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Editors

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Its Molecular Biology and Role in Human Health

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Dedication

This book is dedicated to the pioneers in the selenium field, who provided the foundation for the enormous amount of research that has occurred in recent years, bringing extraordinary insights to selenium biology and its role in health and development of humans and other mammals. The discoverer of selenium, Jöns Jacob Berzelius, a Swedish chemist, deserves special recognition as he identified this element in 1817 as a by-product of sulfuric acid production. As we are now on the verge of the 200th anniversary of the Berzelius discovery, this new edition is particularly timely. The anniversary will also be the prevailing theme of *The 11th International Symposium on Selenium in Biology and Medicine* and *The 5th International Conference on Selenium in the Environment and Human Health*, to be held in Stockholm, Sweden, in August 2017.

One of the early studies that impacted how selenium was viewed in mammalian health was carried out by Kurt Franke. He reported in 1934 that diseases found primarily in livestock and military horses, called alkali disease and blind staggers disease, were caused by these animals ingesting selenium-accumulating plants. These seleniferous plants grow in the Midwest United States in areas where the soil is rich in selenium and the livestock and military horses had grazed [1]. This was an important finding as it demonstrated that high levels of selenium were toxic to animals. An early study that began to change how selenium was viewed in mammalian health occurred 20 years later than the Franke endeavor, when Jane Pinsent showed that selenium is required for formate dehydrogenase synthesis in *Escherichia coli* [2].

Klaus Schwarz and Calvin Foltz subsequently changed the image of selenium forever in 1957, when they demonstrated that selenium protected rats against liver necrosis [3]; and selenium was soon recognized as an essential trace element in mammals. The impact that Schwarz's and Foltz's discovery had on the livestock industry followed shortly after their report and was monumental in that many disorders described in farm animals were recognized as being related to selenium deficiency, such as exudative diathesis and pancreatic degeneration in poultry,

hepatosis dietetica in swine, and white muscle disease in lambs and calves (reviewed in [4]). In fact, supplementing the diets of livestock globally with selenium was reported in 1986 to have saved the livestock industry in the hundreds of millions of dollars [5], which would equate to multi-billions of dollars by today's financial standards. The pioneer work of J.E. Oldfield on white muscle disease in lambs in 1957 provided much of the foundation for the impact of selenium on the livestock industry (described in [4]; and see footnote in [4] regarding James Oldfield's recent death).

Another discovery that occurred early in the developing selenium field was the identification in 1973 of selenium as a covalently bound component of glutathione peroxidase 1 by Leopold Flohé [6] and J.T. Rotruck [7]. The same year, Thressa Stadtman found selenium in Protein A of glycine reductase [8], and Jan Andreesen showed that the reason why formate dehydrogenase synthesis required selenium was that the protein itself incorporated the element [9]. Subsequently, in 1976, Thressa Stadtman showed that the selenium-containing component of selenoproteins was selenocysteine (Sec) [10]. In addition, Chambers et al. [11] and Zinoni et al. [12] demonstrated in 1986 that TGA in the genes of mouse glutathione peroxidase 1 and bacterial formate dehydrogenase, respectively, coincided with the selenocysteine residue in the resulting gene products. These latter studies provided the initial evidence that the genetic code contained 21 amino acids, and the code would need to be expanded. The further elegant work on the genetics of selenocysteine biosynthesis and selenocysteine incorporation into protein in bacteria, primarily by August Böck [13], laid the conceptual framework and was instrumental for later delineating these processes in archaea and eukaryotes. We refer the interested reader to a comprehensive review which encompasses far more details on the early years of selenium research [14].

These and the many other highly significant discoveries from 1817 through the twentieth century have provided the foundation for recognition of how selenium may act at the molecular level. The significant and intricate impact of selenium in many biological processes clearly identifies research on selenium and selenocysteine, the 21st proteinogenic amino acid, as highly important. As the 200th anniversary of the discovery of selenium approaches, we gratefully dedicate this book to the pioneers of selenium research.

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[1] We sadly note that our colleague, dear friend and a true pioneer in the selenium field, James E. Oldfield, died on April 3, 2016.

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Elias S.J. Arnér, Organizer of *Se2017*, *The 11th International Symposium on Selenium in Biology and Medicine* and *5th International Conference on Selenium in the Environment and Human Health*, Stockholm, Sweden, August 13–17, 2017

Foreword

Selenium is a basic element found in the same chalcogen group of the periodic system as oxygen, sulfur, and tellurium. "*What is more, it is in this regard, midway between sulfur and tellurium, and has almost more characters of sulfur than of tellurium*," as its discoverer Jöns Jacob Berzelius cleverly concluded after his very first experiences, in the year 1817, with this element [1] (the author's translation from Swedish). That was indeed an impressively correct description of the chemical character of selenium. Today, as we approach the 200th anniversary of Berzelius' discovery of selenium, knowledge about its functions in biology is extensive. Research for the last couple of decades has revealed how selenium is intricately linked to a wide variety of important reactions in redox biochemistry, with selenoproteins employing the unique chemistry of selenium in the form of selenocysteine, which in turn is co-translationally inserted by highly complex synthesis mechanisms that, in fact, redefine the genetic code. We also know that low molecular weight selenium compounds and metabolites can, just as selenoproteins, have either health-promoting or toxic effects to cells and organisms, with the final outcome depending upon the entity of compounds, their concentrations, and the context of exposure. Conversely, deficiency of selenium can also give rise to disease in humans and other mammals, since in most organisms that express selenoproteins, at least one selenoprotein is likely to be essential. However, it is not fully understood how many other organisms, such as plants and many classes of microorganisms, can survive without selenoproteins and, conversely, why many of those organisms that have selenoproteins seem to depend upon them. Indeed, many questions have remained unanswered and there are many aspects in selenium biology, and in the molecular interactions between selenium, sulfur, and oxygen, that are as thought provoking as they must have been in the very early days of Berzelius.

In this fourth edition of the book *Selenium: Its Molecular Biology and Role in Human Health*, edited by four very well-known scientists in selenoprotein research, Dolph Hatfield, Ulrich Schweizer, Petra Tsuji, and Vadim Gladyshev, 50 chapters introduce and discuss the very rapid development in recent years of research on selenium and selenoproteins. These chapters are organized in five thematic parts. In the first part, the intricate molecular details of selenoprotein synthesis are introduced

and outstanding questions posted. What were the evolutionary mechanisms that led to an alternative usage of the stop codon UGA as a sense codon for selenocysteine, utilizing secondary structures in the mRNA as the decoding signal? What molecular mechanisms yield specificity and efficiency in this decoding process, and exactly how specific or efficient is selenoprotein synthesis? What are the common denominators in selenoprotein synthesis among different classes or kingdoms of life and what are the differences? How is selenocysteine itself synthesized and degraded, and how is the metabolism of selenium-containing compounds, which is highly complex, integrated into a biological context? What are the absolute chemical differences between selenocysteine and cysteine and what biological significance do such differences play? These types of fundamental questions are still mainly unanswered, but knowledge about these topics has nonetheless increased very rapidly in recent years, which is illustrated and covered by the ten chapters in Part I.

Part II of this book focuses on biological roles of selenoproteins with confirmed activities, and also some with yet only proposed or unknown functions. What selenoproteins are expressed in different organisms, how are their expression patterns regulated, and what are their functions, in the forms of either isolated proteins or in a biological context? Can a better understanding of the function of individual selenoproteins contribute to knowledge of the pathogenesis of diseases? If so, can such knowledge form the basis for novel therapy, employing specific targeting of individual selenoproteins? Some selenoproteins have been more studied than others, but for all selenoproteins, there are still many questions left unanswered and the 14 chapters in Part II focus on these topics and the recent developments in this field.

Many epidemiological studies have revealed that selenium intake indeed has a direct effect on human health and disease, but a full, or perhaps even only a partial and superficial, understanding of the casual links in this relation is still missing. As an overly high selenium intake may give rise to toxic effects, while deficiency also leads to disease, it is intriguing that the nutritional intake span for selenium is so narrow that even small variations in food contents of selenium and its long-term intake may possibly be linked to disease. In Part III of this book, 11 chapters discuss these fascinating aspects of selenium intake in relation to human health.

Although biological effects of selenium may be derived from actions of both low molecular weight metabolites and selenoproteins, it is clear that selenoproteins carry out many important functions in humans that may be important in maintaining fitness and health. Thus, several of the epidemiologically observed links between selenium intake levels and disease that are discussed in Part III are likely to be linked to effects on selenoprotein function; and perhaps more so in the case of selenium deficiency that may lead to insufficient expression and function of one or several selenoproteins. Importantly, like for proteins in general, selenoprotein function is tightly linked to control of expression and that, in turn, will be affected not only by selenium availability but also by the integrity of selenoprotein-encoding genes and their transcription and translation machineries. As these genes and factors may be affected by mutations and other gene alterations, selenoprotein function can also be modulated by genetic aberrations. In Part IV, these aspects of selenium and selenoprotein function are further discussed in ten chapters, focusing upon the modulation of the expression patterns and functions of specific selenoproteins in relation to disease.

In the final Part V, five chapters discuss different animal models for studies of selenium and selenoprotein function. These models include deliberate genetic targeting of specific selenoproteins, or factors required for selenoprotein synthesis, in mice as well as unique selenium usage or the effects of selenium, in particular, in other animals, such as the naked mole rat or in livestock. By observations and conclusions from these animal models, important insights into selenium functions in biology at large are clearly gained.

As is powerfully illustrated by the 50 up-to-date chapters in this book, selenium research has made fantastic progress since the initial discovery of selenium by Berzelius, with an almost exponential growth in knowledge in recent years. Today, more than 30,000 articles can be found in PubMed when searching for "selenium or selenocysteine or selenoprotein." Nonetheless, it is painfully obvious how much is still unknown in this research field, with crucial questions clearly remaining to be answered. How can so many classes of organisms live without any selenoprotein, when selenoproteins seem to be essential for life in mammals? What are the functions and biological roles for all those selenoproteins in nature that have thus far only been observed, but not yet studied at any depth? What exact mechanisms can be determined to explain the links between selenium status and disease? How can any causal links between aberrations in selenium biology and disease be exploited for novel and efficient therapy? The chapters in this book provide a most solid foundation as a starting point for the continued research on these questions and topics. As we are now approaching the 200th anniversary of Berzelius' discovery of selenium, the largest-ever international selenium conference is organized in Stockholm on August 13–17, 2017 (see www.Se2017.se), where a majority of the scientists in selenium research are expected to meet. Also at that conference, this book serves to provide a solid foundation for discussions and presentations, with its comprehensive up-to-date coverage of the molecular biology of selenium and the links of this element to human and animal health. With selenium research being more vibrant than ever, we can look forward to an exciting development of this topic, and we hope that the results of all ongoing selenium studies may lead to better measures for improved human health. To facilitate such a goal, it is recommended that the scientists active and interested in selenium research read this book, which will help to give all parties a common platform of informed knowledge in the field. The editors of this book are cordially thanked for taking the significant efforts of compiling and editing its many chapters, and every scientist in the selenium research area is thankfully acknowledged for the continuous efforts to further expand the boundaries in selenium knowledge.

Stockholm, Sweden Elias S.J. Arnér

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Preface

The first edition of *Selenium: Its Molecular Biology and Role in Human Health* was published in 2001 and largely covered the research in the selenium field for the preceding 30 years with special emphasis on "new and surprising insights into biochemical, molecular and genetic aspects of this fascinating element." The book contained 25 chapters with 46 contributors. The selenium field expanded in the ensuing years, which was reflected in the second edition, published in 2006, containing 35 chapters with 71 contributors, and the third edition, published in 2012, which contained 45 chapters with 96 contributors. Marla Berry played a major role in the success of the second and third editions, which she coedited. In the present edition, Ulrich Schweizer and Petra (Peko) Tsuji are serving as coeditors.

The fourth edition of *Selenium: Its Molecular Biology and Role in Human Health*, like the previous ones, reflects the patterns of growth and diversity in the selenium field. The current edition contains 50 chapters and has 119 contributors. Some of the principal areas in the selenium field, which flourished the most during the last 5 years, are the continued in-depth analyses of functions and regulation of selenoproteins, primarily in human health and disease, and in particular, cancer. While many publications emphasize selenium as having chemopreventive activity, it has become apparent in the last few years that this element also has a role in promoting cancer, and this pattern most likely applies to other chronic disorders. In retrospect, selenium's role in driving malignancies is not surprising, since unhealthy cells, and specifically cancer cells, require potent, robust systems that maintain their redox homeostasis and support rapid growth. Selenoproteins manifesting a "Dr. Jekyll and Mr. Hyde personality" in both preventing and promoting cancer, and the interplay of different oxidoreductase systems, are discussed in various chapters.

Interestingly, glutathione peroxidase 4 has recently been shown to have roles in cancer progression and ferroptosis, a form of non-apoptotic cell death, whereas research on thioredoxin reductase has highlighted the ever increasing roles of this important selenoenzyme in redox biology. It is also becoming clear that selenium-dependent deiodinases not only contribute to circulating thyroid hormone homeostasis, but their local regulation bestows on them key roles in organ development and stem cell biology, and thus affects wound healing and cancer progression.

Modern genetics has also played a major role in selenium research. Mouse models were instrumental in identifying physiological functions of selenoproteins. Currently, all but six selenoproteins have been individually inactivated in mice and interesting phenotypes have been discovered in many of the mice lacking a specific selenoprotein-encoding gene. In parallel, several inherited diseases affecting selenoproteins or their biosynthesis have been identified in humans, and the severity of the symptoms highlights the roles that selenoproteins play in human health. Many polymorphic forms detected in the genes of several selenoproteins have been reported to have different consequences on the corresponding selenoprotein function, including a strong association with disease.

Some important areas in the selenium field have largely been solved and/or have progressed slowly, but they have previously provided a wealth of information. A number of these areas, therefore, have also been included in the book to serve as a source for those readers not working specifically in the selenium field. In addition, inclusion of these findings makes this edition as complete as possible in representing most aspects of the selenium biology field.

Due to the discovery of all selenoprotein genes in mammals and elucidation of the roles of the resulting selenoproteins in cellular metabolism, health, and development, much of the selenium field has focused on selenoproteins in the last 15 years as reflected in the present and previous editions. Thus, the debate that existed in the selenium field at the beginning of this century, i.e., whether small molecular weight selenocompounds or selenoproteins were largely responsible for the many health benefits attributed to selenium, shifted the pendulum largely to the side of selenoproteins as the responsible benefactors. However, we anticipate that small molecular weight selenocompounds will, once again, come much more into focus reflecting selenium toxicity.

