice (Figure 4.10A).

ACC1 is a key enzyme of the cholesterol biosynthesis pathway. It is regulated by insulin, its gene expression is activated by heterodimerization of LXRα with RXRα [339], and inactivated by AMPKα [338]. ACC1 levels were analyzed in response to different Se diets. ACC1 mRNA was found to be upregulated on Se deficient livers (Figure 4.10B).

4.3.4. Specific effects of a low Se diet on Scly KO mice

4.3.4.1. Sepp1 and GPx1 expression

Sepp1 mRNA levels increased ~3-fold in Scly KO mice when fed an adequate Se diet (Figure 3.5 in Chapter 3); this increase was even greater (~10-fold) on a low dietary Se intake (Figure 4.11A). Nevertheless, Sepp1 protein levels in the liver did not change between Scly KO and WT mice (Figure 4.11B). At least for the specific case of Sepp1, the upregulation of mRNA was not followed by increases in protein levels. Despite unchanged protein levels in the liver, Sepp1 levels decreased in the serum of Scly KO animals (Figure 4.11C).

GPx1 has also been previously reported to be involved in glucose metabolism when overexpressed in the liver [171]. Increases in hepatic GPx1 mRNA of Scly KO mice liver on a Se adequate diet were previously shown (Figure 3.5 in Chapter 3). In Scly KO mice on low dietary Se intake, hepatic GPx1 mRNA was not increased (Figure 4.11D). In the same mice, however, hepatic GPx1 protein levels decreased (Figure 4.11E), in spite of the metabolic syndrome phenotype.

4.3.4.2. Food consumption

The Scly KO mice on a Se deficient diet displayed a ~15% increase in the caloric intake over a period of 10 weeks when compared with WT mice (Figure
Nevertheless, because the animals were obese, when the value of ingested calories was corrected for their body weights, differences between Scly KO and WT mice were no longer seen (Figure 4.12B).

4.3.5. Aging effect on glycemia and glucose tolerance in Scly KO mice

As described previously in this Chapter (Figure 4.5, Figure 4.7 and Figure 4.9), juvenile, 3-5 months-old Scly KO mice fed an adequate Se diet had hyperinsulinemia, mild hepatic steatosis and attenuation of the insulin phosphorylation cascade, combined symptoms of metabolic syndrome. Such findings led to the question of whether glucose intolerance would develop as the mice age.

One-year-old Scly KO mice fed standard chow containing adequate Se levels were submitted to GTT and their fat depots were weighed. The differences in fat weight seen in 3-5 months-old Scly KO mice were no longer seen in fat depots of one-year-old Scly KO mice (Figure 4.13). Moreover, fasting glucose levels were maintained in one-year-old Scly KO mice (Figure 4.14A), and they did not develop glucose intolerance (Figure 4.14B).

4.4. Discussion

Diet is the main source of Se, provided Se intake is adequate. When dietary Se levels are insufficient, recycling of Se at the expense of some proteins or tissues to prioritize the Se supply where it is most essential (e.g. brain and testis) may become prominent. Mechanisms for Se prioritization, such as Se recycling from Sec, are likely to have evolved in concert with exposure to limited Se availability, allowing organisms to tightly regulate and prevent the deleterious effects of Se deficiency.

This Chapter reports the additional and unique physiological role of the Se recycling enzyme Scly in vivo in hepatic Se metabolism, emphasizing the crosstalk between Se and pathways associated with glucose and lipid metabolism. The present results suggest a strong influence of the Se recycling pathway on glucose and lipid homeostasis.
The disruption of the Scly gene in mice unveiled a major effect on lipid accumulation in fat depots and liver. Because the liver is a major site of Scly production and function, the above mentioned effect was not entirely unexpected [184]. Nevertheless, the lipogenic phenotype caused by Scly disruption was unexpected.

In addition to liver, Scly is highly expressed in the kidneys and testes [197]. These tissues also express significant amounts of Sepp1 and/or rely on Sepp1 as a source of Se [124]. In contrast to the metabolic phenotype observed in Scly KO mice (section 4.3 of this Chapter), Sepp1 KO mice are smaller than their WT counterparts and have increased Se content in the liver [160]. Sepp1 KO mice also have improved glucose tolerance and enhanced insulin sensitivity [267]. The fact that the Scly KO mice characteristics are opposite to those of Sepp1 KO mice suggests that Sepp1 may rely on Scly inhibition to act upon glucose metabolism.

Both Scly and Sepp1 are produced primarily in the liver. Sepp1 is largely secreted into the bloodstream where it transports Se to other organs of the body [124]. Sepp1 is a unique protein, containing 10 Sec residues in rodents and humans, and most of them are clustered in the C-terminal region of the protein [113]. Although Sepp1 mRNA levels were upregulated in the liver of Scly KO mice, an increase in circulating levels of Sepp1 was not observed (Figure 3.3). This suggests that Scly disruption may impair selenoprotein translation locally but does not affect Se status for other organs of the body. In conditions of low dietary Se, plasma Sepp1 has been shown to be decreased [360] and indeed this is what is observed in Scly KO mice on low Se intake (Figure 4.11C). An increase in hepatic Sepp1 mRNA is observed in Scly KO mice on a Se deficient diet, a pattern that is not followed by increases in hepatic Sepp1 protein. On the other hand, GPx1 protein levels are decreased, which in turn could increase cellular oxidative stress. Taken together with Figure 3.5, these results suggest that hepatic selenoprotein translation is less efficient in Scly KO mice. The diminished Se content in the liver of Scly KO mice appears to be an important factor for selenoprotein synthesis, which compounded with the disruption of Scly leads to elevated oxidative stress, as seen when the system is challenged with insulin.

Previous studies have linked hepatic GPx1 overexpression [171, 361] or increased Sepp1 levels [267] to hyperinsulinemia, hyperglycemia and insulin
resistance. In addition, other selenoprotein transgenic mouse models present disturbances in glucose metabolism, such as the Dio2 KO mice [170, 173]. Scly KO mice on an adequate Se diet increased liver GPx1 and Sepp1 mRNA expression (Figure 3.5). Nevertheless, these increases were not followed by increases in protein levels. The lack of changes in these proteins indicates that dietary Se is overcoming the absence of Scly to some extent inside the liver cell. The fact that SPS2 could bind directly to dietary selenite [362], thus circumventing the Scly step, could explain the milder phenotype observed under adequate Se intake. Interestingly, none of these two proteins seem to be involved in the metabolic phenotype observed on low Se diet. GPx1 levels were decreased in the liver (Figure 4.11E), a result contrary to the proposed effect of GPx1 inducing hyperglycemia and hyperinsulinemia cited above. Such discrepancy suggests that GPx1 levels and its physiological role may be closely dependent on Scly action. Moreover, Sepp1 levels were reported to increase in the plasma of type 2 diabetics, which is not what is observed when Scly is disrupted in mice (Figure 4.11C). Thus, the severe metabolic syndrome observed in the Scly KO mice on low Se diet may result from impaired selenoprotein production.

