UNDERSTANDING OF FUNCTIONS OF SELENOPROTEINS AND DIETARY SELENIUM BY USING ANIMAL MODELS

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UNDERSTANDING OF FUNCTIONS OF SELENOPROTEINS AND DIETARY SELENIUM BY USING ANIMAL MODELS

by

Marina Kasaikina

A DISSERTATION

Presented to the Faculty of
The Graduate College at the University of Nebraska
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Under the Supervision of Professors Vadim N. Gladyshev and Dmitri E. Fomenko

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Selenium (Se) is a trace element that is incorporated into proteins in the form of the 21st amino acid, selenocysteine (Sec). Se supplementation was reported to have beneficial roles in prevention of cardiovascular and muscle disorders, cancer prevention and enhancement of the immune function. However, recent studies also showed that excessive dietary Se increases the risk of development of type 2 diabetes mellitus. Thus, better understanding of Se and selenoprotein functions is required. We used three approaches to address this problem.

First, we used high-throughput sequencing to examine composition of the gut microflora in mice maintained on selenium-deficient, selenium-sufficient, and selenium-enriched diets, and then link information to selenoprotein expression and function. Our data indicate that Se supplementation affects both the composition of the intestinal microflora and the colonization of the gastrointestinal tract in germ-free mice, which in turn, influence the host selenium status and selenoproteome expression.

Second, we characterized the phenotype of the 15 kDa selenoprotein (Sep15) knockout (KO) mice. Sep15 is a thioredoxin-like, endoplasmic reticulum (ER)-resident protein involved in the quality control of glycoprotein folding through its interaction with UDP-glucose:glycoprotein glucosyltransferase. We found that Sep15 KO mice develop nuclear cataracts at an early age. We
suggest that the cataracts resulted from improper folding status of lens proteins caused by Sep15 deficiency.

Third, we evaluated the role of Se and selenoproteins in naked mole rat (MR) *Heterocephalus glaber*, a rodent model of delayed aging due to its unusually long lifespan (>28 years). Tissue imaging by X-ray fluorescence microscopy and direct analyses of trace elements revealed low levels of selenium in the MR tissues. Metabolic labeling of MR cells with \textsuperscript{75}Se followed by sequencing and assembly of the MR transcriptome revealed the loss of expression of glutathione peroxidase 1 (GPx1), whereas expression of other selenoproteins was preserved. Thus, MR is characterized by reduced utilization of selenium due to a specific defect in GPx1 expression.

Overall, the use of rodent models allowed us to obtain insights into interplay of dietary selenium, gut microbiota, and expression and function of several selenoproteins.
ACKNOWLEDGEMENTS

I would like to express the sincere gratitude to my academic advisor, Dr. Vadim Gladyshev, for giving me the opportunity to work in his lab, for patience and believing in my abilities, for supporting my ideas and initiative, for being a great advisor, and the perfect example of a successful scientist.

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ABBREVIATIONS

APS, advanced Photon Source
CV, conventionalized
Cys, cysteine
DI1, thyroid hormone deiodinase type 1
DI2, thyroid hormone deiodinase type 2
DI3, thyroid hormone deiodinase type 3
ER, endoplasmic reticulum
ERAD, ER associated degradation
ICP-MS, inductively coupled plasma mass spectrometry
GF, germ-free
GPx1, glutathione peroxidase 1
GPx2, glutathione peroxidase 2
GPx3, glutathione peroxidase 3
GPx4, glutathione peroxidase 4
GSH, glutathione
I, iodine
LOCS II, Lens Opacities Classification System version II
MR, naked mole rat (*H. glaber*)
MsrA, methionine-S-sulfoxide reductase
MsrB, methionine-R-sulfoxide reductase
MEF, mouse embryonic fibroblasts
NMR, nuclear magnetic resonance
PSA, prostate specific antigen

Se, selenium

Sec, selenocysteine

SECIS, selenocysteine insertion sequence

SelH, selenoprotein H

SelI, selenoprotein I

SelK, selenoprotein K

SelM, selenoprotein M

SelN, selenoprotein N

SelO, selenoprotein O

SelP, selenoprotein P

SelS, selenoprotein S

SelT, selenoprotein T

SelV, selenoprotein V

SelW, selenoprotein W

Sep15, the 15 kDa selenoprotein

SOD, superoxide dismutase

SPS1, selenophosphate synthetase 1

SPS2, selenophosphate synthetase 2

UGT, UDP-glucose:glycoprotein glucosyltransferase

UPR, unfolded protein response

UTR, untranslated region
CHAPTER 1

Introduction
1.1 Selenoproteins: Identification and function

The first identified selenoprotein was glutathione peroxidase 1 (GPx1) [1]. Initially isolated from erythrocytes, it was shown to protect hemoglobin from oxidative damage. Later, it was found to be highly dependent on selenium, which was incorporated into GPx1 as the 21st amino acid, selenocysteine (Sec) [2, 3]. In comparison to cysteine (Cys), Sec has a lower pKa and is a stronger nucleophile [4]. Almost all known selenoproteins are enzymes with Sec in the active center. Sec insertion requires the presence of in-frame TGA codon and Sec insertion sequence (SECIS) element, a kink-turn RNA structure located in the 3’ UTRs of selenoprotein mRNAs. Biosynthesis of Sec occurs on its specific tRNA, tRNA\textsuperscript{Ser[Sec]}, which is initially charged with Ser. SECIS-binding protein 2 (SBP2) binds to the SECIS element in the RNA and recruits Sec-tRNA\textsuperscript{Ser[Sec]} along with other factors involved in Sec insertion [5, 6]. Identification of the structure and conserved sequences of the SECIS element allowed development of computational programs for identification of selenoprotein genes in sequence databases [7-9]. The program SECISsearch was designed to recognize structural and thermodynamic parameters of SECIS elements [10]. By searching for SECIS elements, the in-frame TGA codon in the ORF and the presence of Cys orthologs of selenoproteins, it is possible to identify selenoprotein genes in genomic sequences. The human genome contains 25 selenoprotein genes (Table 1). Most of these proteins participate in maintaining cellular redox homeostasis, including three thioredoxin reductases (TRs), five glutathione peroxidases (GPx1), methionine sulfoxide reductase (MsrB1), and three thyroid hormone deiodinases (DIs).

As is clear from Table 1, few selenoproteins have known functions. Besides TRs, GPxs, MsrB1, DIs, SelP, SelN and SPS2, the specific reactions catalyzed by selenoproteins are not known. However, conservation of selenoproteins among species and preservation
of the complicated biosynthesis pathway for their production indicate importance of this class of proteins. So far, the common feature of all selenoproteins with identified functions is their participation in the thiol-dependent reactions. This type of reactions is important in intracellular redox homeostasis and antioxidant defense. GPxs (and the N-terminal domain of SelP) are capable of reducing various peroxides [11, 12]. TRs and MsrB1 participate in the reduction of oxidized cysteine and methionine residues, respectively [13-16]. DIs catalyze removal of iodine (I) from the outer ring of the prohormone thyroxine (T4) yielding various forms of thyroid hormone [17, 18]. Sep15, SelM, SelH, SelS, SelN, SelT, SelW are less characterized, whereas almost studies have been done involving SelV, SelO, SelI and SelK. Most likely, all these proteins are oxidoreductases with Sec in the active site. More than half of mammalian selenoproteins are characterized by the thioredoxin like fold. Thioredoxin-like fold is a two-layer \(\alpha/\beta/\alpha\) sandwich structure that includes a conserved CXXC motif. In some cases, one of the Cys can be substituted with Ser or Thr. This fold is common for enzymes that catalyze formation or isomerization of disulfide bonds. At least 7 out of 25 selenoproteins (DI2, Sep15, SelK, SelM, SelN, SelS, and SelT) reside in the ER. ER enrichment with selenoproteins might indicate the roles of selenoproteins in ER associated processes, such as protein secretion/modification or ER associated degradation ERAD [19].
<table>
<thead>
<tr>
<th>Selenoprotein</th>
<th>Localization</th>
<th>Possible function</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>15 kDa selenoprotein (Sep15)</td>
<td>ER</td>
<td>-Trx-like fold&lt;br&gt;-regulated by ER stress&lt;br&gt;-interacts with UDP-glucose:glycoprotein glucosyltransferase&lt;br&gt;-potentially involved in glycoprotein folding</td>
<td>[20-23]</td>
</tr>
<tr>
<td>Thyroid hormone deiodinase 1 (DI1)</td>
<td>Plasma membrane</td>
<td>-removes iodine from the outer ring of the T4 thyroid hormone to produce plasma T3&lt;br&gt;-catalyzes deiodination and thus inactivation of T3</td>
<td>[24, 25]</td>
</tr>
<tr>
<td>Thyroid hormone deiodinase 2 (DI2)</td>
<td>ER</td>
<td>-converts T4 to T3 locally in tissues</td>
<td>[26]</td>
</tr>
<tr>
<td>Thyroid hormone deiodinase 3 (DI3)</td>
<td>Plasma membrane</td>
<td>-catalyzes deiodination of T4 thyroid hormone to T3 in peripheral tissues</td>
<td>[25, 27]</td>
</tr>
<tr>
<td>Glutathione peroxidase 1 (GPx1)</td>
<td>Cytosol</td>
<td>-GSH dependent detoxification of H$_2$O$_2$ (enriched in liver, kidney, erythrocytes)</td>
<td>[11]</td>
</tr>
<tr>
<td>Glutathione peroxidase 2 (GPx2)</td>
<td>Cytosol</td>
<td>-GSH dependent detoxification of H$_2$O$_2$ (enriched in epithelial tissues, including intestine and lung)</td>
<td>[28, 29]</td>
</tr>
<tr>
<td>Glutathione peroxidase 3 (GPx3)</td>
<td>Plasma</td>
<td>-GSH dependent detoxification of H$_2$O$_2$ (synthetized predominantly in kidneys and secreted to plasma)</td>
<td>[30]</td>
</tr>
<tr>
<td>Glutathione peroxidase 4 (GPx4, PHGPx)</td>
<td>Cytosol, Mitochondria nucleus (testis specific)</td>
<td>-has cytosolic, nuclear and mitochondrial isoforms&lt;br&gt;-protects lipids from H$_2$O$_2$ mediated oxidation</td>
<td>[31]</td>
</tr>
<tr>
<td>Glutathione peroxidase 6 (GPx6)</td>
<td>Cytosol</td>
<td>-GSH dependent detoxification of H$_2$O$_2$ (enriched in the olfactory epithelium)</td>
<td>[29]</td>
</tr>
<tr>
<td>Selenoprotein H (SelH)</td>
<td>Nucleus</td>
<td>-Trx-like fold&lt;br&gt;-protects cells from H$_2$O$_2$, increases mitochondrial biogenesis and CytC production&lt;br&gt;-AT-hook family protein. In response to redox changes facilitates synthesis of genes responsible for de novo glutathione synthesis and phase II detoxification</td>
<td>[32-34]</td>
</tr>
<tr>
<td>Selenoprotein I (SelI)</td>
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<tr>
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<tr>
<td>Selenoprotein M (SelM)</td>
<td>ER</td>
<td>-Trx-like fold&lt;br&gt;-protects neurons from oxidative stress</td>
<td>[36]</td>
</tr>
<tr>
<td>Selenoprotein N</td>
<td>ER membrane</td>
<td>-expressed in skeletal muscle, heart, lung,</td>
<td>[19, 37]</td>
</tr>
</tbody>
</table>
and placenta -controls redox state of the intracellular calcium-release channel (ryanodine receptor (RyR)), and therefore affects Ca^{2+} homeostasis -mutations in SelN gene cause congenital myopathy

<table>
<thead>
<tr>
<th>(SelN, SEPN1, SelN1)</th>
<th><strong>Selenoprotein O</strong> (SelO)</th>
<th>Mitochondria</th>
<th>unknown function</th>
<th>[10]</th>
</tr>
</thead>
<tbody>
<tr>
<td>(SelP)</td>
<td><strong>Selenoprotein P</strong> (SelP)</td>
<td>Plasma</td>
<td>-Se transport to peripheral tissues and antioxidant function</td>
<td>[38-40]</td>
</tr>
<tr>
<td>(SelR, MsrB1)</td>
<td><strong>Selenoprotein R</strong> (SelR)</td>
<td>Cytosol</td>
<td>-reduces methionine-R-sulfoxide residues in proteins to methionine</td>
<td>[16]</td>
</tr>
<tr>
<td>(SelS, SEPS1, Tanis, VIMP, and SELENOS)</td>
<td><strong>Selenoprotein S</strong> (SelS)</td>
<td>ER</td>
<td>-upregulated upon treatment with pro-inflammatory cytokines and glucose deprivation -co-purifies with the ERAD complex</td>
<td>[19, 41]</td>
</tr>
<tr>
<td></td>
<td>SPS2</td>
<td>Cytosol</td>
<td>-synthesis of selenophosphate</td>
<td>[42, 43]</td>
</tr>
<tr>
<td>(SelT)</td>
<td><strong>Selenoprotein T</strong> (SelT)</td>
<td>ER and Golgi</td>
<td>-Trx-like fold -redox regulation -plays a role in cell adhesion</td>
<td>[44]</td>
</tr>
<tr>
<td></td>
<td><strong>Thioredoxin reductase 1</strong> (TR1)</td>
<td>Cytosol</td>
<td>-controls redox state of thioredoxin -has 6 isoforms generated by alternative splicing</td>
<td>[13, 45]</td>
</tr>
<tr>
<td></td>
<td><strong>Thioredoxin reductase 2</strong> (TR2, TGR)</td>
<td>Cytosol</td>
<td>-contains glutaredoxin domain -catalyze a variety of reactions, specific for thioredoxin and glutaredoxin systems -expressed in spermatids</td>
<td>[46]</td>
</tr>
<tr>
<td></td>
<td><strong>Thioredoxin reductase 3</strong> (TR3)</td>
<td>Mitochondria</td>
<td>-reduces mitochondrial thioredoxin and glutaredoxin 2</td>
<td>[47]</td>
</tr>
<tr>
<td>(SelV)</td>
<td><strong>Selenoprotein V</strong> (SelV)</td>
<td>Cytosol</td>
<td>-Trx-like fold -unknown function -expressed in spermatids</td>
<td>[32]</td>
</tr>
<tr>
<td>(SelW)</td>
<td><strong>Selenoprotein W</strong> (SelW)</td>
<td>Cytosol</td>
<td>-Trx-like fold -unknown function -expressed in skeletal muscles</td>
<td>[48]</td>
</tr>
</tbody>
</table>
1.2 Mouse models for studying selenoproteins

Knockout (KO) and transgenic models can be used for evaluating protein functions as well as their impact on health. To evaluate selenoprotein functions, a number of mouse models were developed and characterized. Generally, all models can be divided into two groups. The first group includes animals lacking (or overexpressing) one or more individual selenoproteins. The second group includes various mouse models with altered selenoprotein biosynthesis pathway. These animals develop systemic selenoprotein deficiency. Both groups are discussed in the following sections.
1.2.1 Targeted removal of individual selenoproteins

Several mouse models with targeted inactivation of one or more selenoproteins were developed and characterized [49, 50]. The overview is given in Table 2. So far, three selenoproteins were found to be essential: TR1, TR3 and GPx4. Knockout of cytosolic TR1 leads to embryonic death between E8.5 and E10.5 [51]. While the cardiomyocyte-specific TR1 KO mice developed normally, the neuronal system (NS) specific TR1 knockout caused severe neurological symptoms, such as ataxia and tremor [52]. These symptoms were the result of cerebellar hypoplasia, abnormal foliation, perturbed lamination in the cerebellum and reduced proliferation of granule cell precursors in cerebellum [52]. Disruption of TR1 in B and T cells did not affect viability and functions of immune cells [53]. Mitochondrial TR3 knockout induced embryonic lethality between E13.5 and E15.5. Compared to controls, embryos were smaller, developed anemia and showed high levels of liver apoptosis. NS-specific TR3 KO mice developed normally, without signs of neurodegeneration; however, the cardiomyocyte-specific TR3 KO mice died from heart failure within few hours of birth [54].
GPx4 is another essential enzyme: KO of this gene leads to embryonic lethality at E7.5 [55]. GPx4 has cytosolic (cGPx4), nuclear (nGPx4) and mitochondrial (mGPx4) isoforms. All isoforms are synthesized from alternative translation initiation codons. nGPx4 expression is driven by its own testes-specific promoter, which lies inside the first intron of the cytosolic GPx4 transcript [31]. To access the function of each isoform, several KO/transgenic mouse models were obtained. nGPx4 KO mice developed normally; neither testicular structure nor fertility were affected in these mice; however the delayed sperm chromatin condensation was observed [56]. The sequence between the two alternative translational initiation codons encodes a mitochondrial signal peptide. Thus, introduction of the in-frame stop codon between alternative translation initiation codons resulted in the disruption of the mGPx4 form without affecting expression of cGPx4. mGPx4 KO male mice were infertile [57]. These experiments revealed an essential role of mGPx4 in male fertility.

KO of other GPxs did not affect viability and fertility. The major findings with GPx KO mice are summarized in Table 2. GPx1 KO mice did not show significant phenotypes; however, they were more susceptible to oxidative stress and viral myocarditis [11, 58]. GPx2 is mainly expressed in epithelial tissues, and its disruption affects intestinal cells [59]. Double GPx1 and GPx2 KO mice are characterized by severe colitis when maintained on an atherogenic diet [60]. Recently, GPx3 KO mice were developed [61]. Even though no significant phenotype was observed, this model revealed the specific binding of GPx3 to the basement membranes of renal cortical proximal and distal convoluted tubules.