The current edition, which covers so many aspects of the selenium field by different investigators, naturally has insights and opinions that occasionally are at variance with each other. We consider these dissimilarities an asset to the reader as they illustrate how different investigators approach these issues and provide a better overall view of current research in the selenium field.

We were informed by Springer that the third edition of this book was in the top 25 % of all e-books published in 2014 with regard to copies acquired, views, and downloads. We hope the new edition with its further expanded scope will be as well received by the readers. It is an exciting time to be in the selenium field and contribute to it; and we look forward to what the future brings with regard to new discoveries involving this element, selenoproteins, and their roles in health and development.

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We also acknowledge our spouses Mary J. Wilson (DLH), Stefanie Schweizer (US), Lee Barnhill (PAT), and Helen Gladysheva (VNG) for their patience and support in the many hours taken away from them in our preparing the fourth edition of this book.

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Part I
The Machinery of Selenoprotein Biosynthesis

Selenium is incorporated into protein through the use of highly specialized, evolutionary conserved machinery. The chapters in this section focus on the molecular components involved in biosynthesis of the 21st proteinogenic amino acid, selenocysteine, and its incorporation into protein.

Chapter 1
Selenocysteine tRNA$^{[Ser]Sec}$: From Nonsense Suppressor tRNA to the Quintessential Constituent in Selenoprotein Biosynthesis

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Abstract When selenocysteine (Sec) tRNA$^{[Ser]Sec}$ was originally discovered, it was proposed to be the first nonsense suppressor tRNA found in mammalian and avian tissues, since it exclusively decoded the nonsense codon, UGA, which normally dictates the cessation of protein synthesis. This tRNA was subsequently shown to be Sec tRNA, which inserted Sec into protein as the 21st proteinogenic amino acid. Once it was established that this tRNA was aminoacylated with serine by seryl-tRNA synthetase and served as the scaffold for Sec synthesis, Sec tRNA was appropriately named Sec tRNA$^{[Ser]Sec}$. The mammalian Sec-tRNA$^{[Ser]Sec}$ population consists

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of two isoforms that differ from each other by a single 2′-*O*-methyl moiety on the uridine at position 34, designated Um34. The non-Um34 isoform is involved in the synthesis of a subclass of selenoproteins, called housekeeping selenoproteins, while the Um34 isoform supports synthesis of stress-related selenoproteins. These novel functions and other unique features of Sec tRNA are the subjects of this chapter, supporting the idea that this tRNA is the quintessential constituent in selenoprotein biosynthesis.

Keywords Housekeeping selenoproteins • Selenocysteine • Selenocysteine tRNA • Selenoprotein biosynthesis • Selenoproteins • Stress-related selenoproteins • UGA codon

1.1 Introduction

Selenocysteine (Sec) tRNA was discovered in 1970 when a seryl-tRNA was found to form phosphoseryl-tRNA [1] and decode exclusively the UGA codon [2] in mammalian and avian livers. Subsequently, phosphoseryl-tRNA and the UGA-decoding seryl-tRNA were found to be the same tRNA [3]. However, since the UGA-decoding seryl-tRNA suppressed the UGA termination codon in rabbit β-globin mRNA [3], its earlier proposal as a nonsense suppressor tRNA was retained until the biosynthesis of Sec was shown to occur on this tRNA in prokaryotes and eukaryotes identifying it as Sec-tRNA$^{[Ser]Sec}$ [4, 5]. From its discovery in 1970 until the finding that it is an indispensable component in selenoprotein biosynthesis, many unusual features of this tRNA were reported. Two essential cellular functions of Sec tRNA are to synthesize Sec, the 21st amino acid in the genetic code, using tRNA$^{[Ser]Sec}$ as the scaffold upon which the synthesis occurs (discussed in Chap. 4) and donate Sec to protein in response to UGA Sec codons in selenoprotein mRNAs (discussed in Chaps. 2 and 5). Since the synthesis of selenoproteins is dependent on Sec tRNA$^{[Ser]Sec}$, manipulation of the expression of Sec tRNA has been used to modulate selenoprotein expression in assessing the role of selenoproteins in health and development (discussed in Chap. 46).

1.2 Primary and Secondary Structures of Sec tRNA

Since its discovery as a seryl-tRNA [1, 2], Sec tRNA was shown to occur in very small amounts compared to the corresponding total seryl-tRNA population in all tissues and cells where it was detected (reviewed in [6]). This tRNA existed in two isoforms [7], which differed by a single 2′-*O*-methylribosyl at position 34, designated Um34. The highly modified base at position 34 is 5-methoxycarbonylmethyluridine

(mcm^{5}U), while the nucleoside at this position is 5-methoxycarbonylmethyl-2′-*O*-methyluridine (mcm^{5}Um). Interestingly, the mammalian isoforms are 90 nucleotides long making them the longest tRNAs sequenced in higher vertebrates. Another unique feature about these tRNAs is that they have relatively few modified bases, and thus, are highly undermodified compared to all other known tRNAs that normally contain 15–17 amended bases.

The secondary structures of the two isoforms are shown in Fig. 1.1 in a clover leaf model along with the four modified bases, mcm^{5}U, *N*6-isopentenyladenosine (i^{6}A), pseudouridine (ψ), and 1-methyladenosine (m^{1}A) at positions 34, 37, 55, and 58, respectively [7]. The nucleoside, mcm^{5}Um, is also shown. Unlike any other known tRNA, Sec tRNA exists in a novel clover leaf form having 13 bases in the acceptor and TψC stems compared to 12 in other tRNAs and the acceptor stem contains nine bases, while the TψC stem contains four. Thus, the tRNA resides in a 9/4 cloverleaf form compared to a 7/5 form in other tRNAs [8, 9]. The D-stem of Sec tRNA has more base pairs, five to six, than all other tRNAs, which have three to four. Furthermore, Sec tRNA does not have the dihydrouracil base found in the D-loop in other tRNAs. The long variable arm and the extra base in the acceptor/TψC stems account for the bases that make this tRNA much longer than canonical tRNAs. When comparing Sec tRNA to all other tRNAs, it is indeed the most unique adaptor RNA described to date. These features account for the inability of elongation factors TU or 1alpha to bind tRNA[Ser]Sec and the requirement instead for dedicated elongation factors SelB and EF-SEC in bacteria and eukaryotes, respectively.

Sec tRNA is rightfully named Sec tRNA[Ser]Sec, since it is initially aminoacylated with serine (Ser) by seryl-tRNA synthetase (SERS); but as a result of synthesizing Sec from Ser on the tRNA, it inserts Sec into protein. Historically, tRNAs were named by the amino acid attached to them by their corresponding aminoacyl-tRNA synthetase, and unlike any other known tRNA in eukaryotes, Sec is synthesized directly on its tRNA. The novelty of these events are highlighted by the uniqueness of its name, tRNA[Ser]Sec.

1.3 Um34 Addition to Sec tRNA[Ser]Sec, a Most Highly Specialized Modification

In the maturation of Sec tRNA[Ser]Sec, the final modification is the addition of Um34, which is indeed a highly specialized event. For example, its synthesis depends on the secondary and tertiary structure of Sec tRNA [10], and its inclusion in the tRNA has a dramatic impact on tertiary structure of the molecule [7]. The synthesis is dependent on selenium status [7, 11, 12] and its presence governs stress-related selenoprotein synthesis [13, 14]. The extent of Um34 modification is dependent on selenium status that in turn governs the magnitude of stress-related

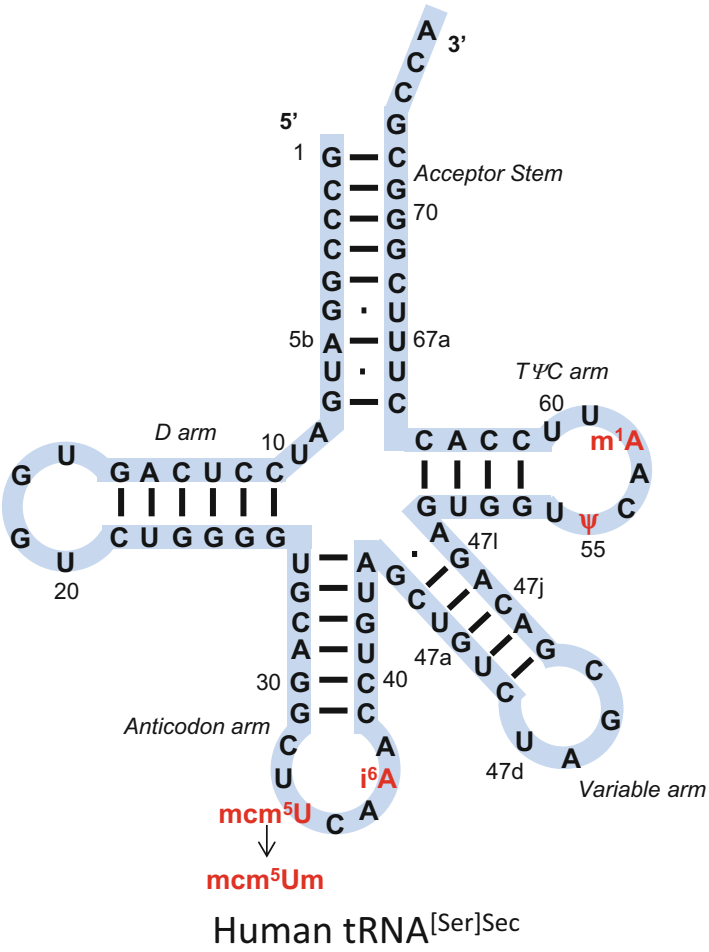

Fig. 1.1 The primary structure of human tRNA[Ser]Sec is shown in a cloverleaf model. There are 90 bases in mammalian tRNA[Ser]Sec and the bases are numbered as shown in the figure (see also [9]). The acceptor stem constitutes the paired 5′ and 3′ terminal bases, the D stem and loop constitute the six paired and four unpaired bases of the left portion of the tRNA, the anticodon stem and loop, the six paired and seven unpaired bases of the lower portion of the tRNA, the variable stem and loop, the five paired and four unpaired bases, and the TψC stem and loop, the four paired and seven unpaired bases of the right portion of the tRNA. Mammalian tRNA[Ser]Sec contains base modifications at positions 34 (mcm^5U), 37 (i^6A), 55 (ψ) and 58 (m^1A) as described in the text. The two isoforms differ from each other by a single methyl group on the 2′-*O*-ribosyl moiety at position 34

selenoprotein expression; i.e., the higher the selenium level, the greater the degree of mcm^5Um occurrence and thus the enhanced expression of stress-related selenoproteins [7, 11, 12].

An unresolved question regarding Um34 addition to the mcm^5U isoform is when exactly does this addition occur during Sec tRNA[Ser]Sec maturation. Synthesis of the

modified bases, Ψ and m^{1}A at positions 55 and 58, respectively, occurs in the nucleus, while the synthesis of mcm^{5}U occurs in the cytoplasm [15, 16]. However, base analysis following injection of the tRNA[Ser]Sec transcript into *Xenopus* oocytes revealed that the i^{6}A modification at position 37 was present in tRNA[Ser]Sec both with and without Um34 [15]. Since only the mcm^{5}Um isoforms, mcm^{5}U and mcm^{5}Um, were found in cells and tissues, we proposed that i^{6}A has to be present on the tRNA for Um34 synthesis to take place and i^{6}A addition was reversible [17]. The fact that tRNA[Ser]Sec apparently must have an amino acid attached, presumably Sec, prior to Um34 addition suggested that the corresponding methylase is highly specific for Sec-tRNA[Ser]Sec and that the i^{6}A modification must be present for Um34 addition. The enzyme that adds i^{6}A, isopentenyl transferase 1 (TRIT1), has been characterized in mammalian cells, and although the reversibility of this enzyme has not been characterized *per se*, it seems highly unlikely that the reaction is sufficiently reversible [18] to accommodate the earlier proposal that addition of the i^{6}A base is reversible (see above and [17]). Sec-tRNA[Ser]Sec must have both Um34 and i^{6}A modifications for stress-related selenoprotein synthesis to occur as the isoform lacking i^{6}A cannot express this subclass of selenoproteins [13, 14]. Thus, it appears that i^{6}A must be present for Um34 synthesis in vivo. These observations raise questions as to where in the cell and at what stage during tRNA[Ser]Sec maturation do Um34 synthesis take place in vivo, if the TRIT1 reaction is poorly reversible and Um34 cannot be synthesized on mcm^{5}U lacking i^{6}A?

Sec-tRNA[Ser]Sec$_{mcm5Um}$ is essential for stress-related selenoprotein synthesis and the presence of Um34 [19] and i^{6}A modifications (see [20] and references therein) enhance the accuracy of codon:anticodon recognition. It should also be noted that mcm^{5}U synthesis at position 34 on tRNA was reported in *Arabidopsis* [21], while the mammalian tRNA methyltransferase, ALKBH8, along with an accessory protein, TRM112, have been found to perform the final methylation step in formation of mcm^{5}U in tRNA[Ser]Sec [22]. ALKBH8 knockout mice lacked the Um34 modification and had reduced GPx1 expression, indicating that mcm^{5}U formation is necessary for Um34 synthesis [22].

1.4 *Trsp*, the Sec tRNA[Ser]Sec Gene

The Sec tRNA[Ser]Sec gene is designated *Trsp*. It is a single copy gene in all organisms examined except zebrafish, which has two gene copies [23]. An RT-PCR technique was developed for identifying and sequencing tRNAs[Ser]Sec from lower eukaryotes [24] and the corresponding tRNAs from *Chlamydomonas reinhardtii* [24] and *Dictyostelium discoideum* and *Tetrahymena thermophila* [25] were sequenced. The longest eukaryotic Sec tRNA[Ser]Sec gene sequenced to date was from *Plasmodium falciparum*, which is 93 nucleotides in length [26, 27]. *Trsp* is present on chromosome 19 in humans [28] and chromosome 7 in mice [29].

1.5 Transcription of *Trsp*

Trsp is transcribed by Pol III as are all other eukaryotic tRNAs (reviewed in [17]), although *Trsp* in *Trypanosoma brucei* has been reported to be transcribed by Pol II [30]. The transcription of *Trsp*, however, is governed largely by three upstream regulatory elements, a *TATA*-like box that resides between positions about −20 and −35, a proximal sequence element (*PSE*) that resides between positions about −46 and −66, and a distal sequence element (*DSE*) that resides between positions about −195 and −210 [31, 32] (Fig. 1.2). On the other hand, transcription of canonical tRNAs is primarily dictated by two intragenic promoter elements, the A and B boxes. *Trsp* also encodes an intragenic A-like box and a B box [33], but the role of these two internal promoters in transcription of the Sec tRNA$^{[Ser]Sec}$ gene is largely not understood [34] and, for the most part, is still controversial [16, 17]. Transcription of *Trsp* begins at the first nucleotide within the gene, and thus, the transcript lacks a leader sequence found in all other tRNAs; however, it has a 3′-trailer sequence characteristic of canonical tRNAs that is removed leaving a transcript consisting of 87 nucleotides in all animals examined, wherein the CCA terminus is then added to complete the tRNA sequence [35]. The 5′-triphosphate at the first nucleotide in Sec tRNA$^{[Ser]Sec}$ remains intact through maturation of the tRNA, but its possible role in Sec biosynthesis and incorporation into protein has not been resolved.