Serum adiponectin levels were expected to decrease in obese animal models. Nevertheless, Scly KO mice did not decrease adiponectin serum levels (Figure 4.7B). Although it is tempting to consider adiponectin a marker of weight loss in obese patients, there are conflicting results from clinical trials leading to reevaluation of adiponectin as a plasma biomarker. From seven clinical trials, five revealed no effect of calorie restriction on adiponectin plasma levels [302]. Interestingly, an unexpected effect of Se restriction was observed in WT mice, which increased adiponectin levels. Nevertheless, since adiponectin serum levels typically decrease in obese mice [363], the maintenance of its levels in Scly KO mice suggest an influence of the recycling mechanism on production and/or secretion of adiponectin at the site where the hormone is synthesized, the WAT depots. In addition, it could indicate an effect of Scly disruption in adipocyte signaling that promotes cell proliferation. Additionally, adiponectin is known to modulate PPARγ and AMPK levels in the tissues targeted by insulin, and to enhance PPARα expression [364]. Scly KO mice showed increased PPARα and PPARγ mRNA expression on an adequate Se diet (Figure 4.10).
The increase in T4 under low Se in the Scly KO mice compared with WT mice (Figure 4.3C) could be explained by an effect of Se deficiency on protein synthesis of hepatic Dio1 [365, 366], the enzyme responsible for conversion of T4 to biologically active T3 in the liver. Decreases in Dio1 levels can be correlated to decreases in the rate of conversion of T4 to T3, an essential reaction for the biological effects of thyroid hormone. Nevertheless, several possibilities remain to be investigated regarding the effect of Scly on thyroid hormone metabolism and function. For example, Scly disruption in the thyroid could be responsible for stimulating T4 production, or could be only affecting the conversion of T4 into T3 in the tissues by decreasing the production of Dio enzymes. The lack of Dio production in the liver could directly influence expression of genes related to energy metabolism, which would ultimately induce the hepatic lipid accumulation [350]. Interestingly, the WT mice decreased their serum T4 levels on Se deficiency, an effect that could be explained by the impaired expression of selenoproteins disturbing thyroid hormone levels [367]. Clearly, further research is required to address some of these questions.

Another possibility to further understand the mechanistic participation of Scly on energy metabolism would be to analyze levels and activity of Scly in the liver of the transgenic mutant tRNA[Ser]Sec mice that express only a subset of selenoproteins not related to stress response. To compensate for the lack of Se-related stress responses, the transgenic tRNA[Ser]Sec mouse could potentially improve its Se utilization by activating recycling mechanisms. Such approach could assist in the clarification of several questions concerning the crosstalk between Se and glucose metabolism. tRNA[Ser]Sec mutant mice were reported to develop a type 2 diabetes-like phenotype [347]. In addition, a detailed metabolic comparison between the insulin resistance developed by the Scly KO mice and mutant tRNA[Ser]Sec mice may provide further insights into the crosstalk between Se metabolism and glucose response.

After insulin challenge, Scly KO mice had increased oxidative stress in the liver compared with WT mice, indicating that disruption of Scly KO mice impairs the antioxidant system by decreasing the GPx activity. Metabolically, disruption of the Scly gene appears to stimulate pancreatic insulin production and secretion, similar to observed following overexpression of GPx1 [172]. In addition, it is widely accepted that alterations of glucose homeostasis can affect lipid metabolism, since in the rodent liver both pathways interconnect at the Acetyl-CoA synthesis step [341] via
downstream activation of AMPKα, and both pathways can be regulated by the action of PTP1B upon high dietary Se intake [327]. Thus, dysregulated carbohydrate metabolism could potentially affect lipid metabolism. Scly action could be one of the molecules that connect Se and energy metabolism in the cell.

Previous studies have shown Se supplementation to be a major regulator of AMPKα phosphorylation in colon cancer cells [368] and skeletal muscle of diabetics rats [369]. In addition, Sepp1 was pinpointed in mice to be the effector protein in the crosstalk between Se and lipid metabolism through the action of AMPKα phosphorylation [267]. Present results indicate that Sepp1 levels are dependent on the presence of Scly, at least to some extent. In addition, the results showing unchanged AMPKα phosphorylation in mice fed an adequate Se diet are in contrast with the reported role of Sepp1 levels in regulating hepatic lipid biosynthesis. This contrast could be attributed to gender differences in Sepp1 expression [370] and hepatic Se content [371] in rodents. Current results were obtained with male mice whereas those used to establish the involvement of Sepp1 on AMPKα phosphorylation were all female [267]. Moreover, in WT mice, Se supplementation could drive a decrease in Scly levels (Figure 2.3), which could in part lead to the metabolic effects observed in this Chapter.

Most notably, the presence of the fat droplets in the liver also suggests a direct effect of Scly on hepatic de novo lipogenesis. Interestingly, Scly was found in a yeast two-hybrid system to directly interact with the liver enzyme farnesyl diphosphate farnesyl-transferase I, also called squalene synthase or SQS [191]. SQS catalyzes the conversion of farnesyl pyrophosphate to the cholesterol precursor squalene, a two-step reaction that releases pyrophosphate. The SQS-catalyzed reaction is the first committed step of the sterol branch of the cholesterol biosynthesis pathway [372] and occurs downstream of AMPKα modulation. The finding that AMPKα phosphorylation was unchanged in the liver of Scly KO mice suggests that the interaction between Scly and SQS may provide a pathway for the involvement of Se on energy metabolism in following Scly disruption. Future studies targeting the nature of the interaction between Scly and SQS may reveal novel insights in the role of Se on energy metabolism.

Under both Se diets Scly KO mice showed increased fat accumulation, but only mice fed low Se developed obesity, suggesting that levels of Se could be
inversely correlated with the extent of obesity developed. The development of several characteristics of the metabolic syndrome phenotype (e.g. obesity, hyperinsulinemia, glucose intolerance, hypercholesterolemia and hepatic steatosis) in Scly KO mice fed a limiting Se diet despite decreased levels of Sepp1 in the serum, suggests that energy homeostasis in this model may be more reliant on proper oxidative stress response than on Sepp1 levels. Moreover, the increase in food consumption by approximately 15% observed in Scly KO mice on a low Se intake (Figure 4.12A) suggest that obesity driven by Se deficiency in these mice could involve leptin-dependent mechanisms under hypothalamic control of satiety. Nevertheless, it should be noted that the elevated food intake in Scly KO mice is not maintained when the ingested kcal is normalized by the BW of each individual mouse (Figure 4.12B). Such result raises the possibility that the observed increase in food intake may be a consequence of increases in fat depot, and not a direct effect of the Scly on feeding behavior.

Despite the hepatic regulation of lipid metabolism, the connection between Se and lipid metabolism may also be uncovered in the WAT. Obesity is characterized by an increase in oxidative stress in WAT cells [373, 374], therefore a role for selenoproteins in this pathology potentially exists. WAT and primary adipocytes from obese mice have lower levels of Sepp1 expression [375]. It must be noted, however, that the detection of Sepp1 in the aforementioned study was performed using a commercially available mouse antibody that recognizes Sepp1 as well as several other proteins (Bellinger F, unpublished data). In addition, selenate, but not selenite, was reported to inhibit adipogenesis via upregulation of SelS in the murine 3T3-L1 pre-adipocyte cell line [376]. The cited report did not include analysis of other selenoproteins and/or selenoprotein synthesis factors that may be involved in adipogenesis and/or adipocyte differentiation mechanisms. Interestingly, SelS is one of the selenoproteins found to be upregulated in the WAT of Scly KO mice (Figure 3.5).