Experiments designed to understand the function of selenoproteins in thyroid gave ambiguous results. Both general and liver-specific knockout of DI1, which is mainly expressed in thyroid and responsible for plasma T3 supply, did not lead to hypothyroidism, indicating a possible additional function in T3 degradation [62, 63]. DI2 is expressed in the
pituitary and is thought to be a T4 sensor that decreases TSH production, being part of the negative feedback loop for thyroid hormone production. DI2 KO mice showed pituitary resistance to T4 [64]. DI2 turned out to be important in conversion of T4 to T3 in peripheral tissues. T3 stimulation is critical for the development of the auditory functions [26, 65]. Lack of DI2 resulted in delayed cochlear differentiation that was the reason for the irreversible deafness of DI2 KO mice [65]. DI1/DI2 KO mice did not augment the phenotype of D1 or D2 KO mice; it was rather the sum of each single KO [66]. DI3 is responsible for inactivation of T3. DI3 KO mice showed mild hypothyroidism, suggesting importance of T3 degradation for maintaining the thyroid hormone axis [67]. D3 KO mice, like DI2 KO mice, were characterized by impaired auditory function, but with different pathogenesis. Unlike DI2 KO, DI3 KO mice displayed accelerated cochlear differentiation, which also resulted in deafness. This finding suggests a protecting role of DI3 in cochlear development [68].

Experiments with SelP KO mice suggested that the major function of SelP is the transport of Se from liver to peripheral tissues [69, 70]. SelP KO mice developed symptoms of general Se deficiency, such as ataxia, seizures and male infertility [71, 72]. Some of these symptoms could be rescued by dietary Se. Apparently, SelP KO mice is a particularly well suited model to study Se deficiency. The majority of SelP is synthesized in the liver. Analysis of the liver-specific Sec tRNA^{Ser[Sec]} KO (liver Trsp KO) mice (these mice lack expression of all selenoproteins in the liver and will be discussed later in this chapter) showed decreased expression and activity of selenoproteins in peripheral tissues, which confirmed the transport function for the hepatic SelP [73]. The levels of Se in the brain remained unaffected in liver Trsp KO mice; also, these mice did not show any
neurological phenotype. These findings suggested another essential SelP function in the brain. Restoration of liver SelP expression in SelP KO mice restored Se transport and removed symptoms associated with Se deficiency [74]. Thus, hepatocyte-derived SelP provides the major Se supply for kidneys, testes and brain. However, under Se deficiency, overexpression of liver SelP was unable to rescue the phenotype of SelP KO mice, which indicates the importance of local SelP production to support selenoprotein biosynthesis under limiting Se conditions [74]. SelP consists of two parts. The N-terminal domain contains a conserved UxxC motif, which is part of the domain characterized by the thioredoxin-like fold [38]. The C-terminal part of SelP contains multiple Sec residues and is involved in providing Se for synthesis of other selenoproteins. Deletion of the C-terminal region of SelP resulted in a milder phenotype compared to the KO of the entire protein. Overall, the C-terminus plays a critical role in Se transport [75].

Recently, two additional KO models were described [76]. A KO of MsrB1 did not lead to strong phenotypes: the KO mice were viable and fertile. However, various tissues of MsrB1 KO mice were characterized by a decreased level of MsrA (methionine sulfoxide reductase specific for Met-SO) and increased levels of malondialdehyde, protein carbonyls, protein methionine sulfoxide, as well as higher levels of oxidized glutathione and reduced levels of free and protein thiols; all this indicates the persistent oxidative stress in MsrB1 KO mice.

Inactivation of the SelK gene also did not affect viability and fertility [35]. However, as a result of the receptor mediated Ca$^{2+}$ flux, SelK KO mice showed compromised functions of the immune cells, including T cell proliferation, T cell and
neutrophil migration, and Fcγ receptor-mediated oxidative burst in macrophages, as well as higher susceptibility to viral infection.

There are a number of selenoproteins, which are still poorly characterized, and which would benefit from developing of knockout mouse models. This list includes SPS2, SelI, SelO, SelS, SelT, SelV, SelW, and GPx6.
<table>
<thead>
<tr>
<th>Gene</th>
<th>Phenotype</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>GPx1</td>
<td>-no gross phenotypes&lt;br&gt;-susceptibility to oxidative stress and viral miocardites&lt;br&gt;-acceleration of cardiac hypertrophy and dysfunction&lt;br&gt;-reduced blood insulin and reduced islet β-cell mass in pancreatic tissue</td>
<td>[11, 58]</td>
</tr>
<tr>
<td>GPx2</td>
<td>-no gross phenotypes&lt;br&gt;-increased apoptosis in colon crypt cells during Se deficiency</td>
<td>[59]</td>
</tr>
<tr>
<td>GPx1+GPx2</td>
<td>-microflora-dependent intestinal colitis&lt;br&gt;-decreased levels of Paneth cells</td>
<td>[60]</td>
</tr>
<tr>
<td>GPx3</td>
<td>-no gross phenotypes</td>
<td>[61]</td>
</tr>
<tr>
<td>GPx4</td>
<td>-embryos die at E7.5</td>
<td>[55]</td>
</tr>
<tr>
<td>TR1</td>
<td>-embryos die at between E8.5 and E10.5</td>
<td>[51]</td>
</tr>
<tr>
<td>TR3</td>
<td>-embryos die at between E13.5 and E15.5</td>
<td>[54]</td>
</tr>
<tr>
<td>DI1</td>
<td>-no gross phenotypes&lt;br&gt;-increased iodine excretion</td>
<td>[63]</td>
</tr>
<tr>
<td>DI2</td>
<td>-pituitary resistance to T4&lt;br&gt;-impaired thermogenic response to cold&lt;br&gt;-at thermoneutral conditions, high fat diet induced glucose intolerance, and exacerbated hepatic steatosis&lt;br&gt;-poor hearing, poorly differentiated sensory epithelium</td>
<td>[26, 65]</td>
</tr>
<tr>
<td>DI3</td>
<td>-reduced levels of circulating T4 and T3&lt;br&gt;-retarded development&lt;br&gt;-deafness with premature cochlear differentiation</td>
<td>[67, 68]</td>
</tr>
<tr>
<td>DI1+DI2</td>
<td>-mild hypothyroidism&lt;br&gt;-the sum of DI1 KO and DI2 KO phenotypes</td>
<td>[66]</td>
</tr>
<tr>
<td>SelP</td>
<td>-neuronal degeneration, leading to ataxia and seizures&lt;br&gt;-reduced selenoprotein expression in peripheral tissues&lt;br&gt;-male infertility</td>
<td>[69-71]</td>
</tr>
<tr>
<td>MsrB1</td>
<td>-no gross phenotypes&lt;br&gt;-increased markers of oxidative stress</td>
<td>[76]</td>
</tr>
<tr>
<td>SelK</td>
<td>-no gross phenotypes&lt;br&gt;-impaired functions of immune cells</td>
<td>[35]</td>
</tr>
</tbody>
</table>
1.2.2 Overexpression of selenoproteins in mice

Besides selenoprotein gene KO, several studies described animals with overexpression of individual selenoproteins. One of the best such models is GPx-overexpressing mouse (GPx1oe). These mice were shown to develop hyperglycemia and hyperinsulinemia, and developed high levels of blood insulin and increased islet β-cell mass [77-79]. It should be noted that similar symptoms are observed in Type 2 diabetes. When maintained on high fat diet, these mice developed obesity and insulin resistance, unlike GPx1 KO mice, which had reduced insulin levels and decreased islet β-cell mass [78]. This phenotype of GPx1oe mice might be explained by insufficient ROS-mediated signaling in islet β-cells. In a different model of diabetes, expression of GPx1 had a beneficial effect. Here, overexpression of GPx1 in islet β-cell of the db/db mice alleviated hyperglycemia at an early age and completely reversed it by 20 weeks of age [80]. These results indicate the importance of controlled GPx1 expression for prevention of Type 2 diabetes.

Another group developed mice with transgenic overexpression of mitochondrial GPx4 (mGPx4) [81]. Compared to littermate controls, these mice developed attenuated cardiac dysfunction in response to ischemia/reperfusion injury. Overexpression of mGPx1 reduced the levels of lipid peroxidation and slightly increased the activity of the electron transport chain (ETC) complexes I, III, and IV.

Another example of overexpression of a selenoprotein in an animal model is overexpression of SelM in rats [82]. These rats showed a better response to oxidant treatment. When fed with high Se diet, transgenic rats showed altered ERK signal transduction in the brain, which was characterized by inhibition of the alpha/gamma-
secretase activity and Tau protein phosphorylation. These observations suggest a possible protective role of SelM in Alzheimer's disease [83].

1.3 Mouse models targeting the selenoprotein biosynthesis pathway

Inactivating a single selenoprotein in mouse can provide information about its function and phenotypes associated with its deficiency. However, targeting the Sec incorporation machinery allows modulation of expression of all selenoproteins. Many such models have been developed.

1.3.1 Selenocysteine incorporating machinery

In eukaryotic cells, Se incorporation is a complex multi-stage process [5, 6, 84, 85]. The overall pathway of Sec incorporation is illustrated in Fig 1. Sec is synthesized on its own tRNA, tRNA\textsuperscript{[Ser]Sec}, which is the product of the Trsp gene. Initially, this tRNA is charged with Ser, forming Ser-tRNA\textsuperscript{[Ser]Sec}. This reaction is catalyzed by seryl-tRNA synthetase (SerRS). Ser-tRNA\textsuperscript{[Ser]Sec} is further phosphorylated by phosphoseryl-tRNA\textsuperscript{[Ser]Sec} kinase (PSTK). The Se donor compound for the Sec biosynthesis, selenophosphate, is synthesized by selenophosphate synthetase 2 (SPS2). O-Phosphoseryl-tRNA:selenocysteinyl-tRNA synthase (SecS) catalyze the pyridoxal phosphate dependent reaction which is resulted in Sec-tRNA\textsuperscript{[Ser]Sec} formation. After that Sec-tRNA\textsuperscript{[Ser]Sec} EFSec and SBP2 protein factors, and this supramolecular complex is translocated to nucleus [86]. SBP2 recognizes the SECIS element, which is located in the 3’-UTR of selenoproteins mRNAs. This complex supports the incorporation of Sec in response to the in-frame-UGA codon. There are several features, which are critical for proper function of the pathway: 1) As shown in Fig 1, SBP2 and EFSec shuttle between the nucleus and cytosol. This allows binding selenoprotein mRNAs in the nucleus and inhibition of the nonsense mediated
decay induced by in-frame stop codon [87]. 2) SPS2 is itself a selenoprotein, forming a positive feedback loop with the Se available in the cell [42]. 3) Recently, it was found that SPS2 can also synthesize thiophosphate, promoting incorporation of Cys in place of Sec. In mice maintained on the Se-deficient diet, insertion of Cys at UGA codon of TR1 equaled that of Sec [88]. 4) Sec tRNA^{Ser\text{Sec}} is a unique tRNA which undergoes multiple modifications, further regulating Sec incorporation. These modifications include isopentyladenosine modification at position 37 and methylcarboxymethyl-5’-uridine (mcm^5U) at position 34. The last step in Sec tRNA^{Ser\text{Sec}} maturation is the methylation of mcm^5U, which is assisted by Secp43 and results in the formation of methylcarboxymethyl-5’-uridine-2’-O-hydroxymethylribose (mcm^5Um) [89]. This process is highly sensitive to the primary, secondary and tertiary structure of tRNA as well as to the overall Se status. mcm^5U supports synthesis of “housekeeping” selenoproteins, such as GPx4, TR1 and TR3, whereas the methylated tRNA is needed for expression of “stress-related” selenoproteins, such as GPx1, GPx3, and MsrB1. This change in selenoproteins expression pattern is commonly observed during Se deficiency, but the precise molecular mechanism is unknown.
Figure 1. Schematic model for Sec biosynthesis and incorporation. See details in the text. Sec tRNA_{Ser}^{[Ser]Sec} is charged with Ser, which is further phosphorylated by PSTK SPS2 to facilitate synthesis of the selenophosphate. SecS catalyze Sec formation. SECp43 catalyze methylation of the Sec tRNA_{Ser}^{[Ser]Sec} at the A34 position. Protein factors, including SBP2 and EFSec binds to the SECIS element, located in the 3’UTR of the selenoprotein mRNA. After translocation to the cytosol protein factors facilitate interaction with the ribosome and Sec incorporation. 1.3.2 Mouse models targeting the selenoprotein biosynthesis pathway
There are several ways to regulate efficiency of Sec incorporation. In order to modulate expression of selenoproteins, the easiest way is to change levels of dietary Se. To examine the effects of dietary Se on various health parameters, one can adjust Se concentration in rodent chow. For example, 0.1 ppm Se in rodent diet approximately corresponds to the human Recommended Dietary Allowance for adults, whereas 0.4 ppm Se may correspond to the diet supplemented with 200 µg Se/day, which is the dose most often used in the clinical trials involving Se [90-92]. This approach was successfully applied to examine Se function in diabetes [93], cancer [94], the immune response [95, 96], etc. The disadvantage of this approach is that, with change in dietary Se, the levels of low molecular weight Se compounds are also changed, which might itself influence certain pathways.

1.3.3 Trsp transgenic models
Stable expression of mutant Trsp was shown to severely affect selenoprotein biosynthesis by interfering with the Sec incorporation pathway by a dominant-negative mechanism. According to this hypothesis, two mouse models were generated. In the first model, A37 was substituted with G37 [97], and in the second, T34 was replaced with A34 [98]. Both models lacked Um34; thus, expression of stress related selenoproteins was severely reduced, whereas expression of housekeeping selenoprotein genes was little affected. The effect of G37 transgene was tissue-specific: it was significant in liver and kidney, but low in tissues testes [97]. The G37 transgenic mice were studied for various health parameters. These mice were found to be more susceptible to viral infection [99], colon cancer [100] and X-ray damage [101]. Crossing of the G37 and C3/Tag mice provided a good model for studying the function of selenoproteins in prostate cancer. Such
mice were found to accelerate the development of prostatic epithelial neoplasia (PIN), suggesting a protecting role of selenoproteins during prostate cancer development [102]. G37 mice demonstrated enhanced muscles growth in experiments modeling the exercise overload. These data correlated with initial activation of the insulin signaling pathway, which includes increased Akt and p70 phosphorylation [103]. Abnormal insulin signaling might be, in part, the reason for glucose intolerance and lead to a diabetes-like phenotype, that had been recently observed in G37 mice [93].

1.3.4 Mouse models of Trsp knockout

Another approach of inactivating selenoprotein function in mice is to target the Trsp gene. The complete KO of Trsp leads to embryonic lethality, but a conditional removal of Trsp is possible [104]. Development of the tissue-specific KO models helped to examine important functions of selenoproteins in heart and skeletal muscles, endothelial cells [105], skin [106], bone [107], neurons [106] and immune cells (macrophages, T cells and hematopoietic tissues) [108-110], but also more dispensable selenoprotein functions in liver [73, 111], mammary gland [104] and podocytes [112]. KO of Trsp in endothelial cells led to embryonic death at E14.5 due to necrosis of the central nervous system, erythrocyte immaturity and subcutaneous hemorrhage. Mice with the myocyte-specific Trsp KO died 12 days after birth from acute myocardial failure [105]. Deletion of Trsp in skin resulted in runt phenotype, epidermal neoplasia, and abnormal development of the hair follicles. Altogether, these abnormalities induced weight loss and early death. Thus, selenoproteins have a function in maintenance of skin integrity [106]. Osteo-chondroprogenitor-specific Trsp KO mice showed multiple skeletal abnormalities, including growth retardation, abnormal epiphyseal plates, delayed ossification, and chondronecrosis of cartilage [107].
The neuron-specific Trsp KO induced severe neurodegeneration in hippocampus and lead to the absence of certain interneurons [106] (similar to that that observed in the neuron-specific GPx4 KO model). Besides, these mice showed degeneration of the Purkinje and granule cells that lead to cerebral hyperplasia. In several studies, Se modulated the immune response. To understand the function of selenoproteins in immune cells, T and B cell-specific Trsp KO mice were developed. The T-cell-specific Trsp KO had decreased pool of mature T-cells and impaired T-cell dependent antibody response. Lack of antioxidant enzymes caused extensive oxidative stress and weak proliferation in response to T-cell receptor stimulation [109]. Macrophage specific-Trsp KO mice showed impaired invasiveness, which might be explained by hyperproduction of ROS and altered expression of extracellular matrix proteins [108]. Ablation of the Trsp gene in hematopoietic tissues resulted in anemia, which led to an increased production of erythroid progenitors in bone marrow as well as to thymus atrophy [110]. The liver-specific Trsp KO induced expression of phase II enzymes, including various GSTs [113]. By preventing SelP synthesis and secretion, the liver-specific Trsp KO dramatically decreased plasma SelP. Thus, these mice showed symptoms of Se deficiency, which could be rescued by increased Se intake. Further characterization of these mice revealed elevated levels of apolipoprotein E (ApoE) in serum as well as plasma cholesterol [98]. The mammary gland-specific Trsp KO mice showed increased levels of p53 and decreased expression of BRCA1 tumor suppressor [104]. Overall, mouse models with conditional Trsp KO turned out to be a powerful tool for understanding functions of selenoproteins in tissues.
1.3.5 Knockout/transgenic mouse models
An additional strategy to investigate the effect of transgene overexpression is to develop KO/transgenic animal models. In the case of selenoproteins, liver Trsp KO mice were crossed with G37 or A34 mice. In both cases, similar expression pattern of housekeeping selenoproteins was observed. Restoration of the housekeeping selenoprotein genes partially decreased elevated levels of ApoE and serum cholesterol that had been observed in the liver-specific Trsp KO. This research directly revealed the function of housekeeping selenoproteins in regulation of lipoprotein biosynthesis and metabolism [98].

Another knockout/transgene mouse model was described in [114]. STAF (Sec tRNA gene transcription activating factor) is a transcription factor for several RNA PolII and RNA PolIII-dependent genes. In this study, the authors overexpressed Trsp lacking the STAF binding promoter region and afterwards removed the WT Trsp. Interestingly, removal of the STAF binding site did not affect Trsp levels in heart and testis, but showed severe reduction of the transgene in liver, kidney, lung, spleen, and brain. Moreover, methylation of Trsp at A34 was significantly decreased, and expression of stress-related selenoproteins was reduced. These mice demonstrated the neurological phenotype similar to that of SelP KO mice. These findings indicated the importance of the STAF binding region in regulation of Sec tRNA^{Ser[Sec]} expression and its proper modification.
CHAPTER 2

Dietary selenium affects host selenoproteome expression by influencing the gut microbiota

Note: The results described in this chapter have been published.