The *TATA*-like box is an essential element for *Trsp* transcription efficiency as observed in vivo in *Xenopus* oocytes [34, 36] and in vitro in HeLa cell extracts [36]. The TATA box and *PSE* encode the elements also found in other Pol III transcribed RNAs that serve as basal promoters for attachment of the regulatory factors governing their transcription [31], albeit variability in sequences exists in these regions and particularly in the *Trsp PSE*, even within different species (reviewed in [17]).

Of the three upstream regulatory regions governing *Trsp* transcription, the *DSE* has sustained more interest. It contains a SPH motif and an octamer sequence that constitute the *Trsp activator element* (*AE*) to which the Sec tRNA transcription activating factor (STAF) binds ([37] and see [38] and references therein). The *AE* is essential for optimal transcription in *Xenopus* oocytes [31], but this regulatory element does not function in *Xenopus* oocyte extracts [39]. A large fragment of DNA was inserted between the *PSE* and *DSE* in mice that resulted in embryonic lethality due to reduced expression of *Trsp* transcription and generation of selenoprotein transcripts [40]. STAF has been characterized in frogs and mice and shown to have

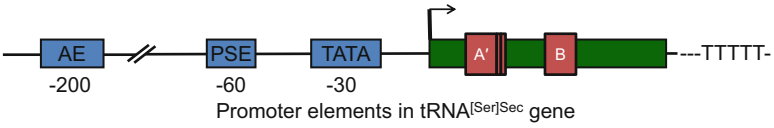

Fig. 1.2 Transcription elements governing *Trsp* transcription. The upstream and intragenic regulatory sites governing transcription of *Trsp* are discussed in the text. The downstream, –TTTTT- sequence, designates the termination signal that dictates the end of transcription leaving a 3′-trailer sequence that must be removed to yield the gene-only transcript

roles in numerous genes transcribed by RNA Pol II and III ([41, 42] and references therein). Mice carrying a deleted *AE* transgene (designated Δ*AE*^{r}) and also lacking *Trsp* (designated Δ*Trsp*) were dependent on the Δ*AE* transgenic mice for expression of tRNA[Ser]Sec [43]. Interestingly, these mice generated tRNA[Ser]Sec populations in dramatically varying amounts in different organs and tissues than their wild type counterparts. Some organs and tissues expressed more total tRNA[Ser]Sec, while others expressed less. One consistent observation in all of the organs and tissues examined was the amount of the mcm^{5}Um isoform was always less in Δ*AE*^{r}/Δ*Trsp* mice than wild type mice [43]. The finding of how the defective *AE* region influenced the levels of the tRNA[Ser]Sec population and in particular the level of mcm^{5}Um is intriguing and must require further work.

1.6 The Genetic Codeword for Sec tRNA[Ser]Sec is UGA

In 1986, two groups reported that TGA occurred in the open reading frames of the genes for mammalian glutathione peroxidase 1 (*Gpx1*) [44] and bacterial formate dehydrogenase (*fdhF*) [45] and their location coincided with Sec in the corresponding proteins. This was a surprising finding as UGA is used as a termination codon in protein synthesis in mammals and *Escherichia coli*. These findings provided the first evidence that the genetic code could contain a 21st amino acid.

Mutation of the *TGA* codeword in *fdhF* to any of a number of other codons resulted in the incorporation of the synonymous amino acid into formate dehydrogenase that in turn was not modified to Sec [46]. This observation further suggested that Sec was most likely the 21st proteinogenic amino acid. Since the minor phosphoseryl-tRNA in eukaryotes was found to specifically decode UGA [3], the possibility that phosphoserine was initially incorporated into selenoproteins and then post-translationally modified to Sec needed to be ruled out. When tRNA[Ser]Sec encoded in *selC* in *E. coli* and mammalian tRNA[Ser]Sec were shown to synthesize Sec on the corresponding tRNAs [4, 5], Sec was unequivocally demonstrated as the 21st amino acid in the genetic code.

1.7 Crystallization of tRNA[Ser]Sec

Numerous elegant studies have been carried out on three-dimensional structures of tRNA[Ser]Sec transcripts from a variety of sources including human [47–49], mice [50], archaea [51, 52] and bacteria [53, 54], either solely as a transcript [47, 50] or in a complex with a protein [48, 49, 51–54]. Although the transcript had to be mutated at several locations to achieve crystallization or to enhance tRNA[Ser]Sec: protein complex formation, these studies revealed important findings about the function of tRNA[Ser]Sec and its interaction with proteins involved in selenoprotein biosynthesis. A major limitation in these studies, however, was that naturally

occurring $tRNA^{[Ser]Sec}$ with its base modifications could not be used. The lack of i^6A in non-modified mouse $tRNA^{[Ser]Sec}$ leads to a disordered anticodon loop involving an illicit A_{37}-U_{33} base pair [50]. The secondary and tertiary structures of $tRNA^{[Ser]Sec}_{mcm5U}$ and $tRNA^{[Ser]Sec}_{mcm5Um}$ are known to be quite different [7] and crystal analyses of the two naturally occurring isoforms would most certainly provide novel insights into how they have such different roles in selenoprotein synthesis.

1.8 Concluding Remarks

A key role of Sec in protein function is certainly evidenced by the appearance and preservation of *Trsp* during evolution, the synthesis of Sec on $tRNA^{[Ser]Sec}$, the selenium-dependent Um34 methylation of $tRNA^{[Ser]Sec}_{mcm5U}$ giving rise to a second isoform, $tRNA^{[Ser]Sec}_{mcm5Um}$, the selective use of these isoforms in synthesizing two subclasses of selenoproteins, housekeeping and stress-related selenoproteins, the additional machinery used specifically in the incorporation of Sec from both isoforms into protein in response to UGA Sec codons and the generation of numerous selenoproteins used in many aspects of cellular metabolism involving health and development.

The appearance of selenoproteins containing selenium in the form of Sec likely occurred early in evolution, prior to the appearance of the three domains of life [55]. The reasons for preservation of Sec utilization during evolution most certainly reside in the fact that Sec is used in catalytic sites of redox-active proteins, thus being an integral and essential part of redox homeostasis. This raises an important question which is why then only some organisms take advantage of such a system? The chemical basis for the selection of Sec in proteins is discussed in Chap. 7.

There are several outstanding questions regarding Sec $tRNA^{[Ser]Sec}$ and its use in cellular metabolism. For example, what are the turnover rates of both isoforms in selenium deficient and replete conditions? What is the identity of the Um34 methylase that converts mcm^5U to mcm^5Um and does methylation occur in the cytoplasm or the nucleus? What are the governing factors responsible for the selective use of mcm^5Um in synthesizing stress-related selenoproteins? Is the $tRNA^{[Ser]Sec}_{mcm5U}$ isoform used exclusively for housekeeping selenoprotein synthesis or is it also used in stress-related selenoprotein synthesis? How does a defective *AE* regulatory region at the −200 position in mice cause such dramatic changes in the levels of the two Sec isoforms in different organs and tissues, always resulting in greater reduction in amounts of mcm^5Um compared to mcm^5U? What are the roles of the intragenic A and B boxes in *Trsp* in transcription? How do the crystal structures of naturally occurring $tRNA^{[Ser]Sec}_{mcm5U}$ and $tRNA^{[Ser]Sec}_{mcm5Um}$ differ from each other? What would their structures divulge about the functions of these two isoforms in interactions with proteins and in donation of the respective Sec to the nascent polypeptide chain (i.e., in decoding UGA)? Are there additional factors in the machinery involved in transcription of $tRNA^{[Ser]Sec}$, including novel ones? Assessing these issues will provide greater insight into the roles of the two Sec $tRNA^{[Ser]Sec}$ isoforms,

elucidate the enigma of how a 2′-*O*-hydroxylmethyl group can selectively govern the synthesis of an entire subclass of selenoproteins and pinpoint how selenium enrichment plays a role in the upregulation of Sec $tRNA^{[Ser]Sec}_{mcm5Um}$ and stress-related selenoprotein expression.

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Chapter 2
Eukaryotic Mechanisms of Selenocysteine Incorporation and Its Reconstitution In Vitro

Mark H. Pinkerton and Paul R. Copeland

Abstract Selenocysteine (Sec) incorporation requires the highly choreographed interplay between a multitude of factors and the elongating eukaryotic ribosome. This chapter focuses on the mechanism by which the known factors deliver Sec-tRNA$^{[Ser]Sec}$ to the ribosomal A-site for both the single Sec incorporation events required for most selenoproteins as well as multiple Sec incorporation events required for the synthesis of Selenoprotein P. The role that in vitro translation systems has played in the determination of these mechanisms is highlighted.

Keywords Ribosome • Rabbit reticulocyte lysate SECIS • SECIS binding protein 2 • Sec-specific elongation factor • Selenocysteine incorporation • Selenoprotein P • Translation • Wheat germ lysate

2.1 Introduction

Selenium (Se) is a trace element found and utilized in organisms across all domains of life and is an essential micronutrient for humans. Se makes its way into proteins via the amino acid selenocysteine (Sec), which was discovered as the twenty-first amino acid in the late 1970s [1, 2]. Sec is structurally similar to the amino acids serine (Ser) and cysteine (Cys) and is synthesized from a Ser precursor as described in Chap. 4. The mechanism of Sec incorporation into proteins is a requirement for many essential functions in humans. The importance of Se is observed as Se deficiency being the underlying cause of numerous diseases in humans. Selenoproteins have a higher enzymatic efficiency and faster chemical reaction rates with electrophiles than their Cys counter parts, which gives selenoproteins high redox potentials [3, 4]. In addition, enzymes harboring Sec instead of Cys in their active sites are much more resistant to oxidative inhibition [5]. Due to these redox characteristics,

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it is not surprising that Sec is found in antioxidant enzymes such as glutathione peroxidases, thioredoxin reductases, and iodothyronine deiodinases in humans and other eukaryotes. However, the importance of Sec and Se as a nutritional requirement varies among species in eukaryotes. For example, fungi and higher plants do not utilize Sec and completely lack the capacity for Sec incorporation. Other organisms, notably *Drosophila*, have the required machinery but synthesize only three selenoproteins that are not essential for survival, fertility or protection from oxidative stress [6].

The molecular machinery for Sec incorporation in eubacteria is well characterized in both synthesis and translation (see Chaps. 3–5), but only in the past two decades has the eukaryotic mechanism of Sec incorporation been deciphered. Sec incorporation in eukaryotes is dependent on *cis* acting factors within the mRNA and *trans* acting factors involved in bringing a selenocysteyl-tRNA (Sec-tRNA$^{[Ser]Sec}$) to the ribosome. Sec-tRNA$^{[Ser]Sec}$ recognizes the UGA stop codon in selenoprotein mRNA, thus representing one of the exceptions to the canonical genetic code. Specifically, recoding will occur when a selenoprotein mRNA contains a stem-loop structure in the 3′ untranslated region (3′ UTR). This element was originally discovered to be required for the synthesis of type 1 iodothyronine deiodinase (DIO1) and was named the Sec insertion sequence element or SECIS element [7]. In a search for SECIS binding proteins, a 120 kDa factor was found to specifically interact with the *Gpx4* SECIS element [8]. This factor, eventually named SECIS binding protein 2 (SBP2 or SECISBP2) was shown to be required for Sec incorporation in vitro [9]. Soon after the discovery of SBP2, a Sec-specific elongation factor (eEFSec) was also found to be a specific Sec-tRNA$^{[Ser]Sec}$ binding factor [10, 11]. Recently, these core factors were shown to be the minimum requirements for Sec incorporation into proteins in a reconstituted cell free in vitro system [2]. There are, however, still underlying fundamental questions that remain about factors that may govern the efficiency and processivity of Sec incorporation. Furthermore, other factors are involved in regulating selenoprotein expression, which will be discussed later in this chapter. Here we review the current state of knowledge regarding the eukaryotic Sec incorporation machinery and discuss in vitro systems and their importance in furthering our understanding of selenoprotein expression.

2.2 UGA Recoding

Recoding is a translational event where the ribosome incorporates an amino acid different than that specified by the cognate codon and the canonical genetic code. This process fundamentally describes the mechanism by which Sec is incorporated. The programmed recoding of codons for different amino acids was once unknown in biology, but studies have discovered numerous ways organisms have altered the canonical genetic code for translation. UAA, UAG, or UGA are the standard termination codons for ribosomes, but only UAG and UGA appear to be the codons that are recoded in organisms [12]. The UGA stop codon appears to be the most

commonly recoded in *Mycoplasma* and mitochondria, both of which use UGA for tryptophan rather than to terminate translation [13, 14], while pyrrolysine is coded by UAG primarily in methanogenic archaea [15]. Typically, stop codon redefinition results from a difference of tRNA specificity, a lack of stop codon recognition by termination factors [16], or suppression due to lack of tRNA-Trp in *E. coli* [17]. In Sec incorporation, however, tRNA specificity and lack of UGA recognition does not play a part, instead it requires the combination of specific factors to work in harmony with the canonical translation elongation system to recode UGA for Sec. As such, this system is unique across all domains of life in the recoding of stop codons [18, 19].

2.3 SECIS

The translational machinery for Sec incorporation requires a mechanism to ensure that only selenoprotein mRNAs have their UGA codons recoded. Selenoprotein mRNAs contain the SECIS *cis* element in the 3′ UTR, which is the only required *cis* acting element for Sec incorporation. SECIS was first reported after sequence alignments of type I iodothyronine deiodinase (*DIO1*) located in the 3′ UTRs containing a loosely conserved sequence that was required for Sec incorporation both in vitro and in *Xenopus* oocytes [7]. It was elaborated further in follow up studies that SECIS elements are found exclusively the 3′ UTR of mRNAs encoding Sec containing proteins [20].

SECIS elements contain highly variable sequences across species and different mRNA 3′ UTRs, but share a similar secondary structure consisting of two helices, an internal loop, and an apical loop. The helices and loops of the SECIS elements are used to define the two forms of SECIS elements that occur. Form 1 contains a relatively large apical loop and a single internal loop separated by a 12–14 base-pair (bp) helix, while Form 2 has another short 2–7 bp helix, an additional internal bulge, and a smaller apical loop [21]. The apical loop contains AAR residues conserved in the apical loop, and the internal loop contains a conserved SECIS core consisting of an unpaired AUGA and UGR along the 5′ and 3′ sides. Both the apical loop and the internal loop are essential for Sec incorporation [20]. The AAR residue is required for Sec incorporation, but its function has not been elucidated and is further confounded by the SECIS elements found in SelM and SelO that contain CCX residues in place of the AAR [22, 23].