Surprisingly, fat weight discrepancies in the Scly KO mice disappeared with aging. When fat depots from one-year-old mice fed a Se adequate diet for their entire lifespan were analyzed, the WAT and BAT weights were not significantly different. Aging is recognized to induce a longitudinal decrease in plasma Se levels in humans [377], which is accompanied by increases in BW. Plasma Se levels were maintained
in mice after one-year-old on adequate Se diet (0.24 ppm). Nevertheless, tissue Se levels are significantly increased in the liver and kidneys after one year [378]. Alternatively, after one year, Scly KO mice may have accumulated enough Se in their livers to restore selenoprotein function. Nevertheless, it is still unclear as to what effects that Scly might directly exert on the cholesterol biosynthesis pathway. It is also possible that WT mice had actually gained more weight as a result of the normal process of aging. Aging is characterized by the shortening of the telomere regions of the genome, exposing the ends of chromosome ([379] reviewed in [380]). Telomere shortening has been linked to several common pathologies, such as diabetes [381], metabolic syndrome, high blood pressure [382], obesity [383], and to responses to psychosocial stress [384], and to patients with NAFLD [385]. The disruption of Scly could instead protect against the increases in BW that are expected with aging.

In the present Chapter the involvement of Se recycling mechanism in lipid and glucose physiology were discussed. It was also shown that adequate dietary Se intake masks and/or compensates some of the lipid disturbances that Scly disruption causes. When challenged with a low Se diet and in the absence of a functional Se recycling mechanism, young Scly KO mice developed obesity and metabolic syndrome. These data implicate that Se metabolism plays a major role in the pathogenesis of obesity possibly via activation of hepatic lipid production. The results presented and discussed in this Chapter broaden our understanding of the function of Scly, and unveiled its unique physiological importance to the Se recycling mechanism. These results reveal new insights into how the Se recycling pathway could be non-essential for Se-dependent functions such as fertility and neurological function, but necessary for a balanced energy metabolism. It also provides in vivo evidence that Scly may potentially be the molecular link that connects Se and energy metabolism pathways in the liver, adding a novel dietary and molecular perspective in the understanding of metabolic syndrome.
4.5. Figures

**Figure 4.1. Illustration showing the progression of type 2 diabetes.** The weight gain combined with a genetic background will induce the production of insulin by β-cells of the pancreas. As insulin rises, insulin resistance in target tissues is developed. The hyperinsulinemic environment allows the establishment of dyslipidemia and cardiovascular problems, as well as impaired glucose tolerance. With time, insulin production decreases and pancreatic β-cells start to fail. This is when glycemia cannot be controlled, and type 2 diabetes ensues. The temporal progression of symptoms is only estimation due to individual variations. Modified from Dagogo-Jack & Bakris, at medscape.org.
Figure 4.2. Development of obesity in male Scly KO mice fed a low Se diet for 3 months. Female Scly KO mice on a low Se diet consistently exhibited a milder metabolic phenotype and were disregarded for the purposes of this chapter’s analysis. (Photo: courtesy of Ann Hashimoto.)
Figure 4.3. Body and fat weights in Scly KO mice fed different Se diets. Animals were fed a low (0.08 ppm) or an adequate (0.25 ppm) Se diet for ~3 months. A) Body weight of mice upon sacrifice. B) Inguinal WAT weight. C) Epididymal WAT weight. D) Interscapular BAT weight. Group comparison was performed by two-way ANOVA (2WA) and $P$ values for variables genotype (g) and diet (d), as well as for the interaction of both, are indicated in the graph. Asterisks represent significance using post-hoc Bonferroni’s test; red line indicate significance after Student’s unpaired $t$-test. *$P<0.05$, **$P<0.01$ and ***$P<0.001$. Sample size is displayed in graphs.
**Figure 4.4.** Macroscopic analysis of Scly KO mice liver on a low Se diet. A) Visual aspect of livers. B) Liver weights. M=male, F=female. *P<0.05 by comparison using Student’s unpaired t-test. (Photo: courtesy of Ann Hashimoto.)
Figure 4.5. Histological analysis of the liver of Scly KO mice on different Se diets. H&E stained sections from WT and Scly KO mice on low (0.08 ppm) or adequate (0.25 ppm) Se diet are shown above. Images on panels depict a representative individual from groups of 3-4 mice. Bars = 100 µm.
Figure 4.6. Cytokine levels in the liver of Scly KO mice fed different Se diets.