2.1 Abstract
Colonization of the gastrointestinal tract and composition of the microbiota may be influenced by components of the diet, including trace elements. To understand how selenium regulates the intestinal microflora, we used high-throughput sequencing to examine the composition of gut microbiota of mice maintained on selenium-deficient, selenium-sufficient, and selenium-enriched diets. The microbiota diversity increased as a result of selenium in the diet. Specific phylotypes showed differential effects of selenium, even within a genus, implying that selenium had unique effects across microbial taxa. Conventionalized germ-free mice subjected to selenium diets gave similar results and showed an increased diversity of the bacterial population in animals fed with higher levels of selenium. Germ-free mice fed selenium diets modified their selenoproteome expression similar to control mice but showed higher levels and activity of GPx1 and MsrB1 in the liver, suggesting partial sequestration of selenium by the gut microorganisms, limiting its availability for the host. These changes in the selenium status were independent of the levels of other trace elements. The data show that dietary selenium affects both composition of the intestinal microflora and colonization of the gastrointestinal tract, which, in turn, influence the host selenium status and selenoproteome expression.

2.2 Introduction
Intestinal microorganisms play an important role in human physiology by regulating such processes as maturation and proliferation of the intestinal cells, food digestion, protection from pathogenic bacteria, and modulation of the mucosal immune response [115, 116]. Growing evidence also links imbalances in the composition of the gut microbiota to complex diseases, including colon cancer, obesity, inflammatory bowel
disease, and Crohn’s disease [117-122], suggesting that dietary interventions affecting the gut microbiota hold substantial promise as alternative approaches to influence human health [123]. One of the main strategies to regulate the gut microbiota through diet is the use of prebiotics, which are defined as nondigestible food ingredients that beneficially affect the host by selectively stimulating the growth and/or activity of one or a limited number of bacterial species in the colon [124]. Because prebiotics promote growth of a select group of microorganisms, other strategies must also be examined, including those that can selectively inhibit or promote growth of species associated with complex diseases.

One such strategy may involve modulation of dietary trace elements. Gut microorganisms, like their hosts, are expected to be sensitive to trace elements. Some species require trace elements such as Se for normal metabolic functions, whereas these same elements can be toxic to other microorganisms, even at very low concentrations [125, 126]. Therefore, changes in dietary trace elements may modulate the composition of intestinal microbiota. For example, reducing dietary iron (Fe) caused an increase in the growth of certain organisms in the small intestine, including anaerobes, microaerophiles, lactobacilli, and enterococci. On the other hand, an increase in dietary Fe suppressed anaerobes, presumably due to elevated oxidative stress [127]. However, the effect of other trace elements on intestinal microbiome is largely unknown. Se is an essential trace element that plays an important role in human health. In particular, it is required for biosynthesis of selenoproteins, which participate in the regulation of cellular redox homeostasis, protection from oxidative stress, immune response, cancer chemoprevention, and other processes [128]. Supplemental Se has been shown to be effective in decreasing incidence and mortality from several forms of cancer, including colon cancer, in both
mouse models and humans [100]. On the other hand, some selenoproteins may promote carcinogenesis, so the role of Se in cancer development is complex.

We hypothesized that Se supplementation may modify composition of mouse microbiota. At the same time, microflora may sequester Se, thus limiting its availability to the host [129]. A better understanding of these interactions might reveal novel aspects of the influence of the microbiota on metabolism of the host. The purpose of the current study was to characterize the impact of Se diets on microbiota and the role of microbiota in influencing Se status of the host.

2.3 Experimental Procedures

**Animals and feeding protocols.** The diets used in the study, including 0-, 0.1-, 0.4-, and 2.25-µg/g (ppm) Se diets, were purchased from Harland TekLad (Madison, WI, USA). As described previously [94], these diets are based on the Torula yeast Se-deficient diet. Se was provided in the form of sodium selenite; 0, 0.1, 0.4, and 2.25 ppm Se indicate the added amounts of Se. In the first experiment, 4-wk-old C57BL/6J male mice (purchased from Jackson Laboratory, Bar Harbor, ME, USA) were subjected to these 4 Se diets (8 mice/group). Ten weeks later, feces were collected and frozen until further analysis. In the second experiment, 10-wk-old germ-free Swiss Webster male mice were subjected to 3 diets: 0, 0.1, and 0.4 ppm Se. One cohort of these animals (5 mice/group) was maintained germ free, and these mice are designated germ-free (GF) mice. Another group (5 mice/group) was placed in a similar germ-free environment, but it was conventionalized with the microflora prepared from the intestine of C57BL/6J mice used in the previous experiment. These mice are designated as conventionalized (CV) mice. Both groups of animals were maintained on the same Se diets, in the same facility and at
the same time, under specific pathogen-free conditions with 12-h light-dark cycle. All animal experiments were approved by the University of Nebraska–Lincoln (UNL) Institutional Animal Care and Use Committee.

**Sample preparation.** After 8 wk on the diets, feces from CV mice were collected and frozen until further analysis. Then, GF and CV mice were sacrificed; their tissues were collected and immediately frozen in liquid nitrogen. For Western blot analysis and activity measurements, tissues were homogenized in PBS supplemented with protease inhibitor cocktail (Sigma, St. Louis, MO, USA). Cellular debris was removed by centrifugation at 13,000 rpm for 15 min at 4°C, and protein concentration was determined with the Bradford assay.

**DNA isolation and pyrosequencing.** Total DNA was extracted from the fecal pellets of 8 mice/feeding group, as described previously [130]. The 16S rRNA gene was amplified using modified F8 and R357 universal primers. These 16S rRNA PCR products were then subjected to pyrosequencing on a Roche/454 GS-FLX instrument (Roche, Basel, Switzerland) in the UNL Core for Applied Genomics and Ecology. The PCR products from each animal were bar coded and then mixed into a single sequencing run on the machine, so as to generate 2000 reads/animal. Taxonomy-dependent analysis was first performed using the naive bayesian Classifier algorithm of the Ribosomal Database Project (RDP; Michigan State University, East Lansing, MI, USA). Phylotypes were determined at 97% cutoffs using CD-Hit, and representative sequences were searched against the RDP database using SeqMatch. Statistical analyses were performed on taxonomic groups from Classifier and CD-Hit phylotypes by normalizing the number of reads per taxon or phylotype by the total number of reads for a given animal. These normalized proportions
were then tested for statistical significance by ANOVA. For rarefaction analysis, phylotypes were assigned using the RDP pipeline (Aligner complete linkage clustering) and rarified using the Web-based rarefaction tools.

**Western blot analyses and activity assays.** Expression of selenoproteins GPx1, SeIP and MsrB1 was analyzed by Western blots with polyclonal antibodies specific for these proteins. To assay for GPx1 activity, a glutathione peroxidase kit was used (Sigma). MsrB activity was measured in an HPLC assay. Briefly, 200 µg of total protein was added to a reaction mixture (100 µl), the mixture was kept at 37°C for 30 min in the presence of 20 mM DTT, and 200 µM dabsyl-methionine-R-sulfoxide was added. After stopping the reaction by adding 200 µl acetonitrile, it was centrifuged at 4°C for 15 min at 13,000 rpm, and the supernatant (50 µl) was injected onto a C18 column (Zorbax Eclipse XDB-C18; Agilent Technologies, Santa Clara, CA, USA) to quantify the resulting dabsylated methionine.

**RNA isolation and quantitative PCR.** To analyze for SelP mRNA expression in the liver, total RNA was isolated by TriZOL extraction (Invitrogen, Carlsbad, CA, USA), according to the manufacturer’s instructions. Genomic DNA was removed using a DNA removal kit (Ambion, Austin, TX, USA). RNA concentration was measured spectrophotometrically, and cDNA was obtained with SuperScript III Reverse Transcriptase (Invitrogen). Real time PCR was performed using a Fast SYBR Green Master Mix (Applied Biosystems, Foster City, CA, USA). Primer sequences for the SelP expression analysis were as described previously [131]: 5’-CCTTGGTTTGCCCTACTCCTCC-3’ and 5’-TTGTTGCGTGGTGGGTGG-3’. SelP mRNA expression level was normalized to that of actin mRNA.
Analyses of trace elements. To measure Se content in biological materials, we applied quantitative inductively coupled plasma mass spectroscopy (ICP-MS). Tissue extracts were digested with the mixture of 15% nitric acid and 15% hydrogen peroxide for 2 h at 70°C. After digestion, samples were diluted 10x with PBS, and the internal standard (gallium) was added to a final concentration of 50 ppb. Each sample was analyzed 3 times and in triplicate. The ICP-MS analysis was performed at the UNL Redox Biology Center spectroscopy core facility, using 7500 Agilent Technologies and Elemental Scientific (Omaha, NE, USA) SC4 autosampler instruments operating with a collision.

2.4 Results
Dietary Se regulates composition of the intestinal microbiota. To assess the effect of dietary Se on the composition of mouse microbiota, 2-mo-old male C57BL/6J mice were subjected to a Se-deficient diet and diets supplemented with 0.1, 0.4, or 2.25 ppm of Se in the form of sodium selenite. Previously, these diets were shown to modulate selenoprotein expression [94]. The expected (i.e., based on what is known about regulation of selenoprotein expression) changes in selenoprotein levels are shown schematically in Fig 2A. After 8 wk on the diets, the Se status of mice was examined by analyzing the levels of SelP, the main selenoprotein in plasma of mammals. SelP decreased dramatically in mice fed the Se-deficient diet, whereas, as expected, the differences in SelP levels among mice fed other diets were minimal (Fig 2B). Feces from animals in the Se diet groups were collected, and the gut microbiota were examined by pyrosequencing of 16S ribosomal RNA tags at a sequencing depth of ~2000 reads/animal. Using a threshold of 97% for phylotype assignment, rarefaction analysis of the data pooled by Se diet group showed a significant effect of Se deficiency on the numbers of phylotypes that were
detected. Overall, Se in the diet increased the diversity of the microbiota (Fig 3A). This Se-induced increase was similar at all supplementation levels. To examine the effects of Se on major taxonomic groups of the microbiota, the proportions of taxa at the phylum, class, and order level from Classifier data were analyzed by ANOVA. No significant differences were observed on the relative proportions of major taxonomic groups (data not shown). The effects on specific phylotypes were then examined using 97% CD-Hit clusters. ANOVA identified several phylotypes showing significant effects of diet (Fig 3B). Remarkably, there were differential effects on related taxa. For example, some phylotypes belonging to the class Bacteriodales showed increases in response to Se (e.g., Porphyromonadaceae phylotypes 1 and 3, *Tanerella* phylotype 2), whereas others, such as *Alistipes* phylotype 1 and *Parabacteroides* phylotype 3 declined. Relative to absolute abundance, the decline in *Parabacteroides* in response to Se in the diet was by far the most significant effect on the microbiota. As with many phylotypes in the Bacteriodetes, several of the Firmicutes also showed modest effects in response to Se, including phylotypes within the *Clostridia* and *Erysipelotrichi*.

**Se supplementation influences bacterial colonization of the gastrointestinal tract.** To determine whether the microbiota have a significant effect on the physiological distribution of dietary Se, GF Swiss Webster mice were treated with the same 3 Se diets (0, 0.1, and 0.4 ppm Se), except that these diets were sterilized. One cohort of these animals was maintained GF, while the gut microbiota was reestablished in the other cohort using fecal materials from the CV C57BL/6J animals used in the previous experiment. Before and after 8 wk of diet treatment and conventionalization, SeLP levels were examined in plasma, and fecal samples were taken at the end of the experiment and subjected to the
analysis of microbiota. Western blots revealed undetectable SelP in all mice fed the Se-deficient diet, whereas little difference was observed between the 0.1- and 0.4-ppm Se groups (Fig 2C). Thus, dietary Se affects selenoprotein expression independent of GF, CV, or colonization conditions in mice. Similar to the conventional animals, pyrosequencing of the microbiota from conventionalized GF animals showed effects on the microbiota. Most notable was an increase in the overall diversity of the microbiota (Fig 3A). While the 0.4-ppm diet in the conventionalized GF animals showed significantly increased diversity, the 0.1-ppm diet did not. Statistical analysis of the phylotype abundances in the conventionalized GF animals showed that several phylotypes were affected by dietary Se (Fig 3B). In general, these phyla belonged to the same taxonomic groups as those affected in the conventional animals, including phylotypes related to *Tanerella* and Porphyromonadaceae. As in the conventional animals, these phylotypes showed differential effects, with some being stimulated by Se and others significantly decreasing. Likewise, one of the phylotypes belonging to the Clostridiales showed significant increases in response to Se in the conventional animals, but another phylotype was significantly reduced in response to dietary Se in the conventionalized GF animals. Collectively, our results show that dietary Se affects the overall diversity of the microbiota and has differential effects on specific taxonomic groups, even in related taxa. These effects were observed whether the microbiota was obtained naturally or by conventionalizing GF animals.

**Microbial colonization affects the levels and activities of selenoproteins of the host.** Gastrointestinal microbiota was recently shown to affect Se status and selenoprotein expression in mice [129]. In that study, conventional FVN/NHanTMHsd maintained for 5
weeks on Se-deficient and Se-adequate diets showed reduced GPx and/or thioredoxin reductase 1 (TR1) activities in plasma, liver, and/or intestine under Se limiting conditions, when compared to the corresponding GF group. In our study, we analyzed the status of stress-related selenoproteins, GPx1 and MsrB1, in tissues of GF mice in comparison with those in CV mice, which served as controls, thus allowing us to directly examine the effect of gut colonization on the selenoprotein status of animals. In this experiment, GF and CV mice were subjected, in parallel, to 0-, 0.1-, and 0.4-ppm Se diets, and expression levels and activities of GPx1 and MsrB1 were analyzed in liver and kidney. Both GF and CV mice showed similar patterns of selenoprotein regulation. Their expression in mice on the 0-ppm Se diet was dramatically lower, and we also observed differences in selenoprotein expression between 0.1- and 0.4-ppm Se diets. Selenoenzyme activities paralleled the expression patterns. Total GPx activity (GPx1 is the main GPx in liver and kidney of mice; thus, this activity largely corresponds to the activity of GPx1), and the total MsrB activity (MsrB1 is the main MsrB in liver and kidney) was decreased several fold in the 0-ppm Se group compared to the 0.1-ppm Se group, whereas the 0.4-ppm Se group showed a statistically significant increase over the 0.1-ppm Se group. This is consistent with the previous findings that expression of GPx1 and MsrB1 is maximized at ~0.15 ppm Se in the diet [131]. However, we were particularly interested in the effect of gut colonization on the regulation of selenoprotein expression (Fig 4). Indeed, within the 0.1-ppm Se groups, we observed higher GPx1 expression in liver and kidney, and higher GPx activity in kidneys of GF mice, compared to CV mice. In addition, within the 0.4-ppm Se groups, higher GPx1 expression and activity were observed in kidneys, and we also found higher MsrB activity in liver and kidneys of GF mice. In the 0-ppm Se group, we did not observe
differences between GF and conventionalized mice, probably because of low expression and activity of selenoproteins, which approached background levels. Overall, there was a clear pattern wherein conventionalization of mice decreased expression and activity of stress-related selenoproteins in liver and kidney.

**Gut colonization and SelP levels in plasma.** Mouse SelP contains 10 selenocysteine residues and is highly expressed in hepatocytes. This secreted protein transports Se from liver to other organs and was also suggested to serve an antioxidant function [38]. Unlike other selenoproteins, SelP showed a trend toward increased levels in CV mice; at least in the mice fed 0.1 ppm Se, as examined by Western blots (Fig 5A). However, its mRNA expression in the liver did not differ between GF and CV mice (Fig 5B). We also examined Se levels in plasma in GF and CV mice (Fig 5C). Whereas the 0-ppm Se group showed several fold lower Se levels, there were no statistically significant differences within each dietary group between GF and CV mice.

**Influence of Se status and gut colonization on other trace elements.** The intestinal microbiota may be sensitive to changes in trace element levels, and, in turn, gut colonization may influence host trace element status, either through competition for certain elements or by modifying the processes of food absorption or digestion. Using ICPMS, we analyzed concentrations of several trace elements in CV and GF mice subjected to the 3 Se diets in liver, kidney, spleen, brain, and testes (Fig 6). Se levels in liver, kidney, and spleen were sensitive to dietary Se, whereas its concentration in brain and testes did not differ with changes in the Se dietary levels. Also, we found no difference in the Se levels between GF and CV mice. Analysis of other trace elements, including Mn, Fe, Zn, Mo, Cu, and As, did not reveal statistically significant differences between Se diets. Cd levels
in the liver were higher in GF mice regardless of the diet, suggesting a possible role of the gastrointestinal microbiota in assisting in detoxification of this element.

2.5 Discussion

Intestinal microbiota is sensitive to dietary Se status. Our results indicate that dietary Se can affect both the composition of the existing microbiota and establishment of gastrointestinal microflora. Particularly, in both experiments, the strongest effect was observed within genus *Parabacteroides* of the phylum Bacteroidetes, which demonstrated the opposite correlation with dietary Se supplementation. This finding might be explained by the use of Se by various microorganisms and Se toxicity to certain organisms. We also observed differential effects of Se across multiple phylotypes belonging to the phylum Bacteroidetes. It is possible that the niches vacated by the effect of Se on *Parabacteroides* were filled by other phylotypes within related taxonomic groups. The fact that multiple groups showed increases while fewer groups, in general, showed decreases is consistent with the overall effect of Se on increasing diversity of the microbiota. The differential sensitivity of various microorganisms to dietary Se is likely due to differences in their ability to uptake, store, use, and remove Se from the cell. In particular, the gut microbiota may sequester Se for expression of its own selenoproteins. About one quarter of all bacteria expresses selenoproteins, and, therefore, they require Se for optimal growth. For example, *Escherichia coli* has 3 selenoproteins. The number of selenoproteins in bacteria varies from 0 to 57. This use of Se by microorganisms decreases the availability of this trace element for the expression of host selenoproteins. As a result, microbiota increases the requirement of the host for Se. However, because of unavailability of the genomes for
the majority of microorganisms that respond to Se status, it is not currently possible to
directly link sensitivity to dietary Se and occurrence of specific Se utilization pathways.