SECIS elements contain a conserved feature that defines their structure and function. The AUGA sequence of the SECIS forms non-Watson-Crick base pairs with a 3′ side, which form a kink turn (K-turn) motif that generates an approximate 120° bend in the helix [24]. K-turn motifs are bound by proteins like SBP2 via the K-turn binding motif designated the L7Ae domain [25–29]. The functionality of K-turns may stem from their flexibility in conformation as they appear to move like a hinge to support variable conformations [30]. In the presence of Mg^{2+}, the hinge is found to be in its fully bent confirmation, which can explain the phenomenon of Mg^{2+}

inhibition of SECIS/SBP2 binding [31]. The importance of the AUGA region is emphasized in a study of a single homozygous point mutation of the AUGA to ACGA in the SECIS element of selenoprotein N (*SEPN1*) in vivo, which causes congenital myopathies because the mutation prevents SBP2 binding to the SECIS element [32].

The SECIS element also has a hard coded ability to regulate Sec incorporation. The regulation is observed by large differences in UGA recoding efficiency both in vitro and in vivo of various SECIS elements in chimeric constructs [2, 33, 34]. Some of the predictors of increased translation efficiency included the presence of a GC base pair in helix 2 of the SECIS element and a U in the 5′-side of the internal loop [33]. SECIS elements also vary in their distance from the UGA, 104-5200 nucleotides away in known transcripts, but there appears to be a minimal spacing requirement of 51 nucleotides downstream of UGA [35]. In addition, the affinity of SBP2 for the SECIS element does not correlate with more efficient translation [33, 34], nor does the concentration of EFSec or SBP2 [36]. Currently, the mechanism by which SECIS elements regulate Sec incorporation efficiency remains a mystery.

2.4 SECIS Binding Protein 2

SECIS elements are made functional, at least in part, by the binding of SBP2, which was originally identified as a 120 kDa protein that specifically cross-linked to the *Gpx4* SECIS element [8]. Subsequent purification and characterization led to the discovery that it is essential for Sec incorporation [9]. The known functions of SBP2 include SECIS binding, ribosome binding and transient interaction with eEFSec. Truncation and site-specific mutagenesis of SBP2 was initially used to demonstrate that SBP2 contains three distinct domains: (1) an N-terminal domain of ~400 amino acids with no known function, a central “Sec incorporation domain (SID)” of about 100 amino acids; and (2) a C-terminal ~300 amino acid domain that contains the conserved L7Ae RNA binding domain [9, 25, 29, 37–39].

Interestingly, the SID and RNA binding domains are sufficient for all known functions of SBP2, thus demonstrating that the SBP2 N-terminal domain is not essential for Sec incorporation in vitro [9, 40]. Evidence for a function for the N-terminal domain comes from the fact that it contains discrete conserved stretches of amino acid sequence [41], and mutations in humans that are predicted to truncate the N-terminal domain (reviewed in [42]). In addition, the N-terminal domain is predicted to contain a lysine-rich nuclear localization sequence (NLS) [9]. The presence of a functional NLS was confirmed in a study of liver cells under conditions of high oxidative stress and when nuclear export was blocked by leptomycin B resulted in reduced selenoprotein levels. The reduction of Sec incorporation suggested that SBP2 may get shunted to the nucleus during oxidative stress perhaps to avoid oxidative damage [43].

The functional relationship between the SID and RNA binding domains is complex. Even when the SID and RNA binding domains are expressed separately as

individual proteins, they form a stable SECIS-dependent complex and retain all of their functions in Sec incorporation in vitro with the exception of stable ribosomal binding [39]. SID has been described as an extension of the RNA binding domain [44], but it does not make contact with the SECIS element and instead plays a role in increasing SECIS binding affinity of the RNA binding domain [39]. The complexity of the SID/RNA binding domain interplay is well illustrated by the fact that mutation of the conserved IILKE$^{526–530}$ to alanine eliminated Sec incorporation and stable interaction with the RNA binding domain, but it did not affect high affinity SECIS binding [39]. This same mutation in the intact C-terminal half of SBP2 results in complete inactivation of SECIS binding. From this, the authors concluded that physical linkage between the SID and RNA binding domain constrains conformational options, but the structural significance of this awaits high resolution structure determination.

As alluded to above, one role of SBP2 that is not well understood is the ability to bind to the ribosome. Recent work that followed the initial characterization of the interaction between SBP2 and the large ribosomal subunit (see review in [45]) included mapping ribosome conformational changes by selective 2′-hydroxyl acylation analyzed by primer extension [46] and mapping of the ribosomal binding sites of SBP2 on 28S rRNA to an expansion segment 7 L [47]. As tantalizing as these data have been, little is known about the actual role that SBP2 plays when stably bound to the ribosome. Resolution of this mechanism will require a substantial effort integrated with structural studies.

2.5 Sec-Specific Elongation Factor

eEFSec is central to the Sec incorporation process as the factor that delivers Sec-tRNA$^{[Ser]Sec}$ to the ribosome. eEFSec was discovered based on sequence similarity to its archaeal counterpart SelB. Much like SelB, eEFSec is a GTP binding protein with roughly equal affinity for GTP and GDP thus lacks the action of a guanine nucleotide exchange factor (GEF) for functionality [10, 11]. Unlike SelB, eEFSec does not bind to the SECIS element. Instead, experiments suggest eEFSec forms a transient complex with SECIS bound SBP2 and delivers Sec-tRNA$^{[Ser]Sec}$ to the ribosomal A-site during translation when the ribosome encounters a UGA codon in selenoprotein mRNA.

In contrast to eEFSec, the canonical translation elongation factor 1a (eEF1A) is the workhorse during protein elongation as it carries aminoacylated tRNAs to the ribosome to allow translation elongation. eEF1A contains 3 domains that are phylogenetically related to eEFSec. The function of the eEF1A domains have been characterized as such: Domain I is required for binding to the ribosome and guanosine-5′-triphosphate phosphatase (GTPase) activity, which drives binding of aminoacyl-tRNA in Domain II, and Domain III is specifically involved in binding the acceptor arm of tRNA as well as interacting with its GEF, eEF1B [48]. This characterization has provided some insight to the function of the similar domains in

eEFSec. However, it does not help explain the functional differences between the two elongation factors. The main functional difference between eEFSec and eEF1A begins with tRNA specificity, as eEFSec can only bind to Sec-tRNA$^{[Ser]Sec}$, while eEF1A binds to the 20 canonically charged tRNAs. Analysis of the structure of archaeal SelB with X-ray crystallography revealed a chalice like structure, which has only been previously reported in IF2/eIF5B and not eEF1A [49]. While much is known about eEF1A, the precise roles for eEFSec domains remain largely unstudied and only speculation is currently possible based on similarity to eEF1A.

The most conspicuous difference between the two elongation factors is the additional unique domain on the C-terminal end of eEFSec, Domain IV, which has been implicated in all of the known functions for eEFSec: i) SBP2/SECIS binding, Sec-tRNA$^{[Ser]Sec}$ binding, and GTP hydrolysis [50]. The interaction between eEFSec and the SBP2/SECIS complex has only been observed in cells when tRNA$^{[Ser]Sec}$ was overexpressed [51], or when an electrophoretic mobility shift assay was used to capture the transient complex [39]. The fact that this interaction requires the presence of Domain IV may suggest that the SBP2/SECIS complex induces a stable conformational change in eEFSec that allows recognition of the UGA codon. This hypothesis remains to be tested.

GTP hydrolysis plays an important role in the proper function of canonical eEF1A and eEF2. However, the role GTP hydrolysis plays in eEFSec seems to be different. In eEF1A and eEF2, GTP hydrolysis is critical for the conformational changes required for stepwise progression through the elongation cycle. In eEF1A and eEF2, a GEF is used after hydrolysis to exchange GDP for GTP to allow for a conformational change to promote tRNA binding. The GTP bound form of SelB has a million-fold higher affinity for Sec-tRNA$^{[Ser]Sec}$ than the GDP bound or apo form. Upon binding GTP, SelB undergoes a conformational change and then a stabilization of the SelB/GTP/Sec-tRNA$^{[Ser]Sec}$ complex occurs [52]. It is thought that Sec-tRNA$^{[Ser]Sec}$ is delivered to the ribosomal A site by SelB in the presence of the SECIS element, and then SelB hydrolyzes GTP, which causes the rapid release of Sec-tRNA$^{[Ser]Sec}$ from eEFSec [53]. While bacterial SelB and eukaryotic eEFSec have many differences, it seems likely that the fundamental mechanism of Sec-tRNA$^{[Ser]Sec}$ accommodation in the ribosomal A-site may be conserved.

2.6 Other Factors

While the core essential factors involved in Sec incorporation have been shown to be sufficient for Sec incorporation [2], there are many other factors that are implicated in Sec incorporation either by direct experimental evidence or phylogenetic relationships. The most striking example of the latter is the SECIS binding protein 2 like protein (SECISBP2L). SECISBP2L was identified via BLAST searches based on its similarity to SBP2 in the C terminal domain where it shares a 46 % amino acid identity [9]. Like SBP2, SECISBP2L has both a RNA binding domain

and Sec incorporation domain and can specifically bind to the AUGA core. SECISBP2L is not functionally active in Sec incorporation in vitro, and there currently is no direct evidence of any function [41]. The lack of a discernable function for SECISBP2L is a puzzle that is not illuminated by phylogenetic analysis of its origins, which seem to suggest that SBP2 and 2 L are orthologues that diverged after a gene duplication event during early evolution of vertebrates [41]. Additionally, vertebrate SECISBP2L seems to be more closely related to invertebrate SBP2 than vertebrate SBP2 based on the number of conserved regions found between them. Many deuterostomes do not have SBP2, but still retain SECISBP2L as the only SECIS binding protein, suggesting in some organisms that SECISBP2L is active in Sec incorporation [41]. Currently, it is believed that the divergence of SBP2 and SECISBP2L in vertebrates caused SECISBP2L to lose its ability to support Sec incorporation. While not functional for Sec incorporation in vitro, it is still possible SECISBP2L might serve some other undiscovered function in vivo. Despite the lack of direct evidence, however, it is striking that mice lacking *Secisbp2* retain substantial selenoprotein synthesis capacity [54], which is possibly supported by an as-yet, undetermined function for SECISBP2L.

Another non-essential SECIS binding protein identified, but not fully understood, is eukaryotic initiation factor 4a3 (eIF4A3). eIF4A3 is an RNA dependent ATPase, ATP dependent RNA helicase, and a DEAD-box protein family member that was found to regulate Sec incorporation [55]. While eIF4A3 is similar to the two other isoforms of eIF4A (I and II), it appears that is functionally distinct. In addition to its function in nonsense mediated mRNA decay [56], eIF4A3 binds to the *GPX1* SECIS element at both the internal and apical loop. Binding to SECIS by eIF4A3 prevents SBP2 binding and therefore inhibits Sec incorporation in vitro. It also appears to have a differential binding affinity, specifically to the *GPX1*, but not the *GPX4* SECIS element, giving it a role in regulating selenoprotein synthesis [57]. In McArdle 7777 rat hepatoma cells, eIF4A3 expression becomes upregulated in the absence of Se, and in turn reduced Gpx1 levels while Gpx4 levels were unaffected [55]. Thus, eIF4A3 has emerged as a potentially key factor in determining the hierarchy of selenoprotein expression when Se becomes limiting [reviewed in [58]].

Ribosomal protein L30 (RPL30) is another SECIS binding protein implicated in Sec incorporation [59]. It is a small (14.5 kDa) protein that is part of the large ribosomal subunit [60]. Like SBP2, RPL30 also contains an L7Ae RNA binding motif and competes with SBP2 binding to the SECIS element in vitro [59, 61]. Although RPL30 was shown to stimulate Sec incorporation in transfected cells, it is not yet known whether it is essential for Sec incorporation or whether it can bind to all SECIS elements. The SECIS binding activity of RPL30 is consistent with phylogenetic analysis, which concluded that the L7Ae motif of SBP2 arose from RPL30 [41]. RNase footprinting assays show the binding of SBP2 and RPL30 have some overlap on the SECIS element, but also have their own individual sites as well [61]. The current model of RPL30 activity is in promoting dissociation of the SBP2/SECIS complex by binding to SECIS allowing for canonical ribosomal elongation to continue.

2.7 Unique Sec Incorporation: Selenoprotein P

The plasma selenoprotein, selenoprotein P (SEPP1), is unique because it possesses multiple UGA codons in the mRNA. In humans and rats, there are 10 Sec residues, but it is highly variable between other eukaryotic species. Most of the Sec residues tend to be concentrated at the C terminal end of the protein, but the position of the first UGA codon is highly conserved. Another unique feature of the *SEPP1* mRNA is a long 800 nucleotide 3′ UTR containing both types of SECIS elements, Form 1 and Form 2. In vitro translation experiments in rabbit reticulocyte lysate have demonstrated the efficiency of Sec incorporation into single UGA transcripts is relatively low at about 5–8 % [40]. With such a low efficiency, it would seem to be impossible to efficiently create proteins with multiple UGAs, but the 26 μg/ml of SEPP1 protein observed in plasma and the high SEPP1 levels found in tissue culture suggest that the efficiency is much higher in vivo (see [62] and Chap. 22). Indeed, recent work has indicated that the efficiency of Sec incorporation at UGA codons downstream of the first UGA is much more efficient [63]. Analysis of UGA redefinition in vivo using ribosomal profiling also supports a higher than 10 % UGA redefinition efficiency in hepatic selenoprotein biosynthesis (see Chap. 3 and [64]). Thus, two questions emerge when considering the requirements for SEPP1 synthesis: how is a protein with 10 Sec codons made efficiently, and how are the multiple Sec residues incorporated processively in order to generate full length SEPP1 protein (See also Chap. 3)?

It has been proposed that SEPP1 translation efficiency and processivity is regulated by *cis* acting factors in the 3′ UTR of *SEPP1* involved with recoding of UGAs for Sec incorporation. Some of these *cis* elements were hypothesized in initial sequence analysis of *SEPP1* looking for conserved regions across species where two regions in the 3′ UTR surrounding and including the two SECIS elements were found to be highly conserved [65]. It was hypothesized that the two SECIS elements were involved in processive Sec incorporation [66]; however, in vitro tests showed neither Form II SECIS nor most of the 3′ UTR is required for *SEPP1* translation [63, 67]. Swapping the *SEPP1* 3′ UTR with other selenoprotein 3′ UTRs resulted in reduced efficiency, but processive production of full length SEPP1 protein in vitro was still observed, indicating clear separation of efficiency and processivity [63, 67].