Mice were fed a low (0.08 ppm) or adequate (0.25 ppm) Se diets for ~3 months. A) IL-6 levels. B) TNFα levels. C) MCP1 levels. Black bars, WT; white bars, Scly KO. P values and sample sizes are displayed in graphs and were calculated by two-way ANOVA with Bonferroni’s post-hoc comparison analysis.
Figure 4.7. Serum hormones and cholesterol levels of Scly KO mice. A) Fasting insulin levels. B) Adiponectin levels. C) Thyroid hormone T4 levels. D) Fasting cholesterol levels. All results were analyzed by two-way ANOVA (2WA) analyzing for diet (d) and genotype (g), and followed by Bonferroni's post-hoc test for group comparison. *, ** and *** represent P values below 0.05, 0.01 and 0.001, respectively. Red asterisks indicate significance by Student’s unpaired t-test with 95% confidence interval.
Figure 4.8. GTT and ITT on Scly KO mice. A) GTT of mice fed Se-adequate diet (0.25 ppm Se). B) GTT of mice fed a low Se diet (0.08 ppm). C) ITT of mice fed a low Se diet. AUC bar graphs are displayed for each curve. **P<0.01 by Student’s unpaired t-test with 95% confidence interval.
Figure 4.9. Akt phosphorylation is attenuated and oxidative stress aggravated, but AMPKα phosphorylation is unchanged in Scly KO mice fed an adequate Se diet and submitted to insulin challenge. A) Hepatic oxidative stress levels as measured by HNE peroxidation method. B) Liver GPx activity upon insulin challenge. C) Protein levels of pAkt Ser473, pAkt Thr308 and total Akt, and their quantification in arbitrary units (a.u.). D) Protein levels of pAMPKα Thr172 and total AMPKα, with its respective quantification in (a.u.). Protein blots are representative of n=4. Black bars = WT; white bars = Scly KO mice. *P<0.05, **P<0.01 and ***P<0.001 by two-tailed Student’s unpaired t-test.
Figure 4.10. Gene expression of transcription factors and proteins involved in lipid or glucose metabolism in Scly KO mice. A) qPCR analysis revealed an upregulation of major transcription factors involved in lipid metabolism activation and glucose translocation, such as PPARα, PPARγ, LXRα, PGC1α, and GLUT2, in Scly KO mice fed a Se adequate diet. B) Liver ACC1 gene expression is upregulated on Se deficiency. *, **, *** represent $P < 0.05$, 0.01 and 0.001, respectively, calculated by Student’s unpaired t-test with 95% confidence interval. ns, non-significant.
**Figure 4.11.** Sepp1 and GPx1 expression in Scly KO mice on low dietary Se intake. A) Hepatic Sepp1 mRNA levels increased more than 10-fold in Scly KO mice upon feeding a low (0.08 ppm) Se diet. B) Sepp1 protein expression in the liver of Scly KO mice. C) Serum levels of Sepp1 in Scly KO mice. D) Hepatic GPx1 mRNA levels. E) Hepatic GPx1 protein levels. Black bars = WT; white bars = Scly KO mice. *P* calculated by Student’s unpaired *t*-test and *, ** represent *P* < 0.05 and < 0.01, respectively.
Figure 4.12. Food consumption of Scly KO mice on low dietary Se intake. A) Total food consumption at a low (0.08 ppm) Se diet for 10 weeks. B) Food intake in kcal corrected by individual BW. n=10, housed in 4 cages. Student's unpaired t-test of the average AUC values was used to compare WT versus KO. *P<0.05.
Figure 4.13. Body and fat weights of one-year-old Scly KO mice. Animals were fed *ad libitum* standard mouse chow, containing 0.25-0.3 ppm Se, for their entire lives. A) Body weight measured upon sacrifice of WT and Scly KO mice. B) Inguinal WAT weight. C) Epididymal WAT weights. D) Interscapular BAT weight. Values are mean ± SEM, and *P* values were calculated by Student’s unpaired *t*-test with 95% confidence interval. Sample size is displayed in the graphs.
Figure 4.14. Fasting glucose and GTT in one-year-old Scly KO mice. Animals were fed ad libitum standard mouse chow, containing 0.25-0.3 ppm Se, for their entire lives. A) Fasting glucose levels. B) GTT. Sample size for A is the same as for B. P value was calculated by unpaired t-test with 95% confidence interval. The overlapping GTT curve between WT and Scly KO mice yielded AUC values that were not statistically significant by Student’s unpaired t-test. Sample size for A is the same as in B.
### Table 5. List of mouse PCR primer sequences used Chapter 4.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward</th>
<th>Reverse</th>
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<td>AAATGACCCATCTGTAATGC</td>
<td>TCAATCTCACATAGCAGCT</td>
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<td>GAPD</td>
<td>TGACATCAAGAAGGTTGTAAGA</td>
<td>CCACTGGTCGAGACATCCTT</td>
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<td>GPx3</td>
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<td>CCACTGGTCGAGACATCCTT</td>
</tr>
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<td>GLUT2</td>
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<td>ATCTTGTGGTACATCCTCA</td>
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</tr>
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<td>PGC1α</td>
<td>AGCAGAAAGCAAATTGAAGAGA</td>
<td>AGGTGTAACGAGTAGGTGATG</td>
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<td>PPARα</td>
<td>GTGTACGACAAGTGATGATCG</td>
<td>TTTCAAGATCTTGCCATTCTT</td>
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<tr>
<td>PPARγ</td>
<td>TTGATTCTCCAGCATTTTCT</td>
<td>TGGTGTAAGCTGGTGTCTTT</td>
</tr>
<tr>
<td>Scly KO genotype</td>
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<td>CTGCGTGTCCTGAAACTAGCTTCATA</td>
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<tr>
<td>Scly WT genotype</td>
<td>CACAGTGCGCGGCGATGAGGG</td>
<td>CTGGGCTGTCCTGAACATGCTTCATA</td>
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<tr>
<td>Sepp1</td>
<td>CCTTGTGTTGCTTACTCTTCC</td>
<td>TTTGTTGTGGTGTTCGTGG</td>
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</table>
CHAPTER 5

INCREASED SUSCEPTIBILITY OF SELENOCYSTEINE LYASE KNOCKOUT MICE TO HIGH FAT DIET-INDUCED OBESITY

5.1. Introduction

In the previous Chapters of this dissertation, the regulation of Scly gene expression and physiological role in energy metabolism were investigated. The disruption of Scly in an animal model was carried out and analyzed following feeding diets varying in Se content. Se deficiency uncovered a metabolic syndrome phenotype in the Scly KO mice, mostly due to interference of Scly in hepatic insulin signaling and cholesterol biosynthesis. Scly KO mice fed a low Se diet were obese and insulin resistant. Such outcomes suggested a role of Se recycling directly in the pathogenesis of obesity.

Besides the well-evidenced effects of diet and exercise, there are aspects in obesity development that still need to be addressed. One of these aspects is the influence of micronutrients such as Se on lipid metabolism, as discussed in Chapter 4. As suggested in the previous Chapter, the direct role played by Scly in obesity pathogenesis may shed light into this epidemic disease.

5.1.1. Obesity and Se

The results of the NHANES III study revealed that increased BMI was associated with Se deficiency in men and women [386]. Several studies have shown that morbidly obese people present significant plasma Se deficiency [387, 388]. Moreover, studies in rats demonstrated that Se deficiency results in increased plasma cholesterol [389, 390]. In contrast, a recent Finnish study reported a positive correlation between serum Se levels and serum lipids [391]. The authors justify the contradictory result by pointing out that baseline serum Se levels in the Finnish
population are lower. Yet, the underlying factors connecting Se metabolism with lipid metabolism in humans are unknown.

5.1.2. Obesity, oxidative stress and selenoproteins

Obesity has been very simply defined as the increased accumulation of lipids in the WAT stores. Nevertheless, this simple definition lacks several important considerations, such as genetic background and lifestyle components [392]. The genetic background sets the tone for the efficiency in which pathways will absorb and degrade lipids, or for how the body will respond to lipid overload. Nevertheless, the environment plays a crucial role in the obesity pathogenesis, as the same dietary intake of lipids can either be fully stored in WAT depots, or consumed by the organism as a source of energy for the chemical reactions required for life.

Obesity is considered a chronic low-inflammatory state [393, 394]. It is also a disease state characterized by increased oxidative stress. The production of ROS is one of the hallmarks of an inflammatory response. Interestingly, lipogenesis is induced by oxidative stress, via activation of genes that contain in their promoters an Nrf2 binding site [395].

One of the most abundant thiols in cells is glutathione. This antioxidant molecule is the main reducing agent of GPx enzymes, as explained in Chapter 1. Rats with chemically induced depletion of glutathione have lower hepatic lipogenesis and triglycerides in the liver, while lipid profile in the bloodstream was maintained [396]. This effect occurred due to changes in the redox and glutathionylation states of PTP1B, leading to increases in activity of the insulin signaling. To corroborate this effect of glutathione on insulin signaling, GPx1 KO mice were reported to have enhanced insulin sensitivity through inactivation of phosphatases of the insulin cascade such as PTEN, followed by increases in phosphorylated Akt [329].

Se-dependent GPx enzymes are the major players in cellular antioxidant response and are involved in diverse aspects of lipid metabolism. Mice overexpressing GPx1 are obese [171]. This effect is consistent with the antioxidant effect exerted by GPx1 on lipid metabolism that is directed by insulin resistance. It was later suggested that the increases in GPx1 would deplete the cells of ROS, which would ultimately lead to oxidation-dependent activation of the phosphatase
PTP1B [324]. GPx3 was found to be lower in the plasma of obese subjects, and such effect was a consequence of abnormal GPx3 expression in the adipose tissue [397]. Therefore, it is possible that GPx3 is involved in systemic and local protection from oxidative damage. Interestingly, GPx4 has lipoxygenase and lipid peroxidase activity, using mostly membrane phospholipids as substrates. In conditions of low Se availability, GPx4 may use other thiols as reducing substrates [398], thus indicating that its lipid peroxidation activity is pivotal to cell viability [399].