**Bacterial colonization decreases total Se status of the host.** Se is an essential
trace element required for synthesis and functions of 24 selenoproteins in mice. GPx1 and
TR1 are among the best studied mammalian selenoproteins. TR1 is an essential
selenoprotein that controls the redox state of thio redoxin. Thus, the thioredoxin system
(which is the main redox regulatory system) is dependent on dietary Se. GPx1 is the most
abundant mammalian selenoprotein that catalyzes glutathione-dependent reduction of
hydroperoxides. It is expressed at particularly high levels in liver and kidney. MsrB1 is
another important oxidoreductase, which repairs oxidatively damaged proteins by reducing
methionine sulfoxide residues to methionines. Various selenoproteins are differentially
regulated by dietary Se in mice. Some selenoproteins, such as GPx1 and MsrB1, are
sensitive to Se status, whereas several other selenoproteins, including TR1, are less
susceptible to changes in dietary Se. Thus, mice and other mammals have a priority for Se
supply that is selenoprotein- and tissue-specific. Under conditions of Se deficiency, GF
mice were shown to possess higher GPx and TR activities in liver and intestine [129].
They also had higher expression of GPx1 and its mRNA in the liver and colon. In addition,
plasma, liver, and cecum of these mice contained higher Se concentration compared to CV
animals [129]. In our study, which compared GF mice with their CV controls, we also
found GF mice to have elevated selenoprotein status, including higher GPx1 and MsrB1
activities and expression in liver and kidney. On the other hand, Se levels were similar in
GF and CV mice subjected to the same Se diets. This observation might be explained by
the fact, that in a previous study [129], GF and CV mice were subjected to the diets for 5
wk, whereas in our work, we conventionalized the GF mice with the intestinal flora prepared from C57BL/6J mice. Perhaps, at different Se levels in the diet, somewhat different populations of microorganisms colonize the gut. In contrast to GPx1 and MsrB1 status in GF and CV mice, we observed a trend toward an increased level of SelP in CV mice. A previous study investigated gene expression in colonic epithelial cells, derived from germfree and bacteria-reconstituted mice [132]. SelP gene expression was found to be down-regulated under these conditions. Moreover, SelP was only detected in the GF mice. Since SelP is a transport form of Se, its expression contributes to the Se status of the whole organism. This protein is regulated by the glucocorticoid receptor [133] and by cytokines during inflammation. Its expression is decreased during certain pathologies linked to chronic inflammation, such as liver cirrhosis and Crohn’s disease [38]. In addition, it is induced by IL-10, an anti-inflammatory cytokine [133], but suppressed by IFN-γ, TNF-α, IL-1, and TGF-β [134-136], which promote inflammation. The majority of plasma SelP comes from the liver. Its increase in CV mice might be explained by the indirect effect of cytokines on the expression of liver SelP or its increased use in organs of GF mice that are dependent on this protein for supplies of Se.

**Microbiota and the host Se status.** Disruption of integrity of the intestinal microbiota may lead to diseases, such as inflammatory bowel disease and cancer. Se supplementation may be beneficial in the prevention of colon cancer and certain other cancer types. Our data suggest that the sensitivity of the microbiota to dietary Se may be relevant to the regulation of the host Se status. Our diets were chosen to mimic the dietary Se intake in the human population. For example, the 0.1-ppm Se diet approximately corresponds to the human recommended dietary allowance for adults, whereas 0.4 ppm Se
may correspond to the diet supplemented with 200 μg Se/d, which is the dose most often used in human clinical trials involving Se. Thus, the effects observed with these diets in mice may be relevant to the effects of the human microbiota in setting the status of trace elements. In our experiments, the CV mice had higher requirements for Se compared to the GF mice, likely due to sequestration of Se by gut microorganisms, which compete for Se with the host. These data may also be relevant for understanding the mechanisms of Se mediated protection against malignant transformation of the colon epithelium, but further studies are required to test this idea.
Figure 2. Mouse models for examining the interrelationships between dietary Se, gut microbiota, and host Se status. A) Schematic illustration of selenoprotein expression in mice subjected to Se-deficient (0 ppm Se), Se-sufficient (0.1 ppm Se), and Se-enriched (0.4 ppm Se) diets. B, C) SelP expression was analyzed by Western blotting (WB) in plasma of conventional (B) and GF and CV (C) mice fed the indicated Se diets for 8 wk (B) or 6 wk (C; top panels); gels stained with Coomassie blue served as loading controls (bottom panels); 2 mice/group were analyzed.
Figure 3. Effect of dietary Se on gut microbiota. A) Rarefaction analysis was performed on pyrosequencing data from conventional and GF conventionalized animals. Sequences were pooled from animals of a similar feeding level and aligned using the complete linkage clustering at 97% cutoff available on the RDP pipeline, and clusters were subjected to rarefaction analysis. B) Box and whisker plots depict relative abundance of selected phylotypes showing statistically significant effects of dietary selenium ($P < 0.05$). The taxonomic relationship of each phylotype is indicated above the relevant graph. Left panels: phylotypes from conventional animals (orange boxes). Right panels: phylotypes from GF conventionalized animals (light blue boxes). Vertical bars indicate range of proportions for each treatment group; boxes indicate upper and lower bounds of the 95% confidence intervals.
Figure 4. Selenoprotein expression in GF and CV mice. Expression (A) and specific GPx1 and MsrB1 activities (B) in livers and kidneys are shown; 3 mice/group were analyzed. A) Expression of GPx1 and MsrB1 was analyzed by Western blots in livers
(top panels) and kidneys (bottom panels) of CV and GF mice fed 0-, 0.1-, and 0.4-ppm Se diets. B) GPx and MsrB activities in liver and kidney lysates of GF and CV mice fed 0-, 0.1-, and 0.4-ppm Se diets. Solid bars, CV mice; open bars, GF mice. *P > 0.05; Student’s t test.
Figure 5. Regulation of SelP expression by dietary Se in CV and GF mice. A) SelP levels in plasma of CV and GF mice fed 0-, 0.1-, and 0.4-ppm Se diets, as analyzed by Western blots. B) Liver SelP mRNA expression analyzed by quantitative PCR. SelP mRNA expression in CV mice fed the 0-ppm Se diet was set to 1, and mRNA levels in other groups were calculated relative to this basal level. Significance was analyzed with Student’s t test. C) Se levels in plasma in CV and GF mice fed 0-, 0.1-, and 0.4-ppm Se diets analyzed by ICP-MS. Values are expressed as means +/- sd.
Figure 6. Trace elements in CV and GF mice maintained on Se diets. Trace elements were analyzed with ICP-MS in tissues from CV and GF mice fed 0-, 0.1-, and 0.4-ppm Se diets, as described in Materials and Methods. Values are means +/- sd. Organs in which trace elements were analyzed are indicated at the bottom of each panel.
CHAPTER 3

15 kDa selenoprotein (Sep15) knockout mice: roles of Sep15 in redox homeostasis and cataract development

Note: The results described in this chapter have been submitted for publication in the Journal of Biological Chemistry:

3.1 Abstract

Sep15 is a thioredoxin-like, ER-resident protein involved in the quality control of glycoprotein folding through its interaction with UGT. Expression of Sep15 is regulated by dietary selenium and the unfolded protein response, but its specific function is not known. In the current study, we developed and characterized Sep15 KO mice by targeted removal of exon 2 of the Sep15 gene coding for the cysteine-rich UGT-binding domain. These KO mice synthesized a mutant mRNA, but the shortened protein product could be detected neither in tissues nor in Sep15 KO embryonic fibroblasts. Sep15 KO mice were viable and fertile, showed normal brain morphology and did not activate ER stress pathways. However, parameters of oxidative stress were elevated in the livers of these mice. We found that Sep15 mRNA was enriched during lens development. Further phenotypic characterization of Sep15 KO mice revealed a prominent nuclear cataract that developed at an early age. These cataracts did not appear to be associated with severe oxidative stress or glucose dysregulation. We suggest that the cataracts resulted from improper folding status of lens proteins caused by Sep15 deficiency.

3.2 Introduction

Sep15 was identified in mammals 13 years ago as a protein of unknown function [137]. The NMR structure of the Drosophila melanogaster Sep15 revealed a thioredoxin-like fold within its oxidoreductase domain, with Sec located in the predicted catalytic position [20]. Previous studies showed that Sep15 resides in the endoplasmic reticulum (ER) and interacts with UDP-glucose:glycoprotein glucosyltransferase (UGT) [138]. The latter protein is a part of the calnexin-calreticulin glycoprotein folding cycle and is known to be responsible for targeting unfolded glycoproteins for calcium-dependent transient
glucosylation. Sep15 contains the ER targeting peptide, but lacks an ER retention signal; the tight binding to UGT allows retention of Sep15 in the ER. These findings suggested that Sep15 may assist UGT function and control folding or secretion of certain glycoproteins.

Recently, Sep15 was found to be regulated by ER stress. Compounds causing mild ER stress, such as tunicamycin and brefeldin A, up-regulated Sep15 expression. At the same time, more robust ER stress agents, such as dithiothreitol (DTT), induced rapid proteasomal degradation of Sep15 [22]. Presumably, disruption of Sep15-UGT interaction due to reduction of disulfide bonds in the Sep15 UGT-binding domain displaced Sep15 from the ER. Expression of Sep15 is higher in tissues with secretory functions, such as liver, kidney, prostate and thyroid [139]. Comparison of expression of Sep15 and other components of the protein folding quality control machinery revealed similar expression patterns in mouse brain, including enriched levels in hippocampus and cerebellum. This observation further pointed to the role of Sep15 in glycoprotein folding [22].

Several studies suggested a role of Sep15 in cancer prevention. First, the human Sep15 gene is located on chromosome 1p31, in the locus often deleted or mutated in human cancers. In addition, Sep15 has two polymorphic sites which differentially regulate expression of this protein in response to selenium supplementation [140]. Moreover, levels of Sep15 were decreased in prostate cancer cell lines and in hepatocarcinoma compared to non-transformed cells and tissues. Sep15 expression is also lowered in malignant lung, breast, prostate and liver [141]. These observations suggest a role of Sep15 in tumor suppression, possibly through regulation of folding and/or secretion of cancer-related glycoprotein substrates; however, the specific function of Sep15 in this process is not
understood. At the same time, recent research demonstrated an opposite role for Sep15 in the promotion of growth of colon cancer cells [142, 143]. Stable knockdown of Sep15 significantly reduced growth of CT26 cells in culture and prevented formation of colonies on the soft agar. Mice, injected with the Sep15-deficient cells developed fewer tumors and did not form pulmonary metastases as did mice injected with control cells. However, Sep15 deficiency had no effect on the growth of lung cells.

In the present study, we describe Sep15 KO mice, lacking exon 2 of the gene. Although the mutant mRNA was detected, the shortened dysfunctional Sep15 form was not present in these mice. Analyses of Sep15 KO mice did not reveal any signs of ER stress and any gross abnormalities. However, livers of Sep15 KO mice showed increased levels of malondialdehyde and protein carbonyls, indicative of mild oxidative stress. During the course of further analyses, we found that Sep15 mRNA was enriched in the developing ocular lens. Consistent with this observation, deficiency in the protein due to Sep15 KO resulted in the development of cataract. This study highlights the roles of Sep15 in redox homeostasis and glycoprotein folding and indicates its function in the maintenance of lens transparency.

3.3 Experimental Procedures

Development of Sep15 KO mice. The Sep15 KO construct was prepared as follows. A 4,202 bp fragment of Intron 1 was amplified from 129/Sv mouse genomic DNA by nested PCR with the following primers: Round 1 5’-CCAGTTAACTTCTACGGGTCTGTTACAAGTGGT-3’ and 5’-GTCCAGGCTGCCCCTGAACACTAGCTCTGTGC-3’; and Round 2 5’-GTGGTCTCGAGTGGAAGCATCATTGACAGTTGATGTACTGAAGTG-3’ and 5’-
CTGTGCTCGAGACGACCTCACCCTGATTCTCCTGTCCAGTTCAGG-3’. The amplified fragment was cloned into the XhoI site of the pPNT vector. A second fragment of 4,246 bp of Intron 2 was amplified from 129/Sv mouse genomic DNA by nested PCR with the following primers: Round 1 5’-GCATAACCAGGATTCCACTGAGGCTGTCC-3’ and 5’-ACCCATCTTTATGGACGGTGTCACATGTC-3’; and Round 2 5’-AGGACCAGGATCCAGGATCACCATTGCCCTAGGCTTCTCGG-3’ and 5’-CCTGTGGATCCTGACTCTGCTGGCTGTGGTTGGTGTAGAGAAGC-3’. The amplified fragment was cloned into the BamHI site of pPNT vector. Correct insert orientation of fragments was confirmed by restriction analysis. The construct was digested with NotI, DNA fragments were separated by agarose gel electrophoresis and the targeting DNA was purified from the gel. 129/Sv embryonic stem cells were transfected with the targeting construct and recombinants were selected by growing stem cells in a neomycin-containing medium. Clones with successful construct recombination were identified by nested PCR with the following primer pairs for the neo-cassette and genomic DNA that was not part of the targeting construct: Fragment 1, Round 1 5’-GGGAAGACAGTACCTTGTCATGTAAGTGG-3’ and 5’-GCTGCTAAAGCGCATGCTCCAGACTGCCTTGG-3’; Fragment 1, Round 2 5’-CCAGTTAACTTCTACGGGTCTGTTACAAGTGG-3’, 5’-CCTACCCGGTGAATTGACCTGCAGG-3’; Fragment 2, Round 1 5’-GAACGAGATCAGCAGCTCTCCACAGTTCCAC-3’ and 5’-ACCCATCTTTATGGACGGTGTCACATGTC-3’. Fragment 2, Round 2 5’-GTTCTAATTCATCAGAAGCTGACTCTAGA-3’ and 5’-
CAGTCATGTTGCGCTGCCTTCCTGCCT-3’. Cells with proper construct integration were microinjected into C57Bi/6 blastocysts. The resulting chimeric mice were then mated with C57Bi/6 mice. Germline transmission was confirmed by nested PCR as described above. The KO allele was transferred to the C57Bi/6 background through five rounds of crossbreeding with C57Bi/6 mice, and homozygous Sep15 KO animals were then generated by crossbreeding of Sep15 KO heterozygous mice. The KO phenotype was confirmed by southern blotting, $^{75}$Se metabolic labeling and immunoblot analyses as described in the Results section. For the RT-PCR experiments, total liver RNA was isolated by TRIzol extraction according to the manufacturer’s protocol (Invitrogen). Genomic DNA was removed using a DNA removal kit (Ambion). RNA was quantified with a spectrophotometer, and 5 µg were transcribed with 50 ng of random primer and 200 U of SuperScript III reverse Transcriptase (Invitrogen). 1 µL of the resulting cDNA was used for analysis with primers specific for various regions of the mouse Sep15 gene.

**Isolation of mouse embryonic fibroblasts (MEFs) and metabolic labeling with $^{75}$Se.** Embryos generated from wild type (WT) and Sep15 KO mice were dissected out at the 13.5 embryonic day and separated from maternal tissues. The embryos were treated with 0.05% trypsin at 4 °C for 30 min and incubated at 37 °C for 1 h. Embryonic cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 20% fetal bovine serum, 2 mM glutamine, 50 µM β-mercaptoethanol and 100 units/ml penicillin/streptomycin. After 4 weeks, an aliquot of cells was frozen, and another aliquot was used for further experiments. For the analysis of Sep15 expression, WT and Sep15 KO MEFs were grown in DMEM supplemented with freshly neutralized $^{75}$Se selenious acid (specific activity 1000 Ci/mmol, final concentration in the medium 1 nM) for 24 h.
was immunoprecipitated using a Protein G immunoprecipitation kit (Sigma) and 5 µl of Sep15-specific antibodies. Selenoproteins were visualized by SDS-PAGE followed by autoradiography using a PhosphorImager.

**Cloning and in vitro expression of Sep15 and Sep15\(^\Delta 2\) forms.** To analyze for the possibility of expression of the Sep15 form lacking the Cys-rich UGT-binding domain encoded by exon 2 (Sep15\(^\Delta 2\)), Sep15\(^\Delta 2\) nucleotide sequence was amplified from Sep15 KO cDNA (prepared from total liver RNA) with primers 5’-ATCACTCGAGATGGCGGCAGGGCAGG-3’ and 5’-TGACTCTAGATATGAATATGATGATGTATGCAGTTCCAATTCTCTG-3’ containing XhoI and XbaI restriction sites, and subcloned into pCI-neo using the corresponding restriction enzymes. The SECIS element was separately amplified with 5’-TGACTCTAGATATGTTTTTATACTAATC-3’ and 5’-ATGACGATCTGCAGCCGCTTTTTTTTTTTAAATCAAACATATTTTGAAAG-3’ primers and cloned into the resulting vector at XbaI and NotI sites. For localization experiments, full-length Sep15 and Sep15\(^\Delta 2\) sequences were cloned into pEGFPN1 vector with primers 5’-ATCAGCTAGCATGGCGGCAGGGCAGG-3’ and 5’-ACTTACAATATTACCGGTATTATGCGTTCCAATTCTCTG-3’ containing NheI and AgeI restriction sites. All constructs were verified by sequencing. Sec was substituted with Cys by site directed mutagenesis with primers 5’-CTTGAAGTCTGCGGATGTAAATTGGGGAGGTTCC-3’ and 5’-GGAACCTCCCCAATTACATCCGCAGACTTCAAG-3’ (the introduced mutations are underlined).
**Cell culture studies.** Human HEK 293, mouse NIH 3T3 cells, and MEFs were cultured in DMEM medium supplemented with 10% FBS, 100 units/mL penicillin, and 100 units/mL streptomycin. Transfections were carried out using *Lipofectamine* (Invitrogen) according to the manufacturer’s instructions. To analyze expression of Sep15 and Sep15\(^\Delta 2\), HEK 293 cells were transfected with pCI-neo vector coding for full-length Sep15 and Sep15\(^\Delta 2\) forms, and, 18 h after transfection, medium was replaced with that containing freshly neutralized \(^{75}\text{Se} \text{selenious acid (specific activity 1000 Ci/mmol, final concentration in the medium 1 nM). Cells were metabolically labeled for 24 h, and expression of selenoproteins was analyzed by SDS-PAGE followed by autoradiography.}

To further analyze expression of full-length and Sep15\(^\Delta 2\) forms, NIH 3T3 cells were grown in 6-well plates and transfected with the corresponding EGFP fusion constructs or with an empty vector. Twenty-four hours after transfection, the original medium was replaced with serum-free medium, and after an additional 24 h, cells and media were collected. Cell lysates were prepared using CellLytic buffer (Sigma) according to the manufacturer’s instructions. The conditioned media were concentrated to 40 µl using Centricon concentrators (Millipore). Cell extracts and concentrated media samples were analyzed by Western blotting using GFP-specific antibodies (Sigma).