2.8 Impact of In Vitro Translation Systems for Studying Sec Incorporation

Cell free in vitro translation systems have been key tools for investigators in determining the factors involved with Sec incorporation. The identification, validation and characterization of both SBP2 and eEFSec has in large measure taken place in two commercially available in vitro translation systems: rabbit reticulocyte and

wheat germ lysates. Both of these systems have their uses and limitations, and have an established record of being reliable ways of solving some of the fundamental questions in translation. Prior to the discovery of SBP2, the rabbit reticulocyte lysate system was used to explore the ability of $tRNA^{[Ser]Sec}$ to suppress translation termination [68, 69]. The use of rabbit reticulocyte lysate expanded significantly when it was found that SBP2 is extremely limiting in the system, thus paving the way for the formal proof that SBP2 is necessary for Sec incorporation [9], and extensive characterization of its domain functions [25, 33, 39, 40, 70]. The physiological basis for the lack of SBP2 in this system is an interesting topic that has not been investigated. In the case of eEFSec, there is no evidence that the factor is limiting in rabbit reticulocyte lysate, and its biochemical characterization required the development of a new system.

The commercially available wheat germ lysate system was used in an attempt to reconstitute Sec incorporation with only the known factors, taking advantage of the fact that higher plants do not utilize Sec and do not have any of the Sec incorporation factors. Initially it was found that the addition of SBP2, eEFSec, Sec-$tRNA^{[Ser]Sec}$ and mammalian ribosomes was sufficient to promote robust Sec incorporation into a luciferase reporter construct harboring *SEPP1* 3′ UTR [2]. Subsequent work with a different batch of wheat germ lysate demonstrated that mammalian ribosomes were not required [67], thus establishing that SBP2, eEFSec and Sec-$tRNA^{[Ser]Sec}$ are sufficient to promote Sec incorporation even with ribosomes that did not evolve to work with these factors.

The wheat germ lysate system has tremendous potential in terms of providing a test bed for all of the fundamental mechanistic questions surrounding both Sec incorporation and Sec-$tRNA^{[Ser]Sec}$ synthesis. For example, three different SECIS elements showed markedly different Sec incorporation efficiencies [2], consistent with those previously observed in rabbit reticulocyte lysate [33]. In addition, it was recently found that while full length SEPP1 protein can readily be made in rabbit reticulocyte lysate, only a single Sec incorporation event was observed in wheat germ lysate [67], thus providing strong evidence for the existence of an as yet unidentified factor required for processive Sec incorporation. As illustrated in Fig. 2.1, this wheat-based system is primed to answer several key questions regarding the mechanism of SEPP1 synthesis, and this is just one example of the extent to which our knowledge of the fundamental mechanism of Sec incorporation may be revealed.

2.9 Concluding Remarks

In the 25 years since the discovery of the SECIS element, great strides in understanding the mechanism and regulation of Sec incorporation in selenoproteins have been made, but questions still remain. The understanding of the essential Sec incorporation factors and their fundamental roles brings more insight to the complexity of selenoprotein regulation. Our greater understanding of these mechanisms has

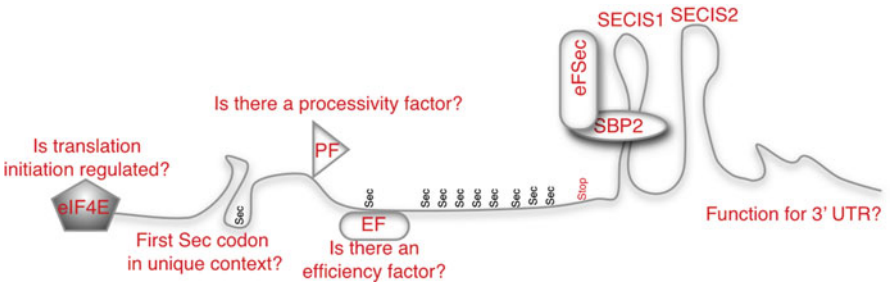

Fig. 2.1 An illustration of the many mechanistic questions that can be answered about the mechanism of SEPP1 synthesis using a wheat-germ lysate based in vitro translation system. The figure shows the SEPP1 mRNA containing ten Sec codons and two SECIS elements along with the known (SBP2, eEFSec) and proposed processivity (PF) and efficiency (EF) factors. The potential role of codon context, regulated translation initiation and a function for the conserved non-SECIS portions of the SEPP1 3′ UTR are also depicted

provided more explanations to the sources of diseases previously unknown, and will continue to do so. Many questions remain in the basic mechanism of Sec incorporation involving eEFSec complex formation with SBP2/SECIS and Sec-tRNA$^{[Ser]Sec}$ delivery to the ribosome. We also do not know how Sec-tRNA$^{[Ser]Sec}$ is accommodated into the ribosome or the role and mechanism of GTP hydrolysis for eEFSec, nor the temporal sequence of events. It is also too early to know if all the factors required for the regulation of Sec incorporation in vivo have been discovered because the difference in efficiency between cell free extracts and tissue culture is not well understood. In vitro translation systems have helped immensely in shedding light on fundamental questions of Sec incorporation so that the molecular mechanism of Sec incorporation can be deciphered.

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Chapter 3
Probing Selenoprotein Translation by Ribosome Profiling

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Abstract Selenoproteins with known functions are oxidoreductase enzymes that serve roles in maintaining cellular redox balance, reproductive health, thyroid hormone metabolism, development, and immune functions. Unique to selenoprotein biosynthesis is the requirement during translation to redefine an in-frame UGA codon to encode for selenocysteine rather than terminate translation. This non-canonical translation event is a bottleneck in selenoprotein synthesis and subject to gene-specific regulation. Here, the application of ribosome profiling, which involves deep sequencing of ribosome protected mRNA fragments, to examine mechanisms of selenoprotein biosynthesis will be discussed. The ability of this technique to quantify ribosome abundance and position, at codon resolution, on selenoprotein mRNAs has provided important insight into long-standing questions regarding the efficiency and regulation of selenocysteine incorporation as well as provoking new questions for future investigations.

Keywords Recoding • Ribosome footprint • Ribosome pausing • Ribosome profiling • Selenium • Selenocysteine • Selenoproteins • Translation

3.1 Introduction

Selenium (Se) is incorporated into a small number of mammalian proteins, encoded by ~25 mammalian genes, in the form of the amino acid selenocysteine (Sec) [1, 2]. In addition to the beneficial physical properties that Sec confers to selenoproteins, it is unique among amino acids in that its incorporation occurs during translation when a UGA codon, normally specifying translational termination, is decoded by Sec-tRNA$^{[Ser]Sec}$ as Sec. The process of UGA redefinition and Sec insertion during translation is thought to be inefficient due, at least in part, to the requirement for

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specialized Sec insertion factors to "reprogram" the ribosome and recruit Sec-tRNA$^{[Ser]Sec}$, in a process that competes with translational termination. Studies by a number of laboratories have uncovered the core components of the Sec incorporation machinery. In eukaryotes, these include the *cis*-acting Sec insertion sequence elements (SECIS) found in the 3′ UTRs of all selenoprotein mRNAs [3–5] and the Sec redefinition elements (SREs), which reside adjacent to a subset of UGA-Sec codons [6–8]. Several *trans*-acting factors are also required including Sec-tRNA$^{[Ser]Sec}$ and the proteins involved in tRNA maturation and Sec synthesis, the Sec-tRNA$^{[Ser]Sec}$ elongation factor (eEFSec) that delivers Sec-tRNA$^{[Ser]Sec}$ to the ribosome [9–11], and finally, the SECIS binding protein 2 (Secisbp2) [12, 13]. The latter is thought to have an essential role in Sec-tRNA$^{[Ser]Sec}$ recruitment to the ribosome as well as an independent role in regulating selenoprotein mRNA stability [14]. In addition to these core components, a number of factors have been implicated as accessory proteins proposed to regulate the efficiency of Sec insertion and/or mRNA stability in response to environmental cues such as Se availability, inflammation, or oxidative stress [15, 16]. Components of the Sec incorporation machinery are depicted in Fig. 3.1.

In addition to the importance of understanding the mechanisms and regulation of selenoprotein biosynthesis for issues related to human health, the process by which Sec is synthesized and incorporated into proteins has broad implications for our understanding of the mechanism of standard genetic decoding. Specifically, selenoproteins are unique biological examples of how the standard readout of the genetic code by the ribosome can be subverted to regulate gene expression, redefine codon meaning, and even encode non-standard amino acids such as Sec.

Ribosome profiling is a new methodology well suited to address questions regarding the mechanisms of UGA "recoding", the efficiency of Sec incorporation, and translational control of selenoprotein synthesis. This technique, which involves

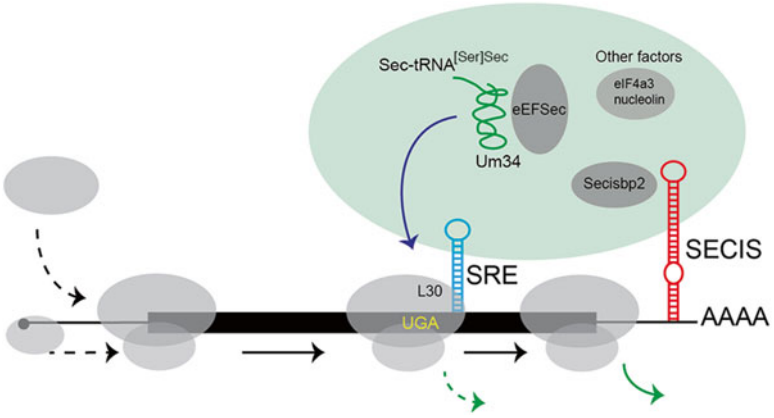

Fig. 3.1 *Cis*- and *trans*-acting features of the Sec incorporation machinery. See text for additional details

the deep sequencing of ribosome protected mRNA fragments (ribosome footprints), provides a means to quantify ribosome density and position on actively translated mRNAs at sub-codon resolution [17]. Specifically, this technique can reveal gene- or region-specific translation rates, sites of ribosome pausing, translation of regulatory 5′ untranslated region (UTR) short open reading frames (ORFs), the use of alternate translation initiation codons, and even ribosome conformational changes [18]. The principles of the approach and specific examples of its application to questions regarding selenoprotein biosynthesis [19] will be presented here.

3.2 Translational Control of Gene Expression Revealed by Ribosome Profiling

Translation is the process by which an mRNA template is read by the ribosome to produce protein. This crucial step in gene expression expends significant energy and is highly regulated. Known means of translational control include a number of mechanisms to vary the rates of translation initiation and elongation along the length of the mRNA (see [20] for review). For example, the length, sequence, and secondary structure of the 5′ UTR can affect ribosome access to the initiation codon, or the use of internal ribosome entry sites to initiate translation can "bypass" cap-dependent initiation and scanning of the 5′ UTR altogether. Some codons and regions within the open reading frame may be translated more slowly than others leading to bottlenecks in translation and sites of ribosome pausing for various purposes. In addition, *cis*- and *trans*-acting factors have evolved in some cases that can alter the very rules of the genetic code [21, 22], such as programmed non-triplet decoding that results in translation of the +1 or −1 reading frame, or alterations in the meaning of a specific codon as occurs during synthesis of selenoproteins.

While the means to globally measure regulation of transcription and changes in mRNA levels have existed for a number of years (e.g., by microarray or RNA-Seq), only recently with the development of ribosome profiling has it become possible to monitor translational control of protein expression at the same scale [18]. This technique is based on the observation that translating ribosomes protect ~30 nucleotides (nts) of the mRNA from digestion with ribonucleases [23, 24]. The method involves isolation of ribosome:mRNA complexes from cells or tissues, digestion of the unprotected mRNA with ribonucleases, and deep sequencing of the ribosome protected mRNA footprints RPFs (Fig. 3.2). Total RNA is also collected and subjected to RNA-Seq in order to normalize the ribosome footprints to changes in mRNA abundance to determine translational efficiency. Counting the number of footprints that map to individual transcripts provides the means to measure ribosome density and provides an approximation of protein synthesis rates. Importantly, additional information lies underneath this gene-level summary of translational activity. Changes in ribosome density at specific positions or regions across an individual

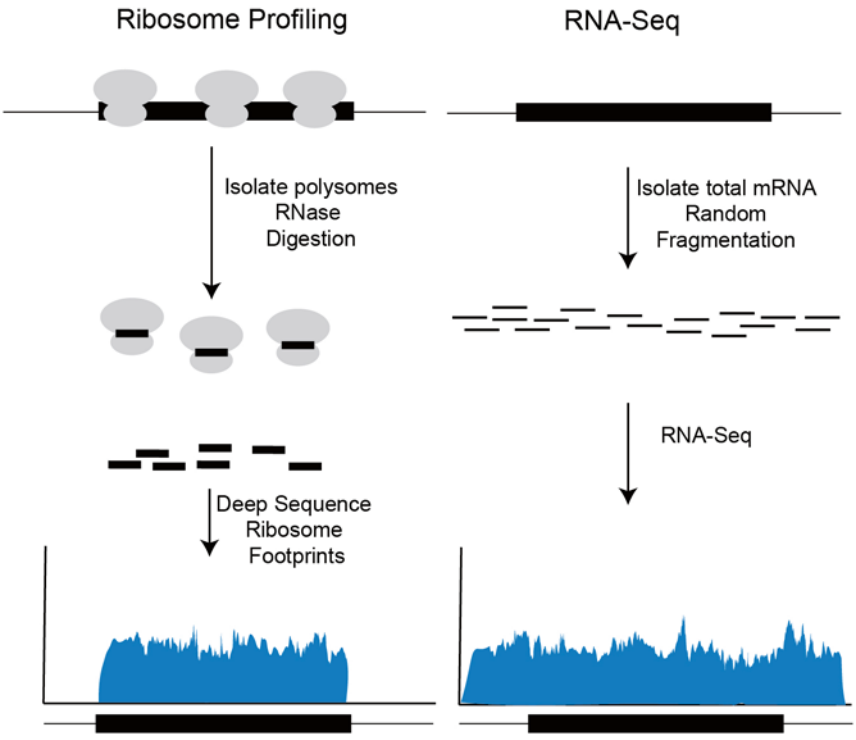

Fig. 3.2 Conceptual view of ribosome profiling and RNA-Seq. In ribosome profiling, total polysomes are isolated, digested with ribonuclease, and the resulting ribosome protected mRNA fragments (RPFs) are isolated and subjected to deep sequencing. Mapping the RPFs to either the reference genome or annotated mRNAs identifies important quantitative translational features. Translational efficiency is determined by normalizing RPFs to changes in mRNA abundance

mRNA can shed light into translational mechanisms. For example, ribosome profiling has revealed the presence of alternative translation start sites and previously unknown protein coding sequences as well as evidence for translation of regulatory upstream ORFs in 5′ UTRs [25]. Sites of ribosome pausing appear as regions with increased ribosome density, and these sites may be mapped to the very codon at which the ribosome is stalled [26, 27]. Sites of translation initiation and termination can be mapped as sites where the ribosome density abruptly increases or decreases, respectively. The inclusion of drugs that inhibit translation initiation, elongation, or termination can be useful under specific circumstances to trap the ribosome at different stages of protein synthesis.