Sepp1 was also demonstrated to possess phospholipid peroxidase activity [113, 400]. Thus, may be considered a good molecular candidate for the connection between Se and lipid metabolism. Sepp1 action was suggested to decrease activity of AMPK, the central regulator of energy metabolism in the liver [267]. The results presented in Chapter 4 showed that for the Scly KO mice, at least when Se is low, Sepp1 plasma levels decreased, despite the increases in plasma cholesterol and BW of the animals. Thus, to exert its effect on lipid metabolism, Sepp1 may depend on the presence of Scly.

In addition to all the mice models described above, transgenic mice lacking tRNA^{Ser}^{Sec} in the liver reduced the overall hepatic selenoprotein production, including Sepp1, but increased their plasma cholesterol, and upregulated hepatic mRNA levels of several genes involved in the cholesterol biosynthesis pathway. This result is similar to what was found in the Scly KO mouse on low dietary Se. Moreover, the tRNA^{Ser}^{Sec} transgenic mouse presents severe liver and WAT necrosis that ultimately led the mouse to death by hepatic failure [174]. It is highly likely that the lack of antioxidant response was the main factor for the liver and WAT necrosis.

The relation between dietary fat intake and selenoproteins has been previously examined. A recent study has shown that mice with targeted disruption of the Dio2 gene, a cellular regulator of thyroid hormone levels, are insulin resistant [170] and develop a fatty liver [321] on standard chow. Both of these traits were also observed in the Scly KO mice. Dio2 KO mice are also more susceptible to obesity induced by high fat diet than their WT littermates [170]. Dio2 has been shown to be expressed in WAT cells [401] and may be involved in the thyroid hormone regulation of lipid metabolism in this organ. This raises the possibility that Scly KO mice on a high fat diet might develop lipid disturbances as a consequence of impaired thyroid hormone regulation. Disruption of deiodinase synthesis and/or activity in target
tissues, such as liver and WAT, as a consequence of Scly disruption could lead to effects on lipid storage and metabolism that are similar to those observed in Dio2 KO mice.

Se levels differentially affect the distribution, expression and action of selenoproteins, as discussed in the section 1.4.2. Moreover, in Chapter 4, the compounding effect of Se deficiency and Scly disruption on lipid metabolism was described and discussed. Thus, obesity was dependent on Se levels. Nevertheless, it remained unclear if the lipid effect observed in the Scly KO mice could be independent of the amount of Se intake. In order to test whether the lipid accumulation was restricted to circumstances of low Se intake, Scly KO mice were submitted to a high fat diet with adequate Se levels. The results of this challenge are described and discussed below.

5.2. Methodology

5.2.1. Animals and diet

The development of Scly KO mice was described thoroughly in Chapter 3 section 3.2.1. Weaned age-matched mice of both genotypes, WT and Scly KO, were assigned to diets containing 45% kcal fat (high fat) for three to four months. High fat diet pellets were branded OpenSource Diets, purchased from Research Diets Inc. (New Brunswick, NJ, catalog number D12451) and the fat content derived from lard and soybean oil. The pellets also contained 35% kcal of carbohydrate, mainly from corn starch, maltodextrin 10 and sucrose, and similar amounts of Se (approximately 0.2 ppm) as sodium selenite, derived from the Mineral Mix S10026 (OpenSource Diets).

Animals were weighed bi-weekly, and when the experiment was terminated, the WAT depots were collected, weighed and frozen in liquid nitrogen. WAT weight was normalized to the BW of the animal at the time of death.
5.2.2. Metabolic parameters

All serum hormones, GTT, serum cholesterol, serum cytokines and serum oxidative stress status were assayed as described in section 4.2, Chapter 4.

5.2.3. Western Blot

Scly KO mice serum (1 µl) was separated by electrophoresis on 4-20% SDS-PAGE and transferred to IPFL membranes as described in section 3.2. Mouse GPx3 antibody (R&D Systems) was diluted according to the manufacturer’s protocols. Rabbit polyclonal Sepp1 antibody (ProteinTech) was used at a 1:2,000 dilution and its development was previously described in section 3.2.

5.2.4. Statistical analysis

Results were plotted and analyzed using Prism software (GraphPad Software Inc., La Jolla, CA). Applied statistical tests varied depending on experimental design and are indicated in figure legends. P values and sample size (n) are indicated in figure legends or graphs. All results are expressed as means ± SEM.

5.3. Results

5.3.1. Effect of a high fat diet on Scly KO mice

5.3.1.1. Body and fat weights

Scly KO mice fed a 45% kcal high fat diet developed severe obesity when compared with WT mice fed the same diet (Figure 5.1). Interestingly, in contrast to the obesity reported on mice fed a low Se diet in Chapter 4, male and female Scly KO mice fed a high fat diet showed the same aggravated obesity phenotype when compared with WT mice. BW differences between Scly KO and WT mice were observed after 5 weeks on diet, and reached statistical significance after 7 weeks on
a high fat diet (Figure 5.2A). IngWAT weight was greater in Scly KO mice than it was in WT mice fed a high fat diet (Figure 5.2B). Nevertheless, only female Scly KO mice showed increased gWAT weight. The weight of the corresponding WAT depot in males, the eWAT, was not different between Scly KO and WT mice after a high fat intake (Figure 5.2C). iBAT of Scly KO mice fed a high fat diet were approximately 20% greater in weight than the same tissue from the WT mice (Figure 5.2D).

5.3.1.2. GTT and insulin levels

Scly KO mice on a high fat diet are also severely glucose intolerant compared with their WT counterparts (Figure 5.3A). Nevertheless, Scly KO and WT mice presented similar fasting glucose levels (Figure 5.3B).

High fat diet induces hyperinsulinemia in WT mice [402, 403]. Nevertheless, Scly KO mice on a high fat diet developed aggravated hyperinsulinemia, with approximately twice as much circulating insulin than WT mice (Figure 5.3C).

5.3.1.3. Serum parameters

Fasting serum cholesterol levels are expected to rise in WT mice with high fat diet-induced obesity. Nevertheless, Scly KO mice fed a high fat diet increased serum cholesterol levels by approximately 30% more than WT mice (Figure 5.4A). Obesity induced by high fat diet accompanied by metabolic syndrome is also known to increase oxidative stress levels in rodents [404, 405]. In accordance with the previous cholesterol results, Scly KO mice presented a more prominent increase in oxidative stress than WT mice (Figure 5.4B).

After high fat exposure, the levels of circulating adipokines IL-6, MCP1 and TNFα are expected to increase in rodents [285, 296, 402]. Interestingly, serum levels of these cytokines were maintained between WT and Scly KO mice on a high fat diet (Figure 5.5), indicating that the effect of a high fat diet overcame the lack of Scly in inducing the production of these cytokines.

Western blot of serum selenoproteins revealed that Sepp1 levels were elevated in Scly KO mice (Figure 5.6A and 5.6B), while circulating levels of GPx3
remained unchanged between the WT and Scly KO mice (Figure 5.6A and Figure 5.6C).