To further characterize expression of full-length and Sep15\(^\Delta 2\) forms, NIH 3T3 cells were grown on cover slips, transfected with the corresponding constructs in pEGFPN1 expression vector, and 24 h after transfection, treated with 1 µM ER-Tracker Blue-White DPX dye (Invitrogen) for 20 min. Then, cells were washed with PBS, fixed in 4% formaldehyde in PBS and mounted with mount media. Images were collected using a BioRad MRC1024ES laser scanning microscope.
Metabolic labeling of WT and Sep15 KO mice. WT, heterozygous and homozygous Sep15 KO mice were injected each with 40 µCi of freshly neutralized $^{75}$Se selenious acid and maintained on a 12 h light/dark cycle and a regular diet. After 48 h, mice were sacrificed and extracts prepared from various tissues in ice-cold PBS, pH 7.6, supplemented with complete protease inhibitor mixture (Roche Applied Science). Extracts from the indicated tissues were subjected to SDS-PAGE, and proteins were transferred onto polyvinylidene difluoride membranes and analyzed with a PhosphorImager.

Tissue and blood preparation. Blood was obtained from sacrificed Sep15 KO and control mice from the heart vein. Brain and prostate tissues were immediately fixed in 10% neutral buffered formalin and washed with PBS. PBS-washed tissues were embedded in paraffin and cut on a standard microtome to 5 µm sections. The sections were deparaffinized in xylene and stained with hematoxylin (Invitrogen) and eosin (Sigma) to reveal the fine tissue structure. Images were taken using a light Olympus AX70 microscope. For prostate-specific antigen (PSA) analysis, erythrocytes were removed by centrifugation, and 30 µg of serum proteins were analyzed by Western blotting with PSA-specific antibodies (Dako). Brains were stained with the indicated antibodies at dilutions of 1:1,000–1:5,000 at 4 °C overnight.

Analyses of oxidative stress parameters. Protein extracts from liver, kidney, heart, testis, and brain were prepared in PBS containing a complete protease inhibitor mixture. Malondialdehyde was measured using the Oxiselect TBARS assay kit (Cell Biolabs) according to the manufacturer's instructions. Protein carbonyl content was assayed using 2,4-dinitrophenyl hydrazine and (molar extinction coefficient of 22 mm$^{-1}$ cm$^{-1}$). Total and protein thiol contents were measured using 5,5′-dithiobis (2-nitrobenzoic acid).
Analysis of SelP secretion in hepatocytes. Hepatocytes of WT and Sep15 KO mice were obtained according to the previously published procedures [144, 145]. Briefly, livers from 3-month old mice were perfused with Ca\(^{2+}\) and SO\(_4^{2-}\) free buffer (6 mM glucose, 115 mM NaCl, 25 mM NaHCO\(_3\), 6 mM KCl, 1 mM MgCl\(_2\)6H\(_2\)O, 1mM NaH\(_2\)PO\(_4\)). After liver was cleared from the blood, collagenase (700 units/ml, Sigma) was added at the concentration of 0.1 mg/ml. Tissues were removed and dissociated by gentle rubbing in perfusion buffer. The suspension was then filtered through a 70 µm cell strainer by gravity flow. Then, hepatocytes were washed 3 times in Williams E medium (Sigma) supplemented with 2 mM L-glutamine (Invitrogen) seeded on collagen-coated 5 cm plates. Fourty eight hours later, the medium was replaced with 3 ml of serum-free medium containing freshly neutralized \(^{75}\)Se selenious acid and supplemented with DMSO or ER stress-inducing agents 1 mM DTT, 10 µg/ml brefeldin A, 5 µg/ml tunicamycin, or 20 µM monensin. Cells and media were collected 24 h later, and media samples were concentrated as described above for NIH 3T3 cells. Cell lysates and concentrated media were analyzed by SDS-PAGE followed by autoradiography.

Tunicamycin injection. WT and Sep15 KO mice were given a single 1 µg/g body weight intraperitoneal injection of a 0.05 mg/ml suspension of tunicamycin in 150 mM dextrose as described [146]. These mice were euthanized 72 h after injection, and their livers were removed. Protein lysates were resolved by SDS-PAGE and analyzed by Western blotting using antibodies specific for Sep15, BiP and β-actin.

Phenotypic screening of cataracts. Phenotypic screening was performed via slit lamp examination as described [147]. Mouse pupils were dilated by 1% atropine, and 15 min later lenses were examined with a slit lamp. Observed opacities were quantified using
Lens Opacities Classification System, version II (LOCS II) [147-149]. For *ex vivo* phenotyping, eyes were dissected for lens phenotyping by *ex vivo* dark field photomicroscopy as previously described [150, 151]. For measurement of glucose levels, mice were fasted for 8 h. Glucose was measured in blood from the tail vein using Elite system (Bayer) glucometer as described by the manufacturer. The data were analyzed with ANOVA (p<0.05 for limit).

### 3.4 Results

**Generation of the Sep15 KO mice.** Sep15 KO mice were generated by homologous recombination. The KO construct was designed to exchange exon 2 with the selection marker followed by a transcription termination signal (Fig 7A, B). Genotypes of mice were confirmed by PCR analysis. However, subsequent RT-PCR analysis showed a signal of Sep15 mRNA in Sep15 KO tissues. To determine the organization of this mRNA, we used primer pairs for various parts of the Sep15 mRNA (Fig 7C). Amplification with primers specific for the first and third exons gave fragments of different sizes in WT and Sep15 KO mice, while the use of primers downstream of exon 3 showed fragments of similar size. Further sequencing of the fragment amplified with primers specific for exons 1 and 5 revealed that the truncated Sep15 KO mRNA lacked exon 2. However, since the reading frame was preserved and the shortened mRNA form had both an in-frame UGA codon and SECIS element in the 3’-UTR, the possibility that the mutant mRNA supported Sec insertion and therefore selenoprotein synthesis had to be considered. To test for possible expression of the shortened protein product in Sep15 KO mice, we prepared MEFs from WT and Sep15 KO mice and subjected them to metabolic labeling with $^{75}$Se (Fig 7D). Selenoprotein patterns were similar in WT and Sep15 KO MEFs, with the
exception of a 14 kDa band that was missing in Sep15 KO MEFs. This band corresponded to the migration property of Sep15 following removal of the N-terminal signal peptide. To verify that the 14 kDa band was Sep15, we carried out immunoprecipitation with Sep15-specific antibodies. This analysis precipitated a 14 kDa protein in WT MEFs, but not in Sep15 KO MEFs (Fig 7D). In addition, we did not detect any selenoprotein bands migrating lower than 14 kDa in either WT or Sep15 KO samples subjected to immunoprecipitation. These data suggested that Sep15 KO mice express neither Sep15 nor its shortened form lacking sequences encoded by exon 2.

**Characterization of the truncated Sep15\(^{\Delta 2}\) form.** Since Sep15 KO mice synthesized a shortened mRNA form, we further examined the possibility that it could code for a protein fragment. The predicted size of Sep15 selenoprotein lacking the region encoded by exon 2 (further designated Sep15\(^{\Delta 2}\)) is 107 amino acids (11.6 kDa) (Fig 8A, B). Transfection of the Sep15\(^{\Delta 2}\) construct into HEK 293 cells did not reveal a band of the expected size when cells were labeled with \(^{75}\)Se. We also tested the possibility that Sep15\(^{\Delta 2}\) was secreted by cells because it lacked the domain that retained the protein in the ER due to interaction with UGT. To exclude this possibility, we prepared constructs for Cys mutants (i.e., Cys replacing Sec) of Sep15\(^{\Delta 2}\) and Sep15 fused with GFP. HEK 293 cells transfected with these constructs showed Sep15\(^{\Delta 2}\)-GFP and Sep15-GFP bands, and these protein forms were absent in the medium (Fig 8D). We also transfected NIH 3T3 cells with the same constructs and examined localization of Sep15 protein forms (Fig 8E). Full-length Sep15-GFP colocalized with the ER tracker, while Sep15\(^{\Delta 2}\)-GFP showed a different localization pattern, wherein the protein seemed to be in granules (it did not correspond to mitochondria or lysosomes). Thus, Sep15\(^{\Delta 2}\) was not expressed in transfected
mammalian cells, based on Western blot data, extremely sensitive $^{75}\text{Se}$ labeling of cell lysates, and immunoprecipitation of Sep15 from $^{75}\text{Se}$-labeled cells. When Sep15$^{\Delta 2}$ was fused to a large globular protein (i.e., GFP) and, in addition, had its Sec replaced with Cys (Sec insertion is very slow and replacing this residue with Cys is a method to dramatically increase protein expression), the protein could be expressed, but it changed localization. We conclude from the above observations that Sep15 KO cells lack functional Sep15.

**Absence of Sep15 does not affect expression of other selenoproteins.** Selenoprotein expression depends on availability of dietary Se [128]. In addition, removal of one selenoprotein may affect expression of other selenoproteins, e.g., due to increased availability of Sec-tRNA or by influencing redox homeostasis. To examine this possibility, WT and heterozygous and homozygous Sep15 KO mice were metabolically labeled with $^{75}\text{Se}$ (Fig 9). However, expression of other selenoproteins was unchanged.

**Oxidative stress in Sep15 KO mice.** We examined parameters of oxidative stress in livers of WT and Sep15 KO mice. Sep15 KO livers showed increased levels of malondialdehyde ($p<0.0001$) and protein carbonyls ($p<0.0001$), while amounts of total thiols and protein thiols were found to be decreased compared to controls ($p<0.05$) (Fig 10). The data show increased lipid and protein oxidation, and suggest mild oxidative stress in Sep15 KO livers.

**Analysis of Sep15 KO mice for prostate and brain pathologies.** Sep15 is expressed at elevated levels in prostate, liver, kidney, testis, and brain. At the same time, Sep15 expression is decreased in malignant prostate cell lines and in hepatocarcinoma [22]. We examined Sep15 KO mice for phenotypes associated with prostate and brain. Prostate was examined in 1.5 year-old males ($n=13$) (Fig 11A. No signs of neoplasia were
observed. PSA is a plasma protein, whose expression is increased during prostate pathologies, such as inflammation and malignant transformation. No differences in PSA levels were observed in serum samples of WT and Sep15 KO mice (Fig 11B).

Selenoprotein deficiency is also known to be associated with neuronal pathology [40]. For example, mice lacking SelP show seizures and ataxia and die before weaning when fed a Se-deficient diet [152]. Comparative analysis of gene expression data in the Allen Brain Atlas revealed expression of all selenoproteins and selenocysteine incorporative machinery in the brain, including high expression of Sep15 [153]. In addition, disruption of neuronal expression of selenoproteins led to neurological symptoms and neurodegeneration [114, 154]. Similar symptoms were observed in glutathione peroxidase 4 (GPx4) KO mice [155] and in Trsp<sup>1AE/1AE</sup> mice expressing hypomorphic tRNA<sup>(Ser)Sec</sup> [114]. Sep15 expression is high in Ammon’s horn and dentate gyrus of hippocampus and in Purkinje cells of the cerebellar cortex [153]. We analyzed brains of Sep15 KO mice (Fig 11). The hippocampal area of Sep15 KO mice showed no signs of astrogliosis, and its levels of glial fibrillary acidic protein and parvalbumin were similar to those in WT mice. Thus, Sep15 deficiency did not lead to the loss of parvalbumin positive interneurons observed in mouse models with general deficiency in selenoprotein expression. Analysis of cerebellum morphology of Sep15 KO mice also did not reveal abnormalities. Thus, Sep15 deficiency does not lead to spontaneous neurodegeneration, consistent with an apparent lack of behavioral phenotypes.

**Sep15 deficiency does not influence SelP secretion.** As a UGT-binding partner, Sep15 may be involved in protein folding and secretion. Sep15 is also regulated by the unfolded protein response (UPR). SelP is a major plasma selenoprotein, synthesized in the
liver and transporting Se to other organs. SelP maturation, intracellular transport and secretion occur through the common ER-Golgi pathway in the liver [156]. We examined the possibility that Sep15 KO affects SelP secretion. Hepatocytes from WT and Sep15 KO mice were isolated and metabolically labeled with $^{75}$Se. The cells were also treated with compounds that compromise function of the ER-Golgi pathway (Fig 12). This treatment did not affect the viability of cells (data not shown). Treatment of hepatocytes with brefeldin A is known to abrogate SelP secretion, which is what we observed in both WT and Sep15 KO hepatocytes, while treatment with tunicamycin and monensin caused accumulation of intracellular non-glycosylated or partially glycosylated SelP forms. However, this difference disappeared after the treatment with tunicamycin, an inhibitor of GlcNAc phosphotransferase. As expected, treatment with this compound induced expression of BiP in Sep15 KO hepatocytes.

**Sep15 deficiency does not lead to activation of the UPR.** To further assess the function of Sep15, WT and Sep15 KO mice were injected intraperitoneally with 1 µg/g body weight tunicamycin or vehicle and sacrificed 72 h after injection. This treatment induced both BiP and Sep15 expression in WT mice (Fig 13), consistent with previous data in cell culture [22]. BiP was elevated similarly in WT and Sep15 KO mice, so the absence of Sep15 did not augment the UPR.

**Sep15 mRNA expression is progressively lens-enriched during mouse development.** Because our data suggested that Sep15 may play a role in the quality control of protein folding, we hypothesized that deficiency in this protein may lead to protein misfolding or aggregation. A prominent human disease associated with altered protein folding is cataract, with opacities caused by aggregation of lens structural proteins, such as
α-crystallins. It has been proposed that genes that are expressed in a lens-enriched manner in the developing mouse lens may have a functional role in the development and maintenance of lens transparency and therefore may represent candidate genes for cataract [157, 158]. In this approach, lens enrichment of a candidate gene is scored against its expression in whole embryonic tissues at various stages in development (Lachke and Maas, unpublished). This approach has been successful in identifying previously described cataract loci. Therefore, we investigated if Sep15 mRNA was enriched in the lens during stages of lens invagination (E10.5) through primary fiber cell differentiation (E12.5). We found expression of Sep15 mRNA to get progressively enriched at E12.5 (Fig 14).

**Development of cataract in Sep15 KO mice.** To directly assess cataract formation in Sep15 KO mice, we carried out slit lamp examination for cataract scoring, which revealed lens opacity in both male (n=10) and female (n=7) Sep15 KO mice (Fig 15A). Observed opacities were quantified using Lens Opacities Classification System, version II. Nuclear changes were classified by scoring the degree of opalescence. In our experiment, clear lens was assigned a grade of 0 and the fully opaque lens a grade of 5. The opacity grade increased with age, based on the comparison of 1.5 and 6-month old females (n=12). Fundus camera examination was also conducted, and representative images are shown in Fig 15B. A group of Sep15 KO mice was examined by slit lamp followed with *ex vivo* phenotyping, including lens dissection followed by microscopy analyses. In this experiment, we used 2.5-month old males (n=3) and females (n=3) as well as 10 month old males (n=4) and females (n=3). Both lenses of each KO mouse showed nuclear opacity, which increased in size with age. Sep15 KO mice predominantly developed nuclear cataract. Representative images of the lenses dissected from 2 month and 10 month Sep15
KO and control mice are shown in Fig 15 C-H and. Although some of the 10 month old control mice developed age-related punctate opacity (Fig 15H), the cataract in Sep15 KO mice was more prominent and occurred in all mice. Moreover, all examined 2 month old Sep15 KO mice had cataract, whereas it was not observed in 2 month old controls. These data show that Sep15 KO mice develop nuclear cataract.
3.5 Discussion

Sep15 is an ER-resident protein implicated in the calnexin-dependent quality control cycle. Our previous studies showed that Sep15 forms a 1:1 complex with UGT, a protein that recognizes improperly folded or partially unfolded glycoproteins and subjects them to the calnexin cycle. In addition to the Cys-rich UGT-binding domain, Sep15 has a thioredoxin-like domain containing a redox active CxU (Cys and Sec separated by another residue) motif, suggesting a thiol oxidoreductase function for this protein. Sep15 is also differentially regulated by ER stress. Mild stress, e.g., caused by tunicamycin or brefeldin A treatments, induces Sep15 expression, whereas acute stress, e.g., caused by treatments with disulfide-reducing DTT or thapsigargin, a compound that disrupts calcium flux from the ER, leads to a rapid proteasomal degradation of Sep15 [22]. However, the specific function of Sep15 in these processes is not known. Sep15 is expressed at elevated levels in secretory organs, such as prostate, liver, kidney, testes, and brain. At the same time, Sep15 expression was shown to be decreased in prostate cancer cells and in hepatocarcinoma. It was also found that stable Sep15 knockdown makes cells more susceptible to oxidative stress.

In the current study, we generated and characterized Sep15 KO mice, which were viable and fertile, and with no obvious phenotypes during development and growth. These mice were obtained by deleting exon 2 coding for the UGT-binding domain of Sep15. Although the mRNA for a shortened Sep15 form was synthesized, the corresponding protein could not be detected under any conditions tested. Moreover, even when this protein form was forced into expression in cell culture by fusion with GFP and replacement of Sec with Cys, it had an abnormal localization. Therefore, Sep15 KO mice lacked functional Sep15.
Initial characterization of Sep15 KO mice revealed no phenotypes with regard to expression of other selenoproteins, susceptibility to treatments that induce ER stress, or protein secretion. Strong interaction between Sep15 with UGT implied the role of Sep15 in the quality control of glycoprotein folding. Thus, we analyzed the effect of Sep15 deficiency on secretion of plasma glycoprotein SelP. However, no differences were found between WT and Sep15 KO hepatocytes subjected or not subjected to ER stress. Apparently, SelP is not a target of Sep15. Previous experiments in cell culture also suggested the involvement of Sep15 in the UPR. Therefore, we examined the levels of BiP (a marker of UPR) in WT and Sep15 KO hepatocytes. Tunicamycin treatment led to a robust increase in BiP expression in Sep15 KO hepatocytes; however, this protein was induced to a similar level in WT and Sep15 KO mice.