The number of fragments that can be sequenced underlies the power of this approach. A typical sequencing lane from the Illumina HiSeq instrument can sequence upwards of 200 million fragments. The resulting sequences are then

mapped back to the reference genome or mRNAs and subjected to bioinformatic analysis to quantify the number of RPFs and their positions across all actively translated coding sequences. Once this is accomplished, several bioinformatic checks should be applied to ascertain whether the nuclease resistant fragments were derived from translating ribosomes. For example, RPFs should be highly enriched in the coding sequences of mRNAs relative to the 5′ and 3′ UTRs and triplet-phased with the codons, as this is the step size of the ribosome. Given the global nature of ribosome profiling, it is possible to examine these features by examining the RPF distributions relative to annotated start and stop codons for all mRNAs collectively. An example from a typical ribosome profiling experiment of mouse liver from the Howard lab is shown in Fig. 3.3a. Here the RPFs for all mRNAs are examined

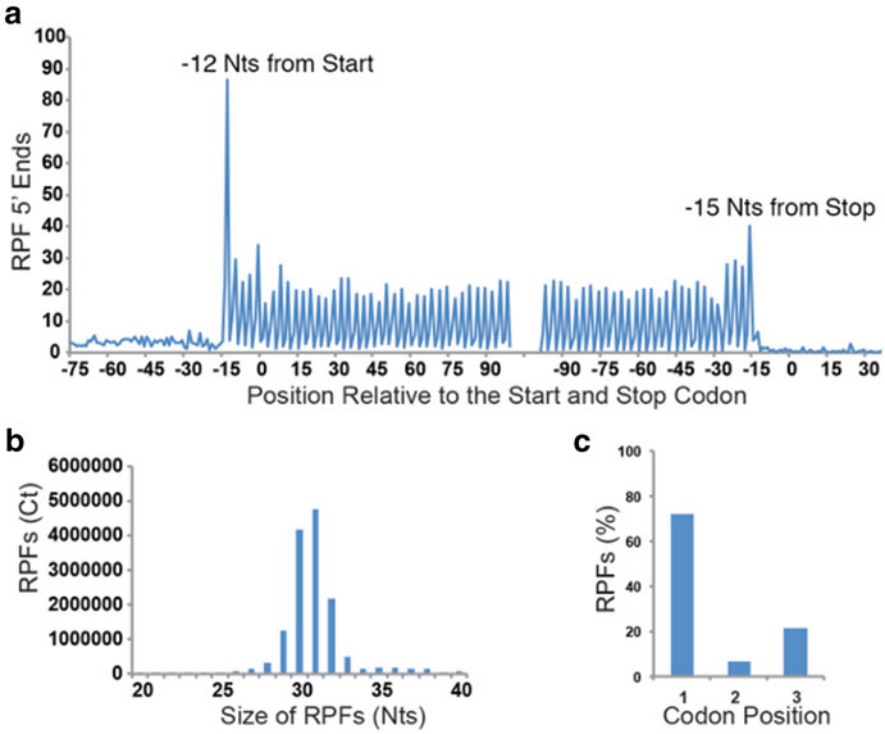

Fig. 3.3 Characteristics of ribosome footprints. (**a**) Meta-analysis: The relative abundance of ribosome footprint (5′ ends) for all mRNAs is shown relative to the start codons (first nt of the start codon is position 0-left) and stop codons (first nt of the stop codon is position 0-right). The 5′ ends begin abruptly 12 nts upstream of AUG start codons and stop abruptly 15 nts upstream of stop codons. This indicates the ribosomal P- and A-sites are located at nucleotides 12–14 and 15–17 on ribosome footprints, respectively. A distinct three-nucleotide repeating pattern is observed within coding sequences corresponding to the codon step size of the ribosome. (**b**) Size distribution of RPFs. (**c**) Percent of RPFs with the 5′ end positioned at the first, second, or third position of a codon indicating phasing of the footprints

together by assigning the first nucleotide of each mRNAs start and stop codon to 0 (start codons 0-left, stop codon 0-right) and the position of the RPF 5′ ends are shown for 100 nts on either side of the start and stop codon. It is clear from this analysis that footprints are highly enriched in the coding sequences relative to the 5′ and 3′ UTRs. Footprints in the 5′ UTR are slightly higher than the 3′ UTR indicating translation of short upstream ORFs. The peaks observed within the coding sequences are exactly 3 nts apart, as expected for the codon step size of the ribosome.

It is also possible from these data to predict the nucleotide positions within the RPFs that occupied the ribosomal P- and A-sites. During initiation, the start codon is located in the P-site of the ribosome, whereas during termination the stop codon is in the A-site. Based on where the 5′ ends of the RPFs abruptly start (position −12 from the first nt of the start codons) and where they stop (position −15 from the first nt of the stop codons), it can be surmised in this experiment that the first nt of the P-site and the first nt of the A-site are 12 and 15 nts downstream from the 5′ end of the RPF, respectively. As expected, the RPF sizes are distributed around ~30 nts (Fig. 3.3b) and the RPFs have triplet phasing consistent with step-wise reading of codons (Fig. 3.3a, c). From these data, we can surmise that the majority of RPFs are derived from translating ribosomes.

3.3 Application of Ribosome Profiling to Investigate Selenoprotein Biosynthesis

It is well known that Se dietary levels can have both tissue- and gene-specific effects on the expression of selenoproteins [28–31]. For example, certain tissues such as brain and testes maintain their Se levels, while others do not when Se is limiting. This effect is most likely due to preferential delivery, uptake, sequestration and recycling of the available Se pools by these tissues. At the gene level, expression of certain selenoproteins with critical functions, e.g., Gpx4 and Txnrd1, are resistant to changes in Se status, whereas expression of other selenoproteins involved in stress-related responses, e.g., Gpx1, Msrb1, and Sepw1, are highly responsive to Se.

Translation and the insertion of Sec in particular, are steps in which control of selenoprotein synthesis has been shown to occur [19, 32]. Se-dependent $tRNA^{[Ser]Sec}$ modifications have been implicated as a direct link between available Se levels and gene-specific selenoprotein production. In mammals, there are two isoforms of $tRNA^{[Ser]Sec}$ that differ by the presence or absence of a Um34 methyl group such that the anticodon loop contains either 5-methoxycarbonylmethyluridine (mcm^5U) or 5-methoxycarbonylmethyl-2′-O-methyluridine (mcm^5Um). The ratio of the two $tRNA^{[Ser]Sec}$ isoforms is tightly linked to Se levels [33, 34] and mutations which prevent Um34 methylation reveal that the housekeeping selenoproteins are preferentially synthesized, whereas synthesis of the stress-related selenoproteins is reduced [35–37]. Further evidence has implicated the identity of the 3′ UTR SECIS in gene-

specific translational control of Sec incorporation. Current models support that Secisbp2 interactions with the SECIS element are required for Sec incorporation and data have been presented that suggest differences in Secisbp2 binding affinity to the 3′ UTR SECIS element of individual selenoprotein mRNAs may in part account for gene-specific differences in mRNA stability [38] or Sec incorporation efficiency [39]. Additional studies have implicated other SECIS binding proteins such as L30 [40] and eIF4a3 [15] as Se-responsive regulators of this process. Intriguingly, a recent study has also shown that a subset of selenoproteins have hypermethylated 5′ caps [41] suggesting that the mode of recruiting ribosomes to selenoprotein mRNAs may be altered under some circumstances.

Numerous studies have examined the efficiency of Sec incorporation using reporter constructs or cDNAs transfected into cultured eukaryotic cells. These studies have consistently concluded that Sec incorporation is inefficient and subject to regulation by Se. Since most selenoproteins carry only one UGA-Sec codon, an inefficient mechanism appears to be a viable hypothesis. Paradoxically, the vertebrate plasma Se transport protein selenoprotein P (Sepp1) carries 7-17 UGA-Sec codons depending upon the species [42]. It is clear from this example that UGA redefinition must be highly efficient at some UGA-Sec codons leading to the speculation that Sec incorporation in vivo may occur with greater efficiency. One study of *Sepp1* translation from our laboratory using Secisbp2-supplemented rabbit reticulocyte lysate and reporter constructs indicated that after incorporation of Sec at a first UGA codon, the efficiency of incorporation at downstream UGA codons occurs with much higher processivity [43]. Similarly, studies with the *Sepp1* cDNA indicate that Sec incorporation is inefficient and that the efficiency of Sec incorporation per UGA codon is higher near the end of the message [32]. The importance of the first UGA has been further implicated in studies of the zebrafish *Sepp1* gene where high termination, analysis of polysomes on sucrose gradients, and mutagenesis of specific elements led to a model in which the first UGA acts as a checkpoint to regulate more processive downstream Sec incorporation [44]. While providing models that may explain the ability of the ribosomes to produce full-length Sepp1, the general conclusions still imply that incorporation of Sec on selenoprotein mRNAs with a single UGA-Sec codon are inefficient in all model systems tested.

Measuring the efficiency of Sec incorporation during selenoprotein expression from mRNAs produced from their native genomic configuration has not previously been possible due to the inability to reliably monitor an unstable termination product [45] relative to full-length protein containing Sec. To address this question in vivo, we proposed that ribosome profiling would be a suitable method to obtain a surrogate measure of Sec incorporation by monitoring the change in ribosome density before and after UGA-Sec codons [19]. While RPFs located 5′ of the UGA-Sec (5′ RPFs) are expected to reflect the number of ribosomes that initiated translation on an mRNA, RPFs 3′ of the UGA-Sec codon (3′ RPFs) are likewise proportional to the number of ribosomes that have successfully incorporated Sec and are translating downstream codons (Fig. 3.4a).

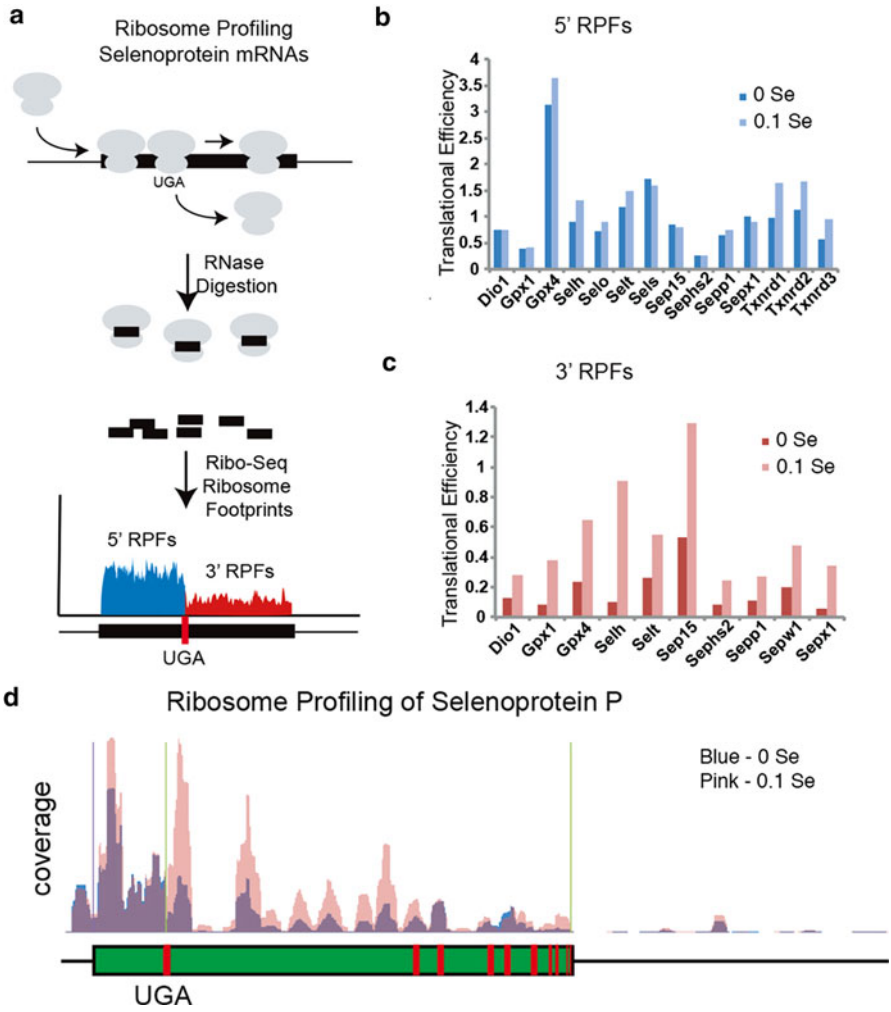

Fig. 3.4 Ribosome profiling and selenoproteins. (**a**) Hypothetical ribosome profiling of selenoprotein mRNAs. Due to competition between Sec incorporation and termination, ribosome density 5′ (5′ RPFs) and 3′ (3′ RPFs) of the UGA-Sec codon are considered separately. Translational efficiencies upstream (**b**) or downstream (**c**) of the UGA-Sec codon are shown for selenoprotein mRNAs isolated from liver of mice fed diets supplemented with 0 or 0.1 ppm Se. Some selenoprotein mRNAs could not be analyzed because the UGA-Sec codon was too near the 5′ or 3′ of the ORF. (**d**) Ribosome footprint coverage of *Sepp1* mRNA in livers of mice fed Se-deficient (0 ppm Se, *blue*) or adequate (0.1 ppm Se, *pink*; overlap appears *purple*) diets. Ribosome coverage was normalized to *Sepp1* mRNA abundance. The *green box* below the plot indicates the ORF and the position of the UGA-Sec codon by the *red vertical bar*

3.3.1 Selenocysteine Incorporation Efficiency

In order to examine translational control of selenoprotein expression in response to changes in dietary Se intake, mice were fed diets with defined levels of Se and livers were isolated and subjected to ribosome profiling and RNA-Seq analysis to monitor translation and mRNA abundance, respectively. Strikingly, the abundance of 3′ RPFs downstream of the UGA-Sec codons is decreased compared to upstream 5′ RPF density [19] (compare Fig. 3.4b, c) for all selenoprotein mRNAs indicating that Sec incorporation is inefficient in vivo. Although changes in mRNA levels in response to Se dietary levels were observed, the changes in ribosome footprints were found to be even greater. Importantly, the RPFs downstream of UGA-Sec were substantially increased in mice fed diets with adequate or supranutritional amounts of Se compared to those on Se-deficient diets (Fig. 3.4c and [19]). This result was found for all selenoproteins measured (except for selenoprotein mRNAs with near C-terminal UGA-Sec codons or those expressed at very low levels that could not be measured) indicating that Sec incorporation in vivo is not efficient, but the levels are tightly regulated by Se status for all selenoproteins.