5.4. Discussion

In Chapter 4, male Scly KO mice were found to be obese and to develop NAFLD when dietary Se was low. The results presented in this Chapter suggest that the increased lipid accumulation of KO mice does not depend solely on Se levels. Scly KO mice challenged with a diet containing adequate levels of Se and 45% kcal of fat developed obesity of greater severity than WT counterparts fed the same diet (Figure 5.1 and 5.2), accompanied of significant glucose intolerance (Figure 5.3A), hyperinsulinemia (Figure 5.3C), hypercholesterolemia (Figure 5.4A), and increased oxidative stress in the bloodstream (Figure 5.4B).

The fact that Scly KO mice on a Se adequate, high fat diet exhibited aggravated obesity suggests a direct effect of Scly disruption on lipid metabolism regardless of Se levels. Interestingly, as discussed in Chapter 4, Scly has been found in a yeast two-hybrid system to bind directly to SQS [191]. SQS is an ER resident protein mostly found in the liver. It plays a key regulatory role in the biosynthesis of cholesterol, catalyzing the first committed step of the sterol branch of the cholesterol biosynthesis pathway, downstream of the ACC1 step [372]. The results of this Chapter suggest that, if a direct interaction between Scly and SQS occurs, Scly would probably function as an inhibitor of SQS, thus downregulating hepatic cholesterol biosynthesis. The disruption of Scly would not allow the enzyme to exert its effect on SQS, and cholesterol biosynthesis would be enhanced. Nevertheless, in the liver of WT mice fed standard chow, Scly was surprisingly localized in the nucleus of the cells [197]. It remains to be investigated if Scly subcellular localization could change upon different metabolic conditions, allowing for interaction with SQS, and Scly participation in lipid metabolism.

SQS is downstream of 3-hydroxy-3-methylglutaryl-CoA reductase, or HMG-CoA reductase, the direct enzymatic target of statins [372]. Statins are a class of drugs widely used in humans as cholesterol lowering agents. Interestingly, statins have been shown to decrease GPx1 and GPx4 abundance and activity in human
hepatocytes [406], and to inhibit expression of TrxR1 the rat liver [407]. Furthermore, HMG-CoA reductase and SQS expression are regulated by different isoforms of transcription factor sterol regulatory element-binding protein (SREBP) [408, 409]. It is possible that expression levels of SREBP could participate in an additional regulatory transcriptional mechanism by which the Scly KO mice respond to a high fat intake.

The effectiveness of treatment with statins may depend on the Se status of the individual, which in turn may rely on the putative interaction between SQS and Scly.

Alternatively, the participation of Scly in lipid metabolism could be to inhibit lipolysis. Instead of activating cholesterol biosynthesis, Scly could be inactivating some step(s) of triglyceride lipolysis. Nevertheless, insulin does not directly regulate the key enzyme for hepatic lipolysis, triacylglycerol hydrolase [410]. Insulin action in adipocytes leads to enhanced lipolysis and subsequent FFA accumulation in the liver [411]. Moreover, increases in hepatic FFA enhance fatty acid oxidation pathways in the mitochondria or peroxisomes [412], which would in turn elevate oxidative stress, requiring the antioxidant action of GPx enzymes. Increased oxidative stress could be further magnified if the liver cannot produce proper levels of GPx enzymes, as occurs with the Scly KO mice upon a low Se intake. Nevertheless, when Se levels are adequate, as in the case of Scly KO mice fed a high fat diet, GPx enzymes are probably being produced at normal levels, thus the liver is possibly able to cope with the enhanced oxidative stress. To pinpoint if the aggravation of obesity upon Scly disruption occurs through direct interference in cholesterol biosynthesis and/or if it is a consequence of the impairment of the oxidative stress response, it would be interesting to feed Scly KO mice a high fat, low Se diet and analyze their livers. The physiological role of Scly in lipid metabolism could be further clarified by such an approach, and it may add Scly to the list of enzymes involved in obesity pathogenesis.

As previously stated, obesity is characterized by increases in fat tissue in several areas of the body. Therefore, it is relevant to understand the molecular intricacies of the WAT physiology that might interfere in energy metabolism.

The expression and physiological role of Scly in the WAT is unknown. The Scly KO mice showed increases in several WAT selenoprotein mRNAs, such as those encoding GPx1, GPx4, SelS and TrxR2 (Figure 3.5) and in the SPS enzymes (Figure 3.6), suggesting that this energy-storage tissue may also be dependent on
Se recycling for proper physiological homeostasis. Besides the unsolved issues of the role of Se in lipid metabolism, it is possible that a new role for Se recycling may emerge from studying adipocytes. Scly may be involved in the disruption of oxidative stress responses seen in WAT of obese models by its interference in selenoprotein synthesis. Thus, the link between Se metabolism and lipid metabolic imbalances of the WAT remains to be further investigated.

The greater fat accumulation in the Scly KO mice fed a high fat diet compared with their WT counterparts would imply a greater production of adipokines by the WAT in these mice. Nevertheless, the circulating levels of adipokines TNFα, MCP1 and IL-6 were maintained constant in Scly KO as in WT mice (Figure 5.5). Insulin resistance has been shown to activate TNFα and IL-6 production in adipocytes [413]. Thus, the result suggests that inflammatory response is neither impaired nor enhanced in Scly KO mice, probably because the Se levels in the diet are enough to allow proper immune responses.

Interestingly, plasma levels of Sepp1 are elevated in the Scly KO mice fed a high fat diet compared with WT mice (Figure 5.6A and 5.6B). Since the Scly KO mice have an adequate Se intake, and hepatic Sepp1 production is probably prioritized among other selenoproteins (section 1.4.2), the Scly KO mice are able to properly produce Sepp1 in the liver, and to secrete it to the bloodstream. Thus, it is improbable that Scly KO mice on a high fat diet are Se deficient in organs that rely the most on Se, such as brain and testis. One possibility is that the Scly KO mice on high fat diet are locally Se deficient, mostly in the liver. Increases in circulating Sepp1 and Sepp1 expression in the liver have been linked to type 2 diabetes [267, 345]. The obesity observed in the Scly KO mice could also be a consequence of the elevated Sepp1 levels. This elevation in Sepp1 could be a consequence of the dysregulated carbohydrate metabolism, which would lead to activation of gene transcription of de novo lipogenesis factors by transcription factors such as PGC1α, which was shown to control Sepp1 transcription in hepatocytes [324, 344]. Nevertheless, obesity did not develop in the Scly KO mice fed a diet with 12% kcal of fat and adequate Se (Chapter 4), showing that the disruption of Scly to allow for progression to severe obesity requires the presence of a high fat intake or low Se levels. It remains to be investigated if these two conditions are affected by each other or if they act independently, which could represent a dual role of Scly in metabolism.
Additionally, it should be noted that GPx3 levels were not different between Scly KO and WT mice (Figure 5.6A and 5.6C), even though the animals had increased oxidative stress in the serum (Figure 5.4B). Combined, these results suggest that Scly KO mice may have an elevated Se status, a condition that was correlated before with increased risk of type 2 diabetes [311, 316, 414]. Moreover, high fat diet exposed Scly KO mice may have impaired oxidative stress response in the serum, even though the GPx3 enzyme is present, which could be due to improper selenoprotein biosynthesis. It should be noted, however, as reported in Chapter 3, that circulating GPx3 levels and total GPx activity were unchanged in Scly KO mice fed standard chow. Thus, it is likely that GPx3 production is not affected when the animals are fed a high fat diet with adequate Se levels, and the defect in oxidative stress response is possibly due to impairment of other enzymes after exposure to elevated lipid intake.