Although the observed phenotypes of Sep15 KO mice were mild, we reasoned that since Sep15 is elevated by the UPR, Sep15 deficiency might modulate development of certain ER stress associated diseases. One such disease is cataract formation resulting from improper protein folding and posttranslational modifications, leading to decreased lens transparency. It has been proposed that lens-enriched genes may represent promising targets associated with cataract formation. Recently, deficiency of a gene with a highly lens-enriched expression pattern was found to cause congenital cataracts in humans, mice and chicken [157, 158]. We found that Sep15 mRNA was progressively enriched in three critical stages of mouse lens development. In addition, transcripts for several proteins functionally linked to Sep15 were found to be enriched in the lens (e.g., UGT1, calreticulin) while others (e.g., UGT2, calnexin, ERp57, SelM) although not enriched are
expressed in the lens. This provides further support that the Sep15 function is important in the lens.

Further analysis revealed that nearly every Sep15 KO mouse developed mild nuclear cataract, some as early as 1.5 months of age. One of the factors that may influence cataract formation is glucose imbalance. We analyzed glucose levels in fasted mice, but they were comparable to those in WT controls (Fig 16). Cataracts may also result from increased protein oxidation and aggregation. Indeed, we found that Sep15 KO mice showed increased levels of both lipid and protein oxidation. To further examine this possibility, we analyzed the ratio of reduced to oxidized glutathione and levels of thiols in lenses. However, these parameters were similar in Sep15 KO and WT lenses (Fig 16 B, C). Therefore, it remains to be determined if Sep15 KO induced cataracts are directly associated with general oxidative stress. Another explanation for cataract formation is the improper folding or disulfide status of Sep15 targets. For example, similar symptoms were previously described in mice lacking connexin 46 [159, 160] Connexins are a family of gap junction proteins, whose mutations are associated with cataract development in human subjects [161, 162]. In the case of Sep15 KO cataracts, further studies are needed to determine the precise function of Sep15 in protein folding, but interaction of this selenoprotein with the protein folding sensor is consistent with the role of protein misfolding in Sep15-dependent cataract development.

Overall, this study shows that the absence of Sep15 does not affect viability and does not lead to obvious phenotypes in mice. To better understand Sep15 function and the role of this selenoprotein in glycoprotein folding, it would be important to identify the subset of Sep15 targets and examine their posttranslational modifications in WT and Sep15
KO mice. Cataract is the major cause of blindness in the world. Several types of cataracts are associated with excessive ER stress and the UPR [163, 164]; however, little is known about the link between defective protein secretion pathways and cataract formation. Future experiments in this direction may lead to a better understanding of the association between abnormal glycoprotein folding/modification and human disease.
Figure 7. Preparation of Sep15 KO mice. (A) Structure of the mouse Sep15 gene. (B) Structure of the KO allele. Coding regions are shown in orange and DNA flanking regions used for homologous recombination are shown in green. The second exon was replaced with the Neo gene containing a transcription termination signal. (C) RT-PCR analysis of Sep15 mRNA in WT and Sep15 KO mice with primers for the indicated regions of Sep15. Aldolase gene was used as a control. (D) Analysis of Sep15 expression in MEFs derived from WT and Sep15 KO mice. Cells were labeled with $^{75}$Se followed by immunoprecipitation with antibodies specific for Sep15. Two lanes on the left show labeled MEFs. Immunoprecipitated (i.p.) and flow through (flow) fractions are shown for each MEF line in the right lanes. Arrow on the right indicates migration of Sep15.
Figure 8. Characterization of a shortened Sep15 form. (A) Amino acid sequence and predicted size of Sep15$^{Δ2}$. (B) Schematic representation of domain organization of Sep15 and its correspondence to gene structure. (C) Sep15 and Sep15$^{Δ2}$ were expressed in HEK 293 cells followed by labeling of cells with $^{75}$Se. Expression of proteins was compared to the selenoprotein expression profile in MEFs derived from WT and Sep15 KO mice. (D) Western blot analysis of Cys mutants of Sep15-EGFP and Sep15$^{Δ2}$-EGFP expressed in NIH 3T3 cells. Protein expression was analyzed in cell extracts and concentrated media. (E) Localization of Sep15 and Sep15$^{Δ2}$. Colocalization of Sep15 forms (green) and the ER marker (blue) is shown.
Figure 9. Metabolic labeling of WT and Sep15 KO mice with $^{75}\text{Se}$. WT (Sep15$^{+/+}$), and heterozygous (Sep15$^{+/-}$) and homozygous (Sep15$^{-/-}$) Sep15 KO mice were injected each with 40 µCi of $^{75}\text{Se}$. Selenoprotein expression was analyzed in indicated organs by SDS-PAGE followed by PhosphorImager.
Figure 10. Oxidative stress in Sep15 KO mice. Levels of malondialdehyde, protein carbonyls, total thiols and protein thiols in livers of WT (white bars) and Sep15 KO (black bars) mice are shown as mean +/- SD. (* p<0.001).
Figure 11. Analysis of prostate and brain pathologies in Sep15 KO mice. (A) H&E staining of prostate sections in WT and Sep15 KO mice. (B) Western blot analysis of PSA in serum of WT and Sep15 KO mice (upper panel). Coomassie Blue staining as a protein loading control (loading). (C) Analysis of brain sections: Nissl, GFAP and parvalbumin staining of the hippocampal area of WT and Sep15 KO mice. Also shown are immunohistochemistry of cerebral cortex area with GFAP antibodies and Nissl staining of cerebellum.
Figure 12. **Sep15 deficiency does not affect SelP secretion.** Hepatocytes were isolated from WT and Sep15 KO mice and metabolically labeled with $^{75}$Se. PhosphorImager analysis of cellular extracts (left panel) and concentrated media (right panel). Membranes were further analyzed with BiP and β-actin (lower left panels) and SelP (lower right panel) antibodies.
Figure 13. Sep15 deficiency does not lead to activation of the UPR. WT and Sep15 KO mice were injected intraperitoneally with tunicamycin (Tun) (1 µg/g body weight) or vehicle. 72 h after injection, mice were euthanized, livers extracted and protein lysates from livers probed by immunoblot assays with polyclonal antibodies specific for Sep15 and BiP, or monoclonal β-actin antibody as a loading control.
Figure 14. Enrichment of Sep15 mRNA during lens development. Sep15 mRNA expression was analyzed at the indicated stages of embryogenesis and compared to the whole body Sep15 mRNA expression (WB).
Figure 15. Development of cataract in Sep15 KO mice. (A) Quantification of slit lamp examination of control and Sep15 KO mice. Opacity was quantified using the Lens Opacities Classification System, version II. The scale was from completely clear (grade 0) to fully opaque (grade 5) lens. L indicates left and R right. (B) Representative images of opacities in Sep15 KO mice using Fundus Camera. Six-month old WT and Sep15 KO
females were used. L indicates left and R right. (C-H) Representative cataracts in Sep15 KO mice. Each panel shows lens from the same animal. Left images represent in vivo slit lamp examination, and the right images show dark field analyses of the same lens examined in vitro. (C, D) Representative lenses from 2-month-old Sep15 KO females. Nuclear opacity is shown with a red arrow. (E) 2-month-old WT female (control), which shows no nuclear opacity. (F, G) 10-month-old Sep15 KO females. Nuclear opacity is shown with red arrows. (H) 10-month-old WT female, which developed age-related punctate opacity.
Figure 16. Comparison of glucose levels and oxidative stress parameters in lenses of WT and Sep15 KO mice. (A) WT and Sep15 KO mice were fasted overnight and blood was analyzed for glucose levels (n=9 for WT and 12 for Sep15 KO mice). (B) Lenses were extracted from WT and Sep15 KO mice. Protein extracts were analyzed for (B) ratio of oxidized to reduced glutathione and (C) total thiol levels. In each group, n=3.
CHAPTER 4

Reduced utilization of selenium by naked mole rats due to a specific defect in GPx1 expression

Note: The results described in this chapter have been published.

4.1 Abstract

Naked mole rat (MR) *Heterocephalus glaber* is a rodent model of delayed aging because of its unusually long lifespan (>28 years). It is also not known to develop cancer. In the current work, tissue imaging by X-ray fluorescence microscopy and direct analyses of trace elements revealed low levels of selenium in the MR liver and kidney, whereas MR and mouse brains had similar selenium levels. This effect was not explained by uniform selenium deficiency as methionine sulfoxide reductase activities were similar in mice and MR. However, glutathione peroxidase activity was an order of magnitude lower in MR liver and kidney than in mouse tissues. In addition, metabolic labeling of MR cells with $^{75}\text{Se}$ revealed a loss of the abundant GPx1 band, whereas other selenoproteins were preserved. To characterize the MR selenoproteome, we sequenced its liver transcriptome. Gene reconstruction revealed standard selenoprotein sequences except for GPx1, which had an early stop codon, and SelP, which had low selenocysteine content. When expressed in HEK 293 cells, MR GPx1 was present in low levels and its expression could be rescued neither by removing the early stop codon nor by replacing its SECIS element. In addition, GPx1 mRNA was present in lower levels in MR liver than in mouse liver. To determine if GPx1 deficiency could account for the reduced selenium content, we analyzed GPx1 knockout mice and found reduced selenium levels in their livers and kidneys. Thus, MR is characterized by the reduced utilization of selenium due to a specific defect in GPx1 expression.

4.2 Introduction

Naked mole rat (*Heterocephalus glaber*; MR) is a unique organism in the mammalian order rodentia. It is an attractive model to study aging due to an extraordinary lifespan (more than 28 years), which is unprecedented for rodents of similar body size.
Aging in MRs is characterized by very slow age-related declines that do not significantly affect breeding capacity, social behavior and daily activity [165-167]. MR evolved a unique eusocial lifestyle with strictly determined social roles and cooperative breeding within the colony. The anatomy of MR has several specific features, which probably evolved as an adaptation to living underground in oxygen limiting conditions, including underdevelopment of the visual system [168], skin insensitivity [169], and ability to tolerate low temperature [170] and low oxygen levels [171]. MRs are also characterized by a decreased metabolic rate, which is associated with reduced levels of thyroid hormones [167]. One of the most remarkable features of MR is that none of the autopsies of dead animals in several colonies revealed cancer incidence [165]. Moreover, primary fibroblasts derived from MR were sensitive to contact inhibition [172], resistant to experimentally induced tumorigenesis and unable to form xenograft tumors [173].

MR poses a challenge to the theories that link aging, cancer and oxidative stress. Since MR lives in low oxygen conditions, it is expected to have low levels of oxidative stress. However, research showed that MR is characterized by significant oxidative damage. Compared to mice, MRs have lower GSH/GSSG ratio, higher rate of lipid peroxidation, high rate of DNA oxidative damage and higher protein carbonylation [174]. It was also demonstrated that MR proteins possess higher levels of protein thiols, increased protein stability, increased resistance to urea-induced denaturation and elevated proteasomal activity, while also showing lower levels of protein ubiquitination. Moreover, unlike the mouse proteome, the MR proteome did not show significant age-related susceptibility to oxidation and ubiquitination [175]. A separate study did not detect age-related changes in the activities of antioxidant enzymes, including Mn superoxide
dismutase (SOD), Cu/Zn SOD, and catalase, and also observed low glutathione peroxidase (GPx) activity [176].

Mammals have eight GPxs, including five selenoproteins. GPx1 is the first identified and one of the best studied selenoproteins [1]. This and other selenoprotein GPxs have selenocysteine (Sec) residues in their active sites. GPx1 is a cytosolic enzyme and the most abundant selenoprotein in mammals. Other GPxs show different cellular localizations and/or tissue and substrate specificities [11, 177]. GPx1 is not an essential enzyme, i.e., GPx1 knockout mice are viable and fertile. However, they are more susceptible to oxidative stress [58, 178] and viral myocarditis [179]. Expression of GPx1 is decreased in prostate cancer and breast cancer cell lines [180], as well as in the mouse model of liver cancer [181]. A single nucleotide polymorphism that changes Pro to Leu at the codon 198 of human GPx1 is associated with lung [182] and bladder [183] cancers.

Currently, there is no information on the utilization of selenium (Se) by MR; however, this is an essential trace element in mammals and its supplementation may be beneficial in cancer chemoprevention [184]. Se functions mostly through incorporation into proteins in the form of Sec. Mice and humans have 24 and 25 selenoprotein genes, respectively [10]. However, nothing is known about the composition of the MR selenoproteome.

In this study, we observed low levels of Se in MR tissues. This observation led to the finding that the reduced utilization of Se by this organism was due to a specific defect in GPx1 expression.
4.3 Experimental Procedures

Animals. Animal experiments were approved by IACUC committees at University of Illinois at Chicago and University of Nebraska-Lincoln. To carry out ICP-MS analyses, western blotting, activity assays and other analyses, animals were sacrificed, their tissues frozen in liquid nitrogen and stored until use. To prepare extracts, tissues were homogenized in PBS supplemented with a protease inhibitor cocktail (Sigma). Cellular debris was removed by centrifugation at 13,000 rpm for 15 min at 4 °C, and protein concentrations were determined by the Bradford assay.

Tissue samples for X-ray fluorescence microscopy (XFM). Tissues from C57BL/6 mice and MRs were extracted and prepared at the same time to match preparation conditions. Mice were fed standard rodent chow diet. MRs were fed either a sweet potato or carrot diet. Tissues from a 1 year old MR were compared with the corresponding samples of a 2 month old mouse to adjust for lifespan differences. All freshly extracted tissues were washed in PBS and placed in 4% neutral buffered formaldehyde in PBS for fixation. After 12 h, tissues were transferred to PBS, paraffin embedded, and cut using a standard microtome into 5 µm sections. Sections were mounted on silicon nitride windows (2 x 2 mm; 200 nm thickness, Silson, Blisworth, UK). Light microscopy images were taken using a Leica DMXR microscope (Leica Microsystems, Bannockburn, IL).

XFM. Trace elements in mouse and MR tissue samples were imaged with XFM at the Advanced Photon Source (APS), Argonne National Laboratory (Argonne, IL). For each pixel, full X-ray fluorescence spectrum was recorded using a single-element silicon drift detector (Vortex EX, SII Nanotechnology, Northridge, CA). Dwell time varied from 1.1 sec to 4 sec per pixel. For quantification, each set of experiments was followed by
recording X-ray fluorescence spectra of thin-film standards NBS-1832 and NBS-1833 (National Bureau of Standards, MD). X-ray fluorescence spectra for each sample were fitted and quantified using data derived from standards. Image processing and analysis were performed using MAPS software [185]. Each X-ray fluorescence image represents 2D distribution of the element.

**Activity assays.** Total GPx activity was measured using a GPx activity kit (Sigma) according to manufacturer’s instructions. Methionine-\(S\)-sulfoxide reductase (MsrA) and methionine-\(R\)-sulfoxide (MsrB) activities were measured in an HPLC assay as described [186]. Briefly, 200 µg of total protein were added to a reaction mixture that was kept at 37 °C for 30 min in the presence of 20 mM DTT and either 200 µM dabsyl-methionine-\(S\)-sulfoxide (to assay for MsrA activity) or 200 µM dabsyl-methionine-\(R\)-sulfoxide (to assay for MsrB activity). After stopping the reaction by adding 200 µl acetonitrile, it was centrifuged at 4 °C for 15 min at 13,000 rpm and the supernatant (50 µl) was injected onto a C\(_{18}\) column (ZORBAX Eclipse XDB-C18) to quantify the resulting dabsylated methionine.

**Splenocyte isolation and \(^{75}\text{Se} \) metabolic labeling.** After dissection, spleen was immediately transferred into ice-cold DMEM and mashed through a 45 µm cell strainer into the 50 ml tube. Cells were centrifuged at 800 g for 5 min. The pellet was resuspended in 5 ml of ASK buffer (GIBCO) and incubated at room temperature for 5 min. Cells were then diluted with DMEM and pelleted at 800 rpm for 5 min. The resulting pellet was resuspended in 15 ml DMEM supplemented with 10% fetal bovine serum (GIBCO), Antibiotic/Antimicotic (GIBCO), 0.4 mM glutamine and freshly neutralized \(^{75}\text{Se} \) selenious acid (specific activity 1000 Ci/mmol, final concentration in the medium 1 nM), and pooled
into a 15 cm plate. After 24 h, cells were washed 3 times with ice-cold PBS, and lysed in CellLytic buffer (Sigma). Protein extracts were analyzed by SDS-PAGE followed by autoradiography.

**Whole transcriptome sequencing and assembly of selenoprotein genes.** Extraction of the total RNA from MR liver was carried out using a RNAqueous kit (Ambion) according to the manufacturer’s instructions. DNA was removed by treatment with DNase I (Ambion). Liver transcriptome was sequenced at the University of Nebraska-Lincoln genomic facility on an Illumina instrument. To assemble 35 bp reads into longer contigs, three different strategies were used. First, mapping reads to reference sequence from mouse or guinea pig genomes was performed using MAQ package 0.7.1 [http://maq.sourceforge.net/]. Second, SOAP package 1.03 [http://soap.genomics.org.cn/] was used for *de novo* assembly of short reads. Finally, we employed an in-house program that was optimized for assembling selenoprotein genes and utilized the variety of information available from MAQ and SOAP output, as well as known sequences from other rodents.

**Inductively-coupled plasma mass spectrometry (ICP-MS).** Quantitative analyses of trace elements in animal tissues were performed using ICP-MS. Freshly frozen mouse and MR tissues were homogenized and sonicated in PBS with Complete protease inhibitor cocktail (Roche, Basel, Switzerland) (1 tablet in 50 ml PBS). Samples were normalized to protein content in each lysate as determined using Bradford Protein Kit (Bio-Rad, Hercules, CA). Samples were digested in 15% nitric acid, 15% hydrogen peroxide for 2 h at 70 °C. 50 ppb gallium were added as an internal control to the digestion mix. After digestion, samples were diluted 10 times with deionized water and analyzed by
ICP-MS. Elemental analysis was performed at the University of Nebraska-Lincoln Spectroscopy Core Facility using an Agilent Technologies ICP-MS Model 7500ce (Santa Clara, CA) and an Elemental Scientific Inc. (Omaha, NE) SC4 autosampler. Each sample was analyzed in triplicate. The carrier and make-up gas flows were 0.95 and 0.15 L/min of Ar, respectively. The collision cell operated with 3.5 mL/min of H\textsubscript{2} and 1.5 mL/min He for reaction/collision mode. Dwell times for all elements were 0.3 s. except \textsuperscript{78}Se, for which the dwell time was set at 0.9 s. Gallium (m/z = 71) was added to all samples and standards as an internal standard at 50 ppb.