Sepp1 is of particular interest because of the presence of 10 UGA-Sec codons that would appear to demand high level Sec incorporation. Measurements of RPFs on *Sepp1* reveal significant loss of ribosomes at the first UGA-Sec codon (Fig. 3.4d). Although it is difficult to distinguish ribosome density between UGA-Sec codons downstream of the second UGA-Sec due to their closeness, ribosome density continues to the end of the message. This is the first view of translation on *Sepp1* mRNA in vivo indicating that for at least the first UGA-Sec codon, Sec incorporation is inefficient and subject to regulation. Experiments to verify and examine the mechanisms involved in regulating Sec incorporation during Sepp1 biosynthesis in vivo are ongoing.

3.3.2 Ribosome Occupancy Near the UGA-Sec Codon and in the 5′ UTR

Slow decoding of the UGA-Sec codon has been postulated to be a kinetic feature of Sec incorporation [44, 46] that would result in ribosome pausing. To address this question, the predicted A-sites of ribosome footprints were mapped near the UGA-Sec codons [19]. Surprisingly, there was little accumulation of ribosome footprints near the UGA-Sec codons for most selenoproteins with the notable exception of four stress-related selenoprotein mRNAs, *Gpx1*, *Msrb1*, *Sepw1* and *Selh*. For these four mRNAs, the number of footprints with A-sites mapping to the UGA-Sec or the five preceding codons amounted to between 20 and 80 % of the total footprints on each mRNA. The vast majority of these did not map with the UGA-Sec in the A-site but rather to the codons just proximal to the UGA-Sec. It is striking that the genes most strongly regulated by Se also contain an abundance of ribosome pausing

upstream of the UGA-Sec codon. These results imply that a delay in translation occurs prior to the ribosome encountering the UGA-Sec codon that is either unique or particularly pronounced for these mRNAs. Further, the number of footprints delayed at these UGA-Sec proximal codons increases with increasing levels of Se. Perhaps this delay is required in order for the ribosome to receive signals for Sec insertion prior to encountering the UGA-Sec codon, or perhaps it is linked to mechanisms controlling mRNA stability. What mechanisms cause this delay in elongation and how it contributes to the mechanism of Sec incorporation needs further investigation.

The ability of 5′ UTRs to regulate translational efficiency through the presence of translated short ORFs is well documented in specific mRNAs. Paradigms that have been experimentally examined through biochemical and genetic means include, among others, the yeast transcription factor GCN4 and the mammalian stress response gene, ATF4 [47, 48]. Translation of upstream ORFs on each of these has been identified in ribosome profiling experiments, demonstrating that ability of ribosome profiling to capture documented cases of translation outside the main coding sequences. Ribosome profiling studies in yeast and mammalian cells have further revealed extensive translation of upstream ORFs, initiated at both AUG and non-AUG codons throughout the transcriptome [17, 26, 49, 50]. Consistent with this observation, RPFs in the 5′ UTR of several selenoprotein mRNAs have been identified including those for *Selh*, *Selt*, *Sephs2,* and *Txnrd1* suggesting the possibility that translation initiation is regulated [19]. However, it seems unlikely that these upstream ribosomes have a Se-dependent effect on translation initiation in liver as the translational efficiency between the start codon and the UGA-Sec codon remained relatively unchanged by dietary Se. For three of these mRNAs, *Selh*, *Sephs2*, and *Selt*, there is a contiguous ORF from the upstream footprints to the first AUG, suggesting an alternative possibility that translation of the main ORF may start upstream of the annotated AUG codon resulting in an N-terminal extension to the predicted protein. Further experiments are required to determine the importance of 5′ UTR translation to selenoprotein synthesis.

3.4 Conclusion: Insights Gained, Limitations, and Future Directions

Ribosome profiling has provided a first view into the quantity and distribution of ribosomes in the process of translating selenoprotein mRNAs in vivo [19]. The results reveal insight into several long-standing questions in the field. RNA-Seq confirms what many have shown before; that dietary Se regulates mRNA levels such that the stress-related selenoprotein mRNAs are reduced when Se is limiting. Perhaps most significant is the finding that Sec incorporation is inefficient in vivo and dynamically regulated in a gene-specific manner by dietary Se levels. Ribosome densities upstream of UGA-Sec codon are mostly unaffected by dietary Se, while

ribosome densities downstream are increased in the livers of mice fed Se- adequate or -supranutritional diets for all measured selenoproteins. The increase in translation past the UGA-Sec codon is particularly dramatic for the stress-related selenoproteins and of much greater magnitude than changes in mRNA levels, suggesting that translation is the predominant factor in the regulation of selenoprotein synthesis by dietary Se. Insight was also gained into how a mRNA with 10 UGA-Sec codons is translated when Sec incorporation is inefficient. The results suggest that Sec incorporation is inefficient and regulated at the first UGA-Sec, but is increasingly processive thereafter. Finally, ribosome footprints are found in the upstream ORF of several selenoprotein mRNAs, suggesting another potential level of regulation, or alternatively, N-terminal extensions to the selenoproteins due to initiation of translation upstream of the annotated initiation codon.

While ribosome profiling provides an exciting new view of selenoprotein mRNA translation, some caution is warranted in interpreting the results. The approach is relatively young, with steadily evolving experimental procedures and data analysis platforms. In general, each step from cell/tissue harvesting to nuclease digestion to library construction can introduce distortions in ribosome profiles. For example, ribosomes must be trapped quickly to prevent ribosome run-on following lysis. The disproportionately high number of ribosome footprints at the initiation codon has been proposed to be an artifact of blocking elongation, but not ribosome scanning and translation initiation. Comparisons of ribosome profiles from drug treated vs. flash frozen samples indicate further differences in ribosome coverage of 5′ UTR ORFs and termination codons [50]. Nuclease digestion and ligase preferences during library construction introduce additional factors that can impact how effectively specific mRNA fragments are captured. Many of these issues are mitigated when examining differential changes in ribosome activity between two different biological samples that are prepared with an otherwise uniform protocol. Nevertheless, this is a reason for some caution in interpreting ribosome profiling data.

In the standard application of ribosome profiling, rates of synthesis measurements for a protein are inferred from the average ribosome density along the mRNA being analyzed. This assumes that all ribosomes finish translation and that the elongation rates are similar between different mRNAs in a cell. Known exceptions include the buildup of footprints near the start codon of many mRNAs, sites of ribosome pausing, and ribosome termination at the UGA-Sec codons. These factors can be compensated by, for example, excluding the initiation region or sites of ribosome pausing from measurements designed to reflect protein expression. In the case of selenoprotein mRNAs, estimates of protein synthesis rates were computed based on the number of ribosomes 3′ of the UGA-Sec codon, as discussed above.

An experimental approach to directly measure elongation rates involves pharmacological inhibition of translation initiation followed by treatment with an elongation inhibitor at different time points [26, 51]. The resulting sequential snapshots of ribosome run-off (progressive clearing of ribosomes from 5′ to 3′) allow for elongation rates to be directly inferred. Using this approach and by grouping genes based on their characteristics (high vs. low expression, length, secreted or cytoplasmic,

codon adaptation index), it was reported that the average rate of elongation was remarkably consistent at ~5-6 codons/second [26]. While in theory, this approach could be applied to individual mRNAs, to date it has only been reported as the average elongation rate calculated by combining the ribosome distributions from multiple mRNAs [26]. Of interest to selenoprotein synthesis is whether incorporation of Sec alters the ribosome in such a way as to affect elongation rates downstream of the UGA-Sec codon. Such an observation may be particularly important in the case of *Sepp1* as it may provide insight into ribosome alterations and the mechanisms responsible for increased Sec insertion efficiency following successful incorporation of Sec at the first UGA-Sec codon.

As methods evolve, ribosome profiling promises to be a powerful tool for scientists investigating the regulation and mechanisms involved in selenoprotein synthesis. Future directions include direct measurements of translation elongation rates across individual selenoprotein mRNAs by the combined use of initiation and elongation inhibitors, and tissue-specific differences in translation. Ribosome profiling also provides a way to examine the subcellular localization and regulation of selenoprotein translation; for example, by cellular fractionation to examine selenoprotein synthesis at the endoplasmic reticulum, or other cellular compartments. Ribosome profiling may also be of therapeutic relevance as a tool to examine the mechanisms of drugs that affect translation, or in the development of new compounds designed specifically to affect selenoprotein expression at the level of translation. Further major advances in our understanding of selenoprotein biosynthesis are likely to emerge from improved methodologies and creative analysis of information-rich ribosome profiling data sets.

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Chapter 4
Pathways in De Novo Biosynthesis of Selenocysteine and Cysteine in Eukaryotes

Vadim N. Gladyshev, Bradley A. Carlson, and Dolph L. Hatfield

Abstract A distinct feature of selenocysteine (Sec) biosynthesis is that this amino acid is synthesized on its tRNA, designated $tRNA^{[Ser]Sec}$. Sec is then inserted into protein in response to the codon, UGA, as the 21st proteinogenic amino acid. In eukaryotes and archaea, Sec biosynthesis involves several steps. Transfer $RNA^{[Ser]Sec}$ is first aminoacylated by seryl-tRNA synthetase with serine. *O*-Phosphoseryl-$tRNA^{[Ser]Sec}$ kinase phosphorylates seryl-$tRNA^{[Ser]Sec}$ forming *O*-phosphoseryl-$tRNA^{[Ser]Sec}$ that in turn reacts with Sec synthase (SEPSECS) in the presence of selenophosphate yielding Sec-$tRNA^{[Ser]Sec}$. Selenophosphate is generated by selenophosphate synthetase 2 (SPS2) from selenide and/or other selenium metabolites and ATP. Interestingly, sulfide can replace selenide in the reaction involving SPS2 yielding thiophosphate which can then form cysteine- (Cys)-$tRNA^{[Ser]Sec}$ in the presence of SEPSECS. The Cys moiety on Cys-$tRNA^{[Ser]Sec}$ can donate Cys to protein in response to UGA codons at internal positions of mammalian selenoprotein mRNAs. Cys/Sec replacement occurs naturally in vivo and the amount of replacement is dependent on the level of selenium in the diet.

Keywords Codon UGA • Cysteine • De novo biosynthesis • Selenocysteine • Selenocysteine tRNA • Selenoprotein biosynthesis • Selenoproteins

4.1 Introduction

The selenium-containing amino acid, selenocysteine (Sec), is biosynthesized unlike any other known amino acid in eukaryotes in that it is synthesized on its tRNA, designated $tRNA^{[Ser]Sec}$. Transfer $RNA^{[Ser]Sec}$ has many unique features that distinguish it from all canonical tRNAs as detailed in Chap. 1. Other than seryl-tRNA

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synthetase (SERS), which initiates the biosynthesis of Sec by aminoacylating $tRNA^{[Ser]Sec}$ with serine, all enzymes involved in the synthesis of this amino acid are exclusive to the Sec pathway (see [1, 2] and references therein).

Interestingly, cysteine (Cys) may replace Sec on $tRNA^{[Ser]Sec}$, and Cys may be incorporated into protein in place of Sec as discussed below. Replacement of Cys with Sec or Sec with Cys in cellular metabolism is not too surprising, since the structures of these two amino acids are so similar, differing only in the presence of a selenium versus a sulfur atom. Furthermore, these two amino acids have similar chemical properties and, when present in active sites of enzymes, may catalyze some of the same reactions. An example of the replacement of sulfur with selenium is also known that involves selenomethionine that may be incorporated into protein in place of methionine (reviewed in [3, 4]). In addition, generation of selenized yeast that largely contains selenium in the form of selenomethionine is widely used in the dietary supplement industry for producing selenium supplements [5–7]. Although sulfur replacing selenium in protein is less common, it has been shown to occur in vitro in mammalian cells in culture and in vivo in livers of mice (see [2] and references therein). The biosynthesis of Sec and the molecular mechanism of how Cys replaces Sec in specific selenoproteins are described below.

4.2 Sec Biosynthesis

The Sec codon, which occurs at internal positions of selenoprotein mRNAs, is UGA. The location of the UGA Sec codon within selenoprotein mRNAs can be anywhere from near the N-terminus to the penultimate codon at the C-terminus. Some selenoprotein mRNAs also employ UGA as a stop codon [8]. The distance between the UGA Sec codon and the Sec Insertion Sequence (SECIS) element, which plays a major role in dictating a UGA codon as Sec, is an important factor in determining the efficiency of Sec insertion into protein (see [9, 10] and references therein). Interestingly, in the ciliate *Euplotes*, UGA can code for either Sec or Cys, even within the same mRNA, and the location of the SECIS element relative to the UGA Sec codon is the major governing factor dictating whether this codon designates Sec or Cys [11].

The biosynthesis of Sec on $tRNA^{[Ser]Sec}$ was initially established in *Escherichia coli* (*E. coli*) by Böck and coworkers (reviewed in [12]). Its synthesis in *E. coli* is detailed in Chap. 5 and will not be further discussed herein. The biosynthesis of Sec on $tRNA^{[Ser]Sec}$ in eukaryotes and archaea is more complex than in eubacteria [1, 13] and involves five major steps: (1) $tRNA^{[Ser]Sec}$ is initially aminoacylated with serine in the presence of SERS to form seryl-$tRNA^{[Ser]Sec}$; (2) a kinase, phosphoseryl-$tRNA^{[Ser]Sec}$ kinase (PSTK), phosphorylates the serine moiety on Ser-$tRNA^{[Ser]Sec}$ to form the intermediate, *O*-phosphoseryl-$tRNA^{[Ser]Sec}$ (pSer-$tRNA^{[Ser]Sec}$); (3) synthesis of the active selenium donor, selenophosphate ($H_2SePO_3^-$), is catalyzed by selenophosphate synthetase (SPS2); and (4) and (5) pSer-$tRNA^{[Ser]Sec}$ is converted to another intermediate, most likely dehydroalanyl-$tRNA^{[Ser]Sec}$, and donation of $H_2SePO_3^-$ to this intermediate forms Sec-$tRNA^{[Ser]Sec}$, wherein both these steps are carried out by SEPSECS (see Fig. 4.1).