The results reported in this Chapter revealed that the Scly KO mice were more susceptible to a high fat diet-induced obesity. This susceptibility could be a result of increased Sepp1 levels, which would inhibit phosphorylation of AMPα [267] a direct role of Scly in inhibiting de novo lipogenesis, or a consequence of the impairment of hepatic selenoprotein biosynthesis. Further investigation using the Scly KO mice may finally elucidate the intricacies of the physiological role of Scly in mammals.
5.5. Figures

*Figure 5.1. Scly KO and WT mice after 4 months on a high fat diet.* Animals were lined up side by side to facilitate visual comparison. M = male; F = female. (Photo: courtesy of Ann Hashimoto.)
Figure 5.2. Body and fat weights of Scly KO mice fed a high fat diet. A) Percentage of body weight increase at indicated times after starting on a high fat diet. B) Inguinal WAT depot weight upon euthanized. C) Gonadal WAT (females) and epididymal WAT (males) weights. D) Interscapular BAT weights. All comparisons were obtained by Student's unpaired t-test, except in (A), where two-way ANOVA (2WA) was applied followed by Bonferroni's post-hoc test. *P<0.05, **P<0.01 and ***P<0.001, other P values are in the graphs, as well as sample sizes.
Figure 5.3. Glucose metabolism parameters of Scly KO mice on high fat diet. A) GTT after a glucose overload. B) Fasting serum glucose levels. C) Serum insulin levels. AUC was calculated for (A) and plotted as a bar graph on the right corner. *P<0.05, ***P<0.001, ns = not significant, by Student’s unpaired t-test with 95% confidence interval.
**Figure 5.4.** Serum cholesterol and oxidative stress levels in Scly KO mice fed a high fat diet. A) Fasting serum cholesterol. B) Serum oxidative stress measurement using the detection of the HNE-his as an indicator of lipid peroxidation. *P<0.05 using Student’s unpaired t-test for comparison with 95% confidence interval.
Figure 5.5. Serum cytokines of Scly KO mice after a high fat diet exposure.
Circulating cytokines were measured by flow cytometry in the serum of Scly KO and WT mice after 4 months in a high fat diet. A) Serum IL-6 levels. B) Serum MCP1 levels. C) Serum TNFα levels. ns = not significant by Student’s unpaired t-test with 95% confidence interval.
Figure 5.6. Levels of serum selenoproteins in Scly KO mice fed a high fat diet. Animals were fed a 45% kcal high fat diet for ~4 months. A) Western Blot analysis of serum Sepp1 and GPx3 protein. B) Sepp1 protein quantification in arbitrary units (a.u.). C) GPx3 protein quantification. **P<0.01 by Student’s unpaired t-test with 95% confidence interval.
CHAPTER 6

CONCLUDING REMARKS

This dissertation attempted to shed light into the regulation by Se and physiological role of Scly, questions that were not previously addressed in the Se research field. Until recently, most Scly studies focused on biochemical and structural aspects of the enzyme, rarely taking into account the participation of Scly in cell physiology and metabolism.

A complex and tissue-specific compartmentalization for Se distribution and action is in place. The effects of Scly are likely to follow this compartmentalization pattern. In Chapter 1, Se research and the known characteristics of Scly were reviewed. Chapter 2 described the regulation of Scly expression by Se levels in hepatocytes and liver. This regulation is of particular interest, when considering that the liver is where highest Scly activity was previously shown [184, 210, 225]. When taking into account the results Scly KO mice described in Chapter 3, it is notable that the liver again emerges as the organ most affected by the lack of Scly. Results reported in Chapter 4 revealed the role of Scly in pathways other than those involved in selenoprotein synthesis and Se metabolism. Scly disruption affected glucose and lipogenic pathways in the liver. Moreover, the effect of Scly KO on hepatic glucose and lipid metabolism were exacerbated in a Se deficient state, when Scly expression and activity are required most. In Chapter 5, a direct effect of Scly on lipid metabolism was observed, but in conditions of adequate Se levels and elevated fat intake. Taken together, the results of this dissertation point to a dual role of Scly in lipid and glucose homeostasis. First, Scly has an impact on selenoprotein biosynthesis, thus playing a role in the maintenance of proper oxidative tonus, mainly in conditions of low Se. The role of Scly in selenoprotein biosynthesis is possibly the primary function of this enzyme. Once the Se recycling pathway is disrupted, a lack of selenoproteins to cope with oxidative stress leads to insulin resistance. Second, Scly may have a direct role in lipid metabolism, independent of Se levels. The
obesity induced by feeding Scly KO mice a high fat, Se-adequate diet suggests that Scly may be in direct control of lipid metabolism. It remains to be elucidated if this effect occurs by activation of de novo lipogenesis, or inactivation of lipolysis. The effect of Scly on lipid homeostasis is possibly a secondary function of this enzyme in cells. It is very likely, however, that the dual role of Scly may depend on the conditions of the cell milieu surrounding the enzyme. The development of the KO mice and the manipulation of their nutritional intake allowed the discovery of such physiological roles of Scly in rodents. Furthermore, the results of this dissertation point to interesting scenarios that will be considered below.

Primarily, these results could improve our understanding of the controversial influence of Se on energy metabolism. Until recently, Se was considered a safe supplement that could potentially prevent several diseases in humans, including cancer. Nevertheless, the clinical trials discussed in section 4.1.2 raised significant questions about Se supplementation, and in light of the results of this dissertation, it is possible that Scly participates in the responses uncovered by these clinical trials.

In the clinical trials in which only men participated, such as SELECT [148, 315, 316], it is possible that Scly activity was downregulated in the liver of these subjects, due to the increase in Se concentration status. In this case, the lower Scly activity could be enhancing the lipid biosynthetic pathway and collaborating to the increases in the incidence of type 2 diabetes reported earlier. Se supplementation would be the driving force to decrease the levels of Scly in the liver of clinical trial subjects. The downregulation of Scly may in turn impair, at least partially, hepatic lipid homeostasis, thus leading to metabolic complications. Because the disruption of Scly in the subjects of these trials was probably incomplete, it is plausible to conjecture that the liver of these subjects was still able to compensate the low Scly activity by another, yet unknown, mechanism that would maintain energy metabolism balanced. In addition, although Se intake of men on the SELECT study was high enough to maintain the Scly recycling pathway slightly downregulated, this high concentration was probably not sufficient to avoid hyperinsulinemia induced by Scly downregulation. Further studies on the regulation of Scly expression by Se in humans are necessary to validate the influence of Scly on insulin levels.