**Expression constructs for mouse and MR GPx1 and their mutants.** Mouse and MR GPx1 cDNAs containing the 3’-UTRs including SECIS elements were amplified by a two step PCR with primers that introduced Myc-tag into ORFs. Primers were designed as follows: mouse: 1\textsuperscript{st} round, 5’-CTCAGAGGAGGATCTCTGTGCTGCTCGGCTCTCCG-3’ and 5’-GCATACTCTAGACTGATATTACGACTTTTTATTCTTTATGATGAAACC-3’; 2\textsuperscript{nd} round, 5’-CTCAGAGGAGGATCTCTGTGCTGGTCGTTTGGCCGCGGCC-3’ 5’-GCATACTCTAGACTGATATTACGACTTTTTATTCTTTATGATGAAACC-3’; MR: 1\textsuperscript{st} round, 5’-CTCAGAGGAGGATCTCTGTGCTGGTCGTTTGGCCGCGGCC-3’ and 5’-GCATACTCTAGACTGATATTACGACTTTTTATTCTTTATGATGAAACC-3’; 2\textsuperscript{nd} round, 5’-GCATACTCTAGACTGATATTACGACTTTTTATTCTTTATGATGAAACC-3’ and 5’-ATGACATCTCGAGATGGAGCAGAAGCTCATCTCAGAGGAGGATCTCTCGC-3’. The PCR products were digested with XhoI and Xba and ligated into a similarly digested pCIneo vector. In the case of mouse GPx1, an early premature stop codon was introduced using site-directed mutagenesis with QuikChange kit (Stratagene) and primers 5’-GAAACCCTGCTGCTCTAGACGTCTGGCAAC-3’ and 5’-
GTTGCCAGACTGCTAGGACAGCAGGGTTTC-3’. The resulting mutant was designated as the M-short form. To rescue the early stop codon in MR GPx1, we performed site-directed mutagenesis with primers 5’-AAGCCCTACTCAGGGGCGCCGCCCCTG-3’ and 5’-CAGGCGGCCGCCCTGAGTGAGTAGGGCTT-3’, and the resulting mutant was designated as the MR-long form. To obtain cysteine mutants of mouse and MR GPx1, we used site directed mutagenesis with primers 5’-TCGCGTCTCTCTGCGGACACCAGAT-3’ and 5’-ATCGTGTTGCCGAGAGACGGA-3’ for mouse GPx1, and 5’-GAATGTGGCTCCCTCTGCGGACCACGGTCGTC-3’ and 5’-GACCGTGGTGCCGAGGATGCGACCATTTC-3’ for MR GPx1. To obtain a pBudGE/GFP construct, EGFP was amplified from pEGPFN3 vector with primers 5’-ATGACAAGGTTATGGAGCAAGGGCGAGG-3’ and 5’-GGACATTCTAGATTACTTTGAGCTCCGTC-3’. The resulting PCR product was ligated into a similarly digested pBudCE4.1 vector. Mouse and MR GPx1 mutants were amplified from pCINeo constructs with primers 5’-GGTACCATGGAGCAGAAGCTCATCTCAGAGG-3’, 5’-GCATACGCATACAGATCTCTGATATTCAGCAAACCT-3’ and 5’-GGTACCATGGAGCAGAAGCTCATCTCAGAGG-3’, 5’-AGATCTACACCCAGCAC-TTTATTAGAGG-3’ respectively. Cys mutants were amplified with 5’-GGTACCATGGAGCAGAAGCTCATCTCAGAGG-3’ and 5’-GCATACGATCTTTAGAGGTGCGACCAGACTG-3’ (mouse) and 5’-GGTACCATGGAGCAGAAGCTCATCTCAGAGG-3’ and 5’-GCATACGATCTCTATGCAGGGCCGCCCC-3’ (MR), and inserted into the vector
without the 3’-UTR. To express mouse and MR GPx1 mutants in a bacterial expression system, GPx1 mutants were amplified with primers 5’-ATGCATCATATGGAGCAGAAGCTCATCTCAGAGG-3’ and 5’-GCATGCTCGAGTTAGGAGTTGCCAGACTGC-3’ (mouse) and 5’-ATGCATCATATGTGCTGTCGTTTGGCCG-3’ and 5’-GCATGCTCGAGCTATGCAGGGCCGCGCCC-3’ (MR) and cloned into pET28a expression vector at NdeI and XhoI sites.

To clone mouse and MR GPx1 into pSelExpress1 (described in [187]), mouse and MR GPx1 coding sequences were amplified with 5’-ATGACATAAGCTTATGGAGCAGAAGCTCATCTCAGAGG-3’ and 5’-GCATACTCTAGATTAGGAGTTGCCAGACTGC-3’ (mouse) and 5’-ATGACATAAGCTTATGGAGCAGAAGCTCATCTCAGAGG-3’ 5’-GCATACTCTAGACTATGCAGGGCCGCGCCC-3’ (MR) primers, digested with HindIII and XbaI restriction endonucleases and ligated into pSelExpress1 vector. To swap mouse and MR 3’-UTRs, we prepared the corresponding constructs on the basis of the pBudCE4.1/GFP vector, which expressed Myc-tagged GPx1 and GFP from separate sites in the construct.

**Cell culture, transfections, metabolic labeling, immunoprecipitation and western blot analyses.** HEK 293 and HeLa cells were cultured in DMEM supplemented with 10% FBS, 100 units/mL penicillin, and 100 units/mL streptomycin. All transfections were performed with Lipofectamin transfection reagent (Invitrogen), according to the manufacturer’s manual. Eighteen hours after transfection, medium was replaced with the one containing freshly neutralized $^{75}$Se selenious acid (specific activity 1000 Ci/mmol,
final concentration in the medium 1 nM). Cells were labeled for 24 h and cellular extracts
analyzed by SDS-PAGE followed by autoradiography. For immunoprecipitation, HEK 293
cells were grown on 10 cm plates, transfected with various GPx1 constructs and
metabolically labeled with $^{75}$Se. Cells were resuspended in PBS, supplemented with
protease inhibitor cocktail (Sigma) and treated with a tissue homogenizer. 600 µg of total
protein were subjected to immunoprecipitation with the Protein G Immunoprecipitation Kit
with mouse anti-Myc antibodies (Invitrogen), according to the manufacturer’s instructions.
For western blot analyses, samples were separated on 10% Bis-Tris gels, transferred onto
PVDF membranes, and incubated with mouse anti-Myc (Invitrogen) or anti-GFP (Sigma)
antibodies.

**RNA isolation and quantitative PCR.** To compare GPx1 expression levels in
mice and MRs, total liver RNA was isolated by TRIZOL extraction according to the
manufacturer’s instructions. Genomic DNA was removed using a DNA removal kit
(Ambion). RNA concentration was measured spectrophotometrically and cDNA was
obtained with Superscript III Reverse Transcriptase (Invitrogen) using the oligo dT primer.
Real time PCR was performed using a Fast SYBR Green Master Mix (Applied
Biosystems). Primer sequences for the GPx1 expression analysis were as follows: mouse,
5’-CAGGAGAATGGCAAGAATGAAG-3’ and 5’-GAAGGTAAAGAGCGGATGTG-3’;
and MR, 5’-GACACCAGGAAAACGCAAAG-3’ and AAGGTGAAGACGGATGTG (primers were based on the assembled transcriptome
sequences). GPx1 expression was normalized to that of aldolase. Primers for aldolase
expression were as follows: mouse, 5’-GTGATCTTTTCTACGAGACC-3’ and 5’-
ACCACAATTCCCTTCTCCTTG-3’; and MR, 5’-AAGATGGGTGTGACTTTGGG-3’ and 5’-GGTACTAGCCCATTCTGTGAC-3’.

**Additional analyses of mRNAs.** RNA was isolated from cells transfected with various GPx1 constructs inserted into pBudCE/GFP vector. It was then treated with RNAse free DNaseI (Fermentas). The resulting RNA samples were reverse transcribed with a SuperScript VILO cDNA synthesis kit (Invitrogen). Quantitative PCR was performed using a Fast SYBR Green Master Mix (Applied Biosystems). To distinguish recombinant GPx1 from the endogenous form, we used the direct primer specific for Myc-tag (5’-TCATCTCAGAGGAGGATCTC-3’) and reverse primers as follows: 5’-TGAGCCTTCTCACCATTCACTTCG-3’ (mouse) and 5’-CGAGCACTACCAGGCCTCTGG-3’ (MR). Expression of GPx1 mutants was normalized to EGFP expressed from the same vector. Primers for EGFP were as follows: 5’-TCAAGGACGACGGAACACTAC-3’ and 5’-TTGTGCCCCAGGATGTTGCC-3’.

**Protein isolation and activity assays.** Recombinant cysteine GPx1 mutants were expressed in BL21(DE3) cells (Novagen). *E. coli* cells were transformed with various GPx1 constructs and the cells grown until an OD of 0.6 at 600 nm. Protein expression was induced overnight with 1 mM IPTG. Cells were harvested by centrifugation, homogenized in 50 ml of PBS supplemented with Protease Inhibitor Cocktail EDTA-free (Roche) and sonicated on ice for 20 min. The suspension was centrifuged at 9,000 rpm and the resulting lysates were assayed for protein concentration and fractioned on TALON™ column (Clontech). Fractions containing GPx1 mutants were dialyzed against PBS and subjected to activity assays using a GPx kit (Sigma).
4.4 Results

**Low levels of Se in MR tissues.** We analyzed the levels and distribution of Se in various tissues of MR, *H. glaber*. Mouse tissues were used for comparison as mice are rodents of similar size. Paraffin-embedded tissue sections were first imaged using synchrotron XFM. Se was uniformly distributed in liver (Fig 17A), heart and lung tissues in both organisms. Mouse testes are known to accumulate Se in elongating spermatids [188]. However, such Se enrichment was not observed in MR testes (Fig 17B). Most MR tissues had considerably lower Se levels (30-75% lower). However, MR and mouse brain samples had similar Se contents. Brain is known to retain Se during deficiency [128].

To verify the XFM observation of low Se in MR tissues, we measured Se in MR and mouse tissues using ICP-MS. All MR tissues analyzed, except brain, had lower Se levels than the corresponding mouse tissues (Fig 17C). The major Se pool in mice is in the liver; however, MR liver had low amounts of Se. Thus, MR is characterized by apparent Se deficiency.

**Low GPx activity in MR tissues.** Since Se mostly occurs in the form of Sec in proteins, the low Se in MR tissues suggested a decreased expression of selenoproteins. To test this hypothesis, we assayed methionine sulfoxide reductase and GPx activities. Mammals have three MsrBs including the selenoprotein MsrB1, which is the major MsrB in mouse liver [16]. Since it is responsible for MsrB activity in the cytosol and nucleus (MsrB2 and MsrB3 are mitochondrial and endoplasmic reticulum proteins, respectively), it is likely the main MsrB in all mammals. MsrB1 expression and activity (as well as total MsrB activity) depend on dietary Se. Mammals also have a single MsrA, which is not a selenoprotein. We found that both MsrA and MsrB activities were similar in mice and
MRs (Fig 18A, B). Thus, low Se in MR tissues cannot be explained by uniform selenoprotein deficiency.

GPx1 is a major mammalian GPx, which is, like MsrB1, easily regulated by dietary Se. We found extremely low GPx activity in MR liver compared to the corresponding mouse tissue (Fig 18C). Low Se in the liver correlated with low GPx activity, which was suggestive of low GPx1 expression.

**Sequencing and analysis of the MR liver transcriptome.** Several rodent genomes were sequenced in recent years, but the genome of MR is not available. To characterize selenoprotein occurrence in MRs, we performed sequencing of the MR liver transcriptome using Illumina technology. Overall, 25,011,515 36-bp-long reads were obtained and assembled into contigs. The length distribution of assembled contigs is shown in Fig 19A. The best results were obtained using k-mer size of 23, wherein 395,038 contigs were generated, with 40,073 contigs longer than 100 bp, and 379 contigs exceeding 1,000 bp.

Occurrence of selenoprotein genes was then examined, and we detected 15 selenoprotein sequences. This analysis suggested that the MR selenoproteome is similar to that of other mammals. Schematic representation of the human selenoproteome (which contains 25 selenoproteins) is shown in Fig 19B, with MR orthologs identified in the current study highlighted. The lack of some selenoprotein genes was likely due to insufficient sequencing depth, but it also could be an indicator of their low expression in MR liver. Overall, even though Se levels and GPx activity were low in MR liver, this organ expressed many selenoprotein mRNAs, and the majority of these sequences had no unusual features.
SelP is the only mammalian selenoprotein that contains more than one Sec and it has two SECIS elements in the 3’-UTR. Mammalian SelPs generally have 10-15 Sec residues, e.g., 10 Sec in human, mouse and rat SelPs [38]. However, we found only 7 Sec residues in MR SelP (Fig 20). Together with SelP from *Cavia porcellus*, this is the lowest Sec content of any vertebrate SelP.

In addition, we detected an unusual feature in MR GPx1: an early stop codon present 5 codons upstream of the position terminating GPx1 synthesis in other mammals (Fig 19C, 21). Thus, MR GPx1 was predicted to be 5 amino acids shorter than other mammalian GPx1s. SECIS elements in MR selenoprotein genes satisfied the requirements of a canonical eukaryotic SECIS model. Overall, it is likely that the MR selenoproteome is not significantly different from that of other mammals, with the exception of SelP and GPx1 sequences.

**Low GPx1 expression in MR.** To test for GPx1 expression levels, we isolated mouse and MR splenocytes, metabolically labeled the cells with $^{75}$Se and they were analyzed by SDS-PAGE followed by autoradiography (Fig 18D). It appeared that the GPx1 band was missing in MR splenocytes. To test if low GPx1 expression and activity were due to a low mRNA expression, GPx1 mRNA levels were examined by real-time PCR. We found that the MR GPx1 mRNA was expressed at much lower levels than mouse GPx1 mRNA in the liver (Fig 18E). Thus, the low GPx1 activity was, at least in part, due to low GPx1 mRNA levels.

**Reduced expression of MR GPx1 in transfected mammalian cells.** An early stop codon in MR GPx1 could also contribute to the low expression and activity of GPx1. To test this possibility, we prepared constructs containing MR GPx1 with the early UAG stop
codon, and the one, where UAG was replaced with CAG (encoding glutamine present in mouse GPx1 at this position). As a control, we prepared mouse GPx1 and its mutant, in which the CAG codon was changed to UAG (Fig 22A). The constructs were transfected into HEK 293 cells, which were then labeled with $^{75}\text{Se}$ and analyzed for selenoprotein expression patterns (Fig 22C, upper panel). The same membrane was also subjected to western blot analysis with anti-Myc antibodies (Fig 22C). To distinguish from the endogenous GPx1, proteins were immunoprecipitated with anti-Myc antibodies and analyzed on a gel (Fig 22C, lower panel). Expression levels of MR GPx1 were much lower than that of mouse GPx1. Introduction of CAG in place of UAG further decreased MR GPx1 expression. At the same time, substitution of CAG with UAG in mouse GPx1 did not decrease GPx1 expression.

In addition, mouse and MR GPx1 forms were cloned into a pBudCE4.1 vector, containing GFP under CMV promoter. In further experiments, GFP was used as an internal control for transfection and protein loading. Inhibition of proteasome in HEK 293 cells did not rescue MR GPx1 expression (Fig 22D). In addition, real time PCR analysis revealed no difference between mouse and MR GPx1 mRNA after transfection (Fig 22B). Thus, MR GPx1 expression was suppressed regardless of the early stop codon. However, substitution of Sec with cysteine partially rescued expression of MR GPx1 (Fig 22E). These data suggest that incorporation of Sec may limit GPx1 synthesis. We also checked if the early stop codon in MR GPx1 affects peroxidase activity of this enzyme by expressing Cys-containing His-tagged MR and mouse GPx1s in bacteria. These mouse and MR GPx1 proteins had similar activities (Fig 23). These data suggest that the early stop codon neither affects expression nor activity of the enzyme, and that the low expression of MR GPx1 is
due to a combination of low mRNA levels and decreased Sec insertion. These two factors may be linked, e.g., inefficient Sec insertion may destabilize GPx1 mRNA.

**SECIS element in MR GPx1 is not responsible for low GPx1 expression.** Sec insertion includes recognition of the SECIS element in the 3’-UTR of selenoprotein mRNAs by SBP2 and subsequent recruitment of additional factors, such as EFSec and tRNA$^{Sec[Ser]}$, which insert Sec in response to the UGA codon. Efficiency of Sec incorporation may depend on additional features, such as the position of the UGA codon within the ORF and the type of SECIS element. Alignment of GPx1 SECIS elements revealed a nucleotide substitution in the conserved SBP2 binding site (Fig 24A). However, comparison of MR (Fig 24B) and mouse (Fig 24C) SECIS elements in GPx1 did not show unusual features in the MR SECIS element, suggesting that the predicted structure may support Sec incorporation.

To directly test if the MR GPx1 SECIS element is responsible for the low GPx1 expression, we cloned coding sequences of mouse and MR GPx1s into pSelExpress1 vector containing a highly efficient eukaryotic SECIS element [187]. Analysis of these expression constructs (containing the same SECIS element) by metabolic $^{75}$Se labeling of transfected HEK 293 cells showed that the MR GPx1 was still expressed at a low level compared to mouse GPx1 (Fig 24D). To further examine the role of SECIS elements as well as the entire 3’-UTRs in GPx1 expression, we prepared constructs that swapped the 3’-UTRs between mouse and MR GPx1s. These constructs were expressed in HEK 293 cells, followed by $^{75}$Se labeling and Western blot analysis (Fig 24E). Substitution of the MR GPx1 3’-UTR with the mouse one did not increase MR GPx1 expression; in addition, replacement of the mouse GPx1 3’-UTR with the MR one did not decrease the expression
of mouse GPx1. Thus, neither SECIS element nor the 3’-UTR were responsible for the reduced MR GPx1 expression. It is an attractive possibility that the overall GPx1 mRNA structure modulates the rate of Sec incorporation.