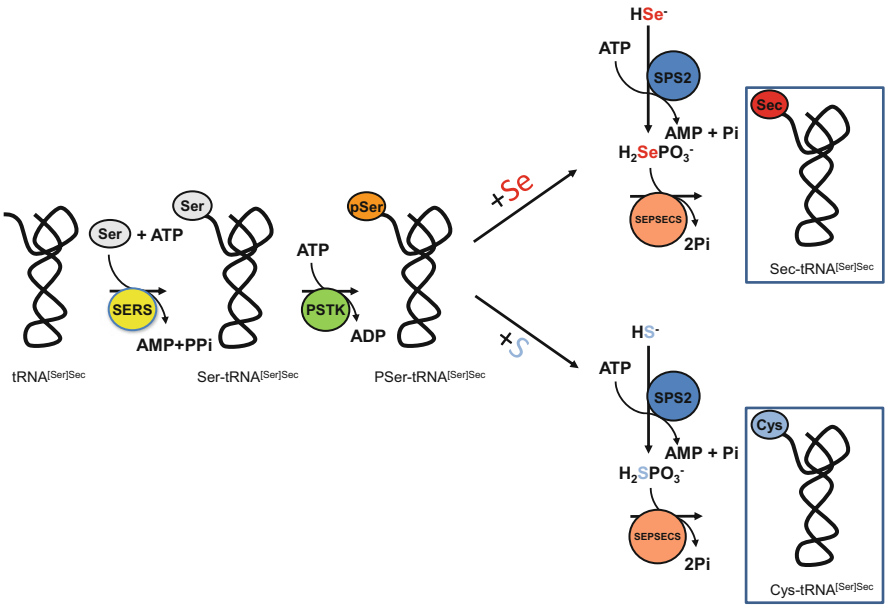

Fig. 4.1 Biosynthesis of Sec in archaea and eukaryotes and de novo biosynthesis of Cys in mammals. The final product of each reaction is boxed. Details are given in the text. Abbreviations: *Pi* inorganic phosphate; *PPi* inorganic pyrophosphate

4.2.1 Discoveries That Provided the Foundation for Sec Biosynthesis

Sec tRNA$^{[Ser]Sec}$ was discovered in 1970 in mammalian and avian livers and initially characterized as a minor seryl-tRNA that either formed pSer-tRNA$^{[Ser]Sec}$ [14] or decoded specifically the termination codon, UGA [15]. Due to the fact that this tRNA recognized only UGA and decoded UGA in protein synthesis [16], it was proposed to be a nonsense suppressor tRNA. It was subsequently shown to have numerous novel features unique to this tRNA (Chap. 1). The important findings in these early studies were the observations that tRNA$^{[Ser]Sec}$ was aminoacylated with serine, decoded specifically UGA and formed pSer-tRNA$^{[Ser]Sec}$.

4.2.2 Step 1: Aminoacylation of tRNA$^{[Ser]Sec}$

As noted above, the first step in the biosynthesis of Sec is the attachment of serine to tRNA$^{[Ser]Sec}$, which is carried out by SERS and requires ATP and Mg^{2+}, as shown in Fig. 4.1.

4.2.3 Step 2: Phosphorylation of the Serine Moiety

PSTK remained elusive for more than 30 years until it was discovered using the methods of comparative genomics. The following rationale was used in identifying the PSTK gene (*Pstk*) [17]. Since SEPSECS, which synthesizes Sec in bacteria, is absent in archaea and eukaryotes, it was assumed that eukaryotes and archaea synthesize Sec by the same pathway that involves pSer-tRNA$^{[Ser]Sec}$ as an intermediate. Bioinformatics analyses for kinase genes present exclusively in those archaea that also encoded selenoprotein genes revealed four possible kinase genes that might be *Pstk*. Analysis of the sequences of these four kinase genes for orthologs in those eukaryotes, which possess the Sec biosynthetic and insertion machinery, revealed a single kinase gene that might encode PSTK. Subsequent biochemical characterization of the protein product demonstrated that it was indeed PSTK [17].

4.2.4 Step 3: Generation of Selenophosphate, the Active Selenium Donor

Two selenophosphate synthetase genes, designated *Sps1* [18, 19] and *Sps2* [20], were discovered in mammals that had homology to bacterial *selD*, and were proposed to also be responsible for synthesizing $H_2SePO_3^-$. Interestingly, *Sps2* had a UGA codon in the position corresponding to a Cys codeword in *selD* suggesting that the expressed protein product synthesized from *Sps2* self-regulates selenoprotein synthesis [20]. Biochemical analyses revealed that SelD and the SPS2 mutant in which Sec is replaced with Cys, synthesized $H_2SePO_3^-$, but SPS1 did not, demonstrating that SPS2 is the functional selenophosphate synthetase in mammals and that SPS1 must have another role in cellular metabolism [1, 21].

4.2.5 Sec Synthesis

Since no ortholog of bacterial *selA*, which synthesizes Sec on tRNA$^{[Ser]Sec}$, was found in mammals, a comparative genomics approach was also used to identify this protein. Eukaryotic and archaea genomes were scanned for co-occurrence of candidate genes involved in Sec biosynthesis, selenoprotein genes and known components of Sec machinery [1]. This search revealed a gene that matched a protein identified previously in patients with autoimmune chronic hepatitis and named soluble liver antigen (SLA) [22]. SLA had been shown to co-precipitate with tRNA$^{[Ser]Sec}$ and to exist in a complex with other proteins involved in Sec metabolism, providing further evidence of its possible role in Sec biosynthesis.

The corresponding gene of SLA is now called *Sepsecs* [1]. PSer-tRNA$^{[Ser]Sec}$ was found to bind strongly to the putative SEPSECS, while tRNA$^{[Ser]Sec}$ bound less well, seryl-tRNA$^{[Ser]Sec}$ bound poorly, and tRNA^{Ser} and seryl-tRNA^{Ser} did not bind. The facts that (1) *O*-phosphoserine was efficiently hydrolyzed from this substrate by SEPSECS, and (2) the resulting intermediate readily accepted the active selenium donor to form Sec-tRNA$^{[Ser]Sec}$ further supported the hypothetical function of SECSEPS. Overall, the data demonstrated that SEPSECS functioned as the Sec synthase in eukaryotic and archaeal Sec biosynthesis [1].

4.3 De Novo Synthesis of Cys and Cys/Sec Replacement In Vitro and In Vivo

As noted above, Cys and Sec are structurally similar. Their genetic language is different, however, in that Cys is decoded by the codewords, UGU/UGC, while Sec is decoded by UGA. Replacement of Sec with Cys in thioredoxin reductase 1 (TXNRD1) has been observed in the livers of selenium deficient rats [23]; however, the mechanism was not determined, and thus, we assessed the mechanism of insertion of Cys into TXNRD1 in lieu of Sec as discussed below.

4.3.1 In vivo Studies

When thiophosphate ($H_2SPO_3^-$) was added to the media of NIH 3T3 cells and the intracellular selenoproteins analyzed, Cys was found to have replaced Sec in TXNRD1 virtually completely [24]. On the other hand, when mice were maintained on selenium deficient (0 ppm selenium), selenium adequate (0.1 ppm selenium), or selenium enriched (2.0 ppm selenium) diets, and TXNRD1 and thioredoxin reductase 3 (TXNRD3) in liver subsequently isolated, purified and analyzed by mass spectrometry [24], about 50 % of liver TXNRD1 and 3 were found to contain Cys in place of Sec in selenium deficient animals, about 10 % in selenium-adequate animals and no replacement in selenium-enriched animals. These studies provided the background for determining the precise mechanism of how such replacement occurs.

4.3.2 In Vitro Studies

To establish the replacement of Sec with Cys in vitro, we initially rationalized that sulfide could replace selenide in synthesizing Cys catalyzed by SPS2 [24]. Thus, the enzymes and other components required for synthesizing Sec-tRNA$^{[Ser]Sec}$ from

pSer-tRNA$^{[Ser]Sec}$ were prepared and Sec biosynthesis carried out. Addition of $H_2SPO_3^-$ to the reaction with pSer-tRNA$^{[Ser]Sec}$ and SEPSECS yielded Cys-tRNA$^{[Ser]Sec}$ as did incubation of pSer-tRNA$^{[Ser]Sec}$, SEPSECS, sodium sulfide, ATP and SPS2(Cys). These two reactions demonstrated that $H_2SPO_3^-$ could replace $H_2SePO_3^-$ yielding Cys-tRNA$^{[Ser]Sec}$ [24]. De novo synthesis of Cys on tRNA$^{[Ser]Sec}$ in mammals is shown in Fig. 4.1 (lower panel).

4.4 Concluding Remarks

The biosynthetic pathway by which the essential element selenium is incorporated into Sec, the 21st amino acid in the genetic code, has been established in eukaryotes and archaea (Fig. 4.1). As shown in the figure, Sec biosynthesis occurs on its tRNA, tRNA$^{[Ser]Sec}$, which represents the only known amino acid in eukaryotes whose synthesis takes place on its tRNA, and was the last proteinogenic amino acid in mammals whose biosynthesis was resolved. Sec-tRNA$^{[Ser]Sec}$ donates its Sec moiety to the nascent polypeptide chain in response to the UGA codon in mRNA generating a selenoprotein product (Chap. 2). Selenium and selenoproteins play major roles in the many health benefits attributed to selenium and some of these roles are: (1) serving as a cancer chemopreventive agent (Chap. 27); (2) delaying the onset of AIDS in HIV positive patients (Chap. 28); (3) reducing the incidence of heart disease, (4) boosting immune function (Chap. 42); (5) and regulating the aging process. Thus, assessing the pathway of how selenium makes its way into protein provides an important step in understanding the overall function of this element in health.

Two of the genes involved in Sec biosynthesis are *Pstk* and *Sepsecs* (Fig. 4.1). They were both identified in mammals using computational, comparative genomics approaches, followed by biochemical analyses. These data suggested that the selenoprotein biosynthetic pathway shown in Fig. 4.1 is the same used in all archaea and eukaryotes that synthesize selenoproteins.

The issue whether SPS1 or SPS2 or both enzymes were used to synthesize the active selenium donor, $H_2SePO_3^-$, was resolved largely by biochemical studies. SPS2 was found to be the enzyme that synthesizes $H_2SePO_3^-$, while the exact role of SPS1 is still elusive [1, 21]. However, the latter has recently been shown to be an essential protein, with a role in regulating redox homeostasis in mammals [25]. Cys in place of Sec in TXNRD1 and TXNRD3 was found to occur in vivo in both cells in culture and in mice, and the mechanism of how this replacement occurred was established using in vitro studies [24]. Thus, Cys can be synthesized de novo and the replacement of Sec with Cys suggests novel roles of Cys in mammalian metabolism.

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Chapter 5
Prokaryotic Selenoprotein Biosynthesis and Function

Michael Rother

Abstract Selenoproteins, i.e., proteins containing selenocysteine (Sec), are found in organisms of all three domains of life, *Eukarya*, *Bacteria* and *Archaea*. Sec is mostly present in the catalytic site of redox-active enzymes with diverse biological functions. The pathway for Sec synthesis and incorporation into proteins during translation differs from that of the 20 canonical amino acids, as Sec is synthesized on its transfer RNA (tRNA) and encoded by UGA, which normally signals termination of translation. The pathway of Sec biosynthesis and incorporation is well understood in Gram-negative bacteria, but considerable gaps of knowledge still exist in the respective systems of Gram-positive bacteria and *Archaea*. This chapter summarizes prokaryotic selenoprotein synthesis and function with a focus on emphasizing the differences between bacterial and archaeal pathways.

Keywords *Archaea* • *Bacteria* • Recoding • Selenocysteine • Selenoprotein • Translation

5.1 Introduction

Selenium was first isolated in lead chambers of a sulphuric acid production factory and named after the Greek goddess of the moon, Selene, by the Swedish chemist Jöns Jacob Berzelius in the early nineteenth century [1]. Initially, selenium was considered to be toxic before its beneficial qualities were recognized in the 1950s. Today, selenium is regarded as an essential trace element for many organisms due to the important roles of selenium-containing cellular macromolecules.

Despite selenium's narrow biologically beneficial "window" for most organisms (deficiency below approximately 0.05 ppm and toxicity above approximately 5 ppm in mammals [2]) and the low environmental concentrations of bioavailable selenium species (typical environmental concentrations of selenate ranging from 0.1 to 20 nM), it is still not clear if organisms requiring selenium possess a dedicated transporter and what selenium species is transported into the cell. For *Escherichia* (*E.*)

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coli, the specific incorporation of selenium into macromolecules is already saturated at 0.1 μM selenite in the medium [3]; still, it is not known how selenium is transported across the cytoplasmic membrane in *E. coli*. It was recently suggested that the putative membrane protein, YedE, which contains two sulfur transport domains and is encoded in an operon with predicted sulfurtransferase, YedF, may serve as a selenium-specific transporter because it occurs only in selenium-utilizing organisms [4]. However, this possibility needs to be experimentally verified. At higher concentrations, *E. coli* metabolizes selenium via the sulfur assimilation pathway [5].

Biologically active selenium occurs in three forms: (1) as a constituent of a base modification (5-[(methylamino)methyl]-2-selenouridine) in certain transfer RNAs (tRNAs, [6]; (2) as a non-covalently bound selenium-containing cofactor [7]; and (3) as the cotranslationally inserted amino acid selenocysteine (Sec). In this chapter, only Sec will be considered.

5.2 Selenocysteine

Sec is by far the best-investigated form of biologically active selenium. It was discovered in 1976 [8] and only 10 years later it was found that Sec is co-translationally inserted into growing polypeptides at the position of an in-frame UGA (opal) nonsense codon in mRNA [9, 10], which led to its designation as the 21st genetically encoded amino acid.

Proteins containing Sec, designated selenoproteins, are found in members of all three domains of life, *Bacteria*, *Archaea*, and *Eukarya*, with Sec almost exclusively located in the catalytic site of redox-active enzymes. Why organisms employ Sec is still not fully understood because: (1) the majority of known organisms do well without Sec; (2) the specific functions of Sec during catalysis are still unknown for most selenoproteins; and (3) homologous proteins with cysteine (Cys) at the respective position exist for all but one selenoprotein, clostridial glycine reductase, in prokaryotes [11]. Still, the use of Sec might be explained by its physico-chemical properties [12]. Its high nucleophilicity and the fact that the selenol group is mostly deprotonated at physiological pH due to its lower pk_a value (5.2 for Sec, 8.3 for Cys) makes Sec more reactive than Cys.

5.3 Selenoprotein Synthesis in Bacteria

5.3.1 Sec Biosynthesis and Incorporation in E. coli

The mechanisms of Sec biosynthesis and its incorporation into selenoproteins (Fig. 5.1a) were first elucidated in *E. coli*. Via genetic