Interestingly, insulin clearance primarily occurs in the liver [415]. In hyperinsulinemic states with established insulin resistance, degradation rates of
insulin possibly increase, as a compensatory response to maintain normal glucose levels. The observed hyperinsulinemia in Scly KO mice may also be caused by diminished insulin degradation rates at the hepatocytes. Even in conditions of normal dietary Se intake, Scly KO mice displayed elevated serum insulin. Nevertheless, Se metabolism has not been directly connected to the insulin degradation pathway. The only report that marginally connects Se and insulin degradation discusses, in fact, the degradation of beta-amyloid 42 (Aβ42). In healthy humans, the degradation of Aβ42 is mediated by Insulin-degrading Enzyme (IDE; [416]). Aβ42 accumulation is the hallmark of Alzheimer's disease. Nevertheless, Se levels have not been shown to correlate with accumulation of Aβ42 in cerebrospinal fluid [417]. Interestingly, IDE is a thiol-sensitive enzyme, and oxidized glutathione has been shown to inhibit its activity [418]. Since oxidized glutathione is the byproduct of the action of GPx enzymes, it raises the possibility that Scly (or Scly-mediated selenoprotein regulation) may interfere with IDE in the liver as well.

Another question raised by the present Scly studies regards the observed gender differences in the Scly KO mice in response to different diets. Male Scly KO mice fed a low Se diet developed severe obesity, whereas females only showed mild fat accumulation. To our surprise, following a high fat, Se adequate diet for three months, both male and female Scly KO mice progressed similarly to severe obesity. These observations raise several questions regarding the influence of gender on Se metabolism.

The impact of gender on Se biomarkers has been observed in clinical trials since the late 60's [419, 420]. Nevertheless, few reports have addressed the effect of gender in Se responses at the molecular level. Under a state of Se deficiency, females have been demonstrated to uptake Se in a more efficient manner than males [421]. Furthermore, female rats have a lower Se requirement than males [420]. Se has also been shown to be differentially uptaken by the reproductive organs. While the retention rate for Se uptake is highly efficient in the testes [421], the female reproductive system does not appear to uptake or retain significant levels of Se [422].

The most studied effect of a selenoprotein in connection to gender is the role of GPx4 in spermatozoa structure and viability [176], and male fertility [423]. Furthermore, it has been observed that female C57BL/6 mice have higher Dio1
mRNA expression in the liver than males; however, Dio1 activity in the liver of females is drastically lower than in male mice [370]. Under a state of Se deficiency, Dio1 mRNA levels in females is 2-fold greater than in males, while Dio1 activity is unchanged between both genders. Thus, Se levels appear to exert a significant impact on the gender dependence of Dio activity in the liver, possibly by affecting translational efficiency and/or protein stability. Plasma GPx3 activity has been reported to be higher in male mice compared with females [370]. Nevertheless, in humans, GPx3 levels and activity is increased in females [424]. On the other hand, hepatic Sepp1 mRNA levels are higher in male rodents [370], and male Sepp1 KO mice show a much stronger neurological impairment than females [261]. Interestingly, the expression levels of Sec incorporation factors EFSec and SBP2 were shown in the same study to be independent of gender, indicating that if the gender influences translational efficiency, it probably does so through other members of the Sec incorporation complex [370].

When considering the gender differences observed in Scly KO mice fed a low Se diet, it is possible that the effect of Scly on selenoprotein production may predominantly occur in a subset of selenoproteins that are strongly regulated by gender. Alternatively, male mice may cope differently with cellular oxidative stress, possibly relying more on Se-dependent mechanisms for the proper antioxidant response. Male Scly KO mice may be more sensitive to increases in oxidative stress, thus facilitating the development of hyperinsulinemia. Both possibilities may explain the gender effect observed in Scly KO mice on a low Se diet. Nevertheless, the observation that high fat diet induces obesity in both genders of Scly KO mice also suggests that a role of Scly in lipid metabolism might not be gender-dependent, unless Se levels are lowered. To assess the gender dependence of obesity in Scly KO mice fed a low Se diet, one possibility would be feeding the same diet and/or a high fat diet to ovariectomized females or orchidectomized males. Such approach would help to clarify the metabolic effects that are gender-dependent from the ones that are restricted to Scly disruption. Another possibility would be the development of a tissue-specific Scly KO mouse model. Due to the main effects on liver, the liver-specific Scly KO mouse would allow sorting of the systemic from the hepatic, localized effects of the Scly disruption. On the other hand, the development of testes-
or ovary-specific Scly KO mice would help to further elucidate the different gender effects observed upon feeding a low Se diet or a high fat diet.

The results of this dissertation also bring a new perspective that may relate to the role of Scly in human metabolism and health. For instance, in the published genome sequence of Khoisan and Bantu African populations [425], the last surviving hunter-gatherer tribes and considered to be one of the oldest lineages of modern humans, 62 single-nucleotide polymorphisms (SNPs) were identified inside the Scly gene sequence found in the NCBI database. Only eighteen of these SNPs are silent mutations, all others being missense mutations and 5 of them validated in the Khoisan group. It is unknown if these missense mutations allow more efficient or less efficient activity of the Scly enzyme. Interestingly, the Khoisan people live in the Northern Kalahari Desert area of Namibia, where Se levels in the soil are low [426, 427]. These Northern Namibian tribal populations have been studied for their weight and nutritional intake, and it has been shown that women in that region are severely thin compared with other Namibian populations [428, 429]. It would be interesting to find out if mutational variations in the Scly gene in these populations are involved at least partially in the thin phenotype. Additionally, higher Scly activity would presumably allow populations subjected to low Se intake to metabolize glucose and lipids in a more efficient manner.

In conclusion, the data presented in this dissertation revealed that the Se recycling mechanism is involved in lipid and/or glucose physiology (Figure 6.1). It was also demonstrated that adequate dietary Se intake masks and/or compensates some of the disturbances in lipid metabolism that Scly disruption causes. When challenged with a low Se diet and in the absence of a functional Se recycling mechanism, the Scly KO mice developed obesity and metabolic syndrome. Moreover, when challenged with a high fat diet, obesity was further aggravated. Scly is potentially implicated in Se metabolism and has a major role in the pathogenesis of obesity possibly through participation in hepatic lipid production. The results also provide in vivo evidence that Scly is an enzyme involved in the crosstalk between Se and energy metabolic pathways, adding a new nutritional and molecular perspective for understanding the pathogenesis of metabolic syndrome, specifically obesity.

Further investigation of the physiological role of Scly in lipid metabolism could help clarify the pathogenesis of obesity, a global epidemic. In human populations
where Se levels are elevated and high fat intake is prevalent, such as in most areas of the United States, a compounding effect of Se and lipid metabolism could take place in the liver, thus worsening the outcome of obesity. Understanding the mechanisms behind the crosstalk between Se and metabolic pathways could lead to a molecular perspective that would improve dietary guidelines and ultimately, improve the quality of nutritional choices for obese people.

6.1. Figure

**Figure 6.1.** Possible physiological role of Scly integrating Se to glucose and lipid metabolism. The dual role of Scly (orange circle, center) is pictured in selenoprotein translation and energy metabolism. Scly may have an impact on glucose metabolism as a consequence of impairing selenoprotein synthesis, mainly GPx1 production. The impact of Scly on lipid metabolism would possibly occur through activation of the enzyme ACC1 (green arrow) or direct inhibitory interaction with SQS, highlighted by the question mark. DBE = DNA binding element; IR = Insulin receptor.
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