**Absence of GPx1 decreases Se levels in mice.** GPx1 is the most abundant selenoprotein in mouse liver and kidney. We tested if removal of this protein could affect Se levels in tissues by examining WT and GPx1 knockout mice. Se levels in GPx1 knockout mice were almost twice lower than those in WT livers (Fig 25) \( (p = 0.00387) \) and kidneys \( (p = 0.0409) \). However, in tissues characterized by lower GPx1 levels, such as spleen, heart, lung, and brain, Se levels were not affected by GPx1 knockout. Thus, the absence of GPx1 could indeed be responsible for the reduced Se use by MRs.

**GPx1 expression in long lived rodents.** To examine if the aberrant expression of MR GPx1 has evolutionary aspects, we analyzed its levels in primary skin fibroblasts derived from long-lived rodents, including white footed mouse, chipmunk, vole, single fox squirrel, beaver and porcupine, and also examined fibroblasts from another long-lived mammal, little brown bat [189]. Cells were metabolically labeled with \(^{75}\)Se, and proteins analyzed by SDS PAGE followed by autoradiography (Fig 26). HEK 293 cells were used as a positive control, and mouse embryonic fibroblasts derived from GPx1 knockout mice as a negative control. All analyzed species, except porcupine, had a strong band corresponding to GPx1. In the case of porcupine, we observed a weak \(^{75}\)Se signal in the GPx1 position and a stronger band that migrated faster than GPx1. Sequence analysis of GPx1 in these rodents should await the availability of their genome sequences. However, examination of the phylogenetic tree indicated that MR and porcupine share a common ancestor (Fig 26B), whereas other examined mammals are more distant. Thus, it appears
that the defect in GPx1 expression may not be limited to MR and may occur in a subset of rodents.

**4.5 Discussion**

GPx1 is an abundant glutathione peroxidase in mammals. It plays an important role in the protection of cells from oxidative stress by reducing hydrogen peroxide with glutathione. Surprisingly, we found that GPx1 is essentially absent in MR tissues, while other selenoproteins are expressed at normal levels and/or are enzymatically competent. Sequencing and analysis of the MR liver transcriptome detected GPx1 mRNA, but it was present at low levels and had an early termination codon. Further analyses revealed that the low expression was likely due to a combination of low mRNA levels and decreased insertion of Sec rather than due to compromised GPx1 activity caused by premature termination.

It was reported that GPx1 is the most abundant liver selenoprotein. In rat liver, it accounts for 63% of the total Se [190]. Se deficiency induces rapid degradation of GPx1 mRNA (and several other selenoprotein mRNAs), while the expression levels of several additional selenoproteins are preserved under these conditions [191]. GPx1 was proposed as a biomarker of Se utilization, since its activity is very sensitive to Se dietary status. It was also suggested that GPx1 is a “Se buffer”, wherein this enzyme stores Se and provides this element for biosynthesis of selenoproteins during Se deficiency [192]. On the other hand, GPx1 knockout does not affect expression of other selenoproteins [78, 193]. This enzyme is also not essential: GPx1 knockout animals are viable; however, they are more susceptible to oxidative stress and show defects in redox signaling [194]. These data correlate with previously reported increases in oxidative stress parameters in MR,
including a low GSH/GSSG ratio, high rate of DNA oxidative damage, and increased protein carbonylation and lipid peroxidation [174]. Moreover, both GPx1 knockout mice and MRs showed abnormal blood glucose regulation [78, 193]. These mice had a normal fasting glucose, but reduced blood insulin levels [194, 195]. However, unlike GPx1 knockout mice, MR also showed abnormal glucose tolerance (glucose levels remained elevated for prolonged periods of time) and insulin sensitivity (glucose levels immediately decreased upon insulin administration and remained low for prolonged periods of time) [176, 196]. In addition, GPx1 knockout mice had reduced islet β-cell mass in pancreatic tissue [195], while MR had different abundances of the four distinct types of islet cells. Even though the abnormal glucose regulation may be relevant to MR adaptation to its specific lifestyle, it could be, at least in part, due to an abnormal GPx1 function.

Se levels in animal tissues vary depending on many factors, including the requirements of the organism for this trace element and the diet. Numerous studies reported on the role of dietary Se in human health. For example, Se deficiency may cause endemic cardiomyopathy (Keshan disease), liver degeneration observed in rats and pigs, and white muscle disease in ruminants and turkeys [184, 197]. None of the Se deficiency-induced pathologies have been reported for MR. In this regard, it is particularly interesting that MRs have never been observed to develop cancer [166]. We compared trace element profiles in control and GPx1 knockout mice, and found a significant decrease of tissue Se levels in liver and kidney. Somewhat similar differences in Se levels were observed between mouse and MR. GPx1 is the most abundant selenoprotein, especially in liver and kidney, and the GPx1 knockout data suggest that this protein accounts for approximately half of Se in these organs.
We also observed significant differences in Se in spleen, lungs and testes (these tissues depend on Se provided by SelP). This selenoprotein is the only mammalian protein that contains multiple Sec residues [38]. Previously, we suggested that the number of Sec residues in SelP might be used as an indirect genetic marker of Se utilization [198]. In rodents and primates, the Sec content of SelP is almost twice as low as in aquatic vertebrates. Comparison of rodent SelPs shows that C. porcellus and MR SelPs have 7 Ser residues, which is the lowest number of SelP Sec residues in vertebrates. MR, porcupine and C. porcellus are closer to each other and are more distant to other rodents, such as mice, rats and rabbits [199]. Our analysis of GPx1 expression suggested a decreased GPx1 expression in MR and porcupine, and previous research revealed low GPx1 activity in C. porcellus [200]. Overall, these data suggest a possible loss of GPx1 function in this specific group of rodents and, as a consequence, a reduced requirement for Se by these organisms. It is remarkable that such an important selenoprotein is compromised in these animals, which survived for tens of millions of years essentially without GPx1.
Figure 17. XFM and ICP-MS analysis of Se in mouse and MR tissues. XFM scans of mouse and MR livers (A) and testes (B). Each element and its maximum and minimum threshold values are given above each image in ng/cm$^2$. The rainbow-colored scale bar relates to the signal intensity measured as ng/cm$^2$ in each spot with dark pixels representing areas of low concentration and a gradient to bright pixels depicting increasing concentrations. A scale bar is shown below the elemental maps. (C) Se was analyzed by ICP-MS in mouse and MR tissues. Values are means +/- SD. Organs in which trace elements were analyzed are shown below each panel (*$p<0.05$).
Figure 18. MR tissue extracts have low GPx1 expression and activity. Total MsrB (A), MsrA (B) and GPx (C) activities were measured in indicated mouse and MR tissues. (D) Primary splenocytes derived from MR and mice were metabolically labeled
with $^{75}$Se and protein extracts were analyzed by SDS-PAGE followed by autoradiography. The band corresponding to GPx1 is shown with an arrow on the right. (E) Relative expression of GPx1 mRNA in MR and mouse liver (* p<0.05).
Figure 19. Transcriptome analysis and characterization of the MR selenoproteome. (A) Length distribution of assembled contigs of the MR liver transcriptome. (B) Selenoproteins identified in MR. The human selenoproteome is used as a reference to represent the MR selenoproteome. Selenoproteins identified by sequencing the MR liver transcriptome are highlighted. Location of Sec residues is indicated by a red line. Seven Sec residues were found in SelP. GPx1 has an early termination codon (see text for details). (C) Schematic representation of MR GPx1 in comparison with mouse GPx1.
Figure 20. Protein sequence alignment of SelP. The following sequences were used for alignment construction (Genbank accession numbers): *Homo sapiens* (AAH58919.1); *Canis familiaris* (NP_001108590.1); *Sus scrofa* (NP_001128295.1); *Mus musculus* (AAH01991.2); and *Rattus norvegicus* (NP_062065.2). Selenocysteine residues are highlighted in red, and cysteines in the positions corresponding to selenocysteine are...
shown in blue.

Figure 21. Protein sequence alignment of GPx1. The following sequences were used for alignment construction (Genbank accession numbers): *Homo sapiens* (NP_000572.2); *Sus scrofa* (NP_999366.1); *Canis familiaris* (NP_001108591); *Rattus norvegicus* (AAB95647.2), *Mus musculus* (CAA27558) and *Cavia porcellus* (AAKN02012894.1). The selenocysteine residue is highlighted in red and indicated by an asterisk. The early stop codon in the MR sequence is also highlighted in red.
Figure 22. GPx1 is poorly expressed in mammalian cells. (A) Schematic representation of MR and mouse GPx1 mutant constructs. Positions of Sec and glutamine (CAG) codons, the stop signal (TAG) and the SECIS element in the 3′-UTR are shown. (B) mRNA levels were assessed by real time PCR and normalized to GFP expressed from the same vector. Results are given +/- SD. (C) MR and mouse Myc-GPx1 constructs were transfected into HEK 293 cells. Cells were labeled with $^{75}$Se followed by SDS-PAGE and autoradiography (upper panel). Migration of ectopically expressed GPx1 is shown on the left. Same lysates were probed with anti-Myc and anti-β-actin antibodies (two bottom panels). Myc-GPx1 mutants were also immunoprecipitated with anti-Myc antibodies, followed by PhosphorImager analysis of the $^{75}$Se-labeled GPx1. (D) Mouse and MR constructs were transfected into HEK 293 cells followed by treatment of cells with 10 µM MG132 for 12 h. Cells were labeled with $^{75}$Se followed by protein analysis by SDS-PAGE
and autoradiography (upper panel). The same membrane was stained with Myc and GFP antibodies. (E) Expression of mouse and MR cysteine mutants of GPx1 in HEK 293 cells was analyzed by Western blotting. IP, immunoprecipitation. WB, western blotting.
Figure 23. Expression of mouse and MR GPx1 in *E. coli*. Cysteine mutants of MR and mouse GPx1 were cloned into pET28a and expressed in *E. coli*. (A) Proteins were isolated from identical amounts of bacterial culture and aliquots were analyzed by SDS-PAGE. (B) Glutathione peroxidase activity of MR and mouse GPx1 was measured and normalized to the amount of protein in each sample. Results are given +/- SD.
Figure 24. SECIS element does not decrease GPx1 expression level. (A) Alignment of mammalian GPx1 SECIS elements. Nucleotides critical for the SECIS function are underlined. (B) MR GPx1 SECIS element as predicted by SECISearch. Functional sites are shown in bold. (24). (C) Mouse GPx1 SECIS element. Functional sites are shown in bold. (D) Mouse and MR GPx1 coding sequences were cloned into pSelExpress1 containing an efficient eukaryotic SECIS element and expressed in HEK 293 cells. Cells were labeled with $^{75}$Se followed by SDS-PAGE, autoradiography and Western blotting with Myc antibodies. Coomassie staining is a loading control. (E) Coding sequences of mouse GPx1 containing the MR GPx1 3’-UTR (M-long-MR SECIS) and coding sequence of MR GPx1 containing mouse GPx1 3’-UTR (MR-short-M SECIS) were
expressed in HEK 293 cells. Cells were labeled with $^{75}\text{Se}$ followed by SDS-PAGE, autoradiography and Western blotting with Myc and GFP antibodies.

Figure 25. Reduction in Se levels in tissues of GPx1 KO mice. Se was determined by ICP-MS in tissues from wild type (WT) and GPx1 knockout (KO) mice. Values are means +/- SD. Organs in which Se was analyzed are shown below each panel (** p<0.005, *p<0.05).
Figure 26. Expression of GPx1 in skin fibroblasts of rodents and long lived mammals. (A) Cells were labeled with $^{75}$Se followed by SDS-PAGE and autoradiography. HEK 293 cells and MEFs derived from GPx1 KO mice were used as positive and negative controls, respectively. Migration of the band corresponding to GPx1 is shown with an arrow. (B) Schematic representation of the phylogenetic tree of rodents (adapted from [199]).
CHAPTER 5

Conclusions and future directions
5.1 Roles of trace elements in regulation of the gastrointestinal microbiome

Our results indicate the importance of the trace element Se in regulation of the gastrointestinal microflora. Dietary Se increased diversity of microflora and differentially affected various groups of microorganisms. These effects were more pronounced in conventionalized mice, which might be explained by the fact that the contribution of the pre-existing microflora was removed. The observed differential sensitivity to dietary Se might be due to differences in Se utilization pathways among microorganisms [201]. On the other hand, our study demonstrates that the intestinal microflora is an essential intermediate between dietary Se and the host selenoproteome. This observation leads to several questions that might be addressed in future studies. First, intestinal microflora plays an important role in protecting a rapidly renewing intestinal epithelium from various pathological processes, such as through induction of Toll like receptor (TLR) mediated signaling. Induction of this pathway is critical in protecting intestinal cells from apoptosis, which might be induced by exogenous injury or pathogenic infection [202]. At the same time, GPx1 and GPx2 play a role in protecting intestinal cells from oxidative stress and their removal is associated with severe pathology [203, 204]. Thus, a better understanding of Se distribution between the host and microbiota may explain the beneficial role Se supplementation under certain conditions. Second, it would be important to follow the effect of Se-modified intestinal microflora on health parameters, which depends on intestinal microflora, such as susceptibility to IBD, various intestinal infections, food digestion and obesity. These experiments might be carried out using similar models we applied in our study. Intestinal microflora in GF mice can be reestablished by intestinal
extracts obtained from Se-deficient and Se sufficient mice. Finally, the observed effects might not be limited just for Se. Other trace elements, such as Cu, Fe, Mn and Zn may also play an important role in regulation of the intestinal microflora, and it might be an interesting field in nutritional studies. Se has been the first trace element for which the role of microbiota has been addressed, and these studies should be extended to other trace elements.

5.2 Function of Sep15 in cataract formation

In our study, we characterized Sep15 KO mice and found them to consistently develop nuclear cataracts. However, the underlying mechanism is still unclear. In addition, Se-deficient mice (that are maintained on the Se deficient diet for one year) were not found to develop cataracts (unpublished results). Sep15 participates in the glycoprotein folding cycle, and Sep15 mRNA is enriched during lens development. One of possible explanations of the nuclear cataracts, observed in the Sep15 KO mice, is the improper folding of Sep15 targets, which may play a role in maintenance of lens integrity and transparence. The next step in our understanding of Sep15 function in cataract formation will be the identification of Sep15 targets in lens epithelial cells. To identify Sep15 targets, we will apply in vivo target search model, since Sep15 is characterized by thioredoxin-like fold and has a redox motifs, UxC (U is Sec). We will take advantage of the previously developed reaction mechanism of this class of enzymes. One of Cys in the redox motif serves as a nucleophilic attacking group, leading to the formation of a covalently-linked intermediate. The other Cys has a resolving function: it releases the reduced product and forms an intramolecule disulfide. A standard approach to trap the substrates of thiol
oxidoreductases is to mutate the resolving Cys to serine, that would allow a formation of the intermolecular intermediate, but would prevent substrate release (Fig 27). We will employ this approach to trap Sep15 targets and further sequence them by mass spectrometry. Introduction of FLAG and HA tags on N- and C-termini will allow us to perform large scale purification using FLAG/HA tandem purification kit, designed by Sigma, i.e., I.P for one tag followed by Western blot identification with antibodies for another tag. These mutants will be prepared in a vector that allows efficient expression in mammalian cells. Previously, a similar approach was applied for Drosophila Sep15, which contains a Cys in place of Sec. When such redox-inactive mutants were purified from insect cells and immobilized on the column, no targets were identified. A possible explanation is that, for proper function, Sep15 needs to be localized in the ER and/or interact with UDP glucose:glycoprotein glucosyltransferase. To examine the interactions under more natural conditions, we will express Sep15 (Fig 27B) in lens epithelial cells. The Sep15 mutant proteins will contain the HA tag upstream of the ER-targeting signal and the FLAG tag at the C-terminus. In SelM (homolog of Sep15) mutants, the FLAG tag will be inserted upstream of the ER retention signal. Then, we will perform immunoprecipitation with anti-HA antibodies. Samples will be applied to SDS-PAGE under non-reducing conditions. This will allow us to identify mixed disulfides by Western blotting with anti-FLAG antibodies. Later, the I.P. samples will be reduced with 10 mM DTT, separated by SDS-PAGE, stained with Coomassie blue, and subjected to mass spectrometry analyses. Targets identified during this procedure will be verified in subsequent in vitro experiments, and the interactions will be confirmed in a cell culture system by co-immunoprecipitation. Both Cys- and Sec-containing versions of
selenoproteins will be used. These proteins will be co-transfected into mammalian cells, selenoproteins will be immunoprecipitated with antibodies against the tag, and the samples will be probed with antibodies against the tag in the target protein, and, if available, against the target protein itself.
Figure 27. Experimental design for identification of substrates of Sep15. (A) The active site of the thioredoxin-like fold protein Sep15 includes a CxU motif. In the CxU motif, one residue (Sec) is the attacking residue, whereas the other residue (Cys) likely completes the reaction by resolving the intermolecular disulfide. (B) The mutants of Sep15 designed for the in vivo target search experiment.
5.3 Reduced utilization of Se by long lived animals

In our study, we found low levels of Se in MR tissues. Such reduced utilization of Se can be explained by the low levels of the major mammalian selenoprotein, GPx1. However, the exact reason for such dramatic reduction in expression is still unclear. We examined several hypotheses, including the role of the premature stop codon and an atypical SECIS structure; however, these factors did not explain the observed low expression of GPx1. Interestingly, substitution of Sec with Cys rescued GPx1 expression, by increasing the rate of translation. One additional hypothesis is associated with the presence of rare codons in the sequence of the MR GPx1. The analysis of GPx1 expression in long lived mammals, demonstrated the low expression of GPx1 in the porcupine. Porcupine, MR and guinea pig are in the same branch of the evolutionary tree. Thus, analysis of expression of porcupine and guinea pig GPx1 may be predicted to be low as well. If so, comparison of the sequences of MR, porcupine and guinea pig GPx1 can reveal common features (e.g., rare codons), that drastically decrease protein expression. Such studies may help identify novel mechanisms of regulation of protein expression at the translational level.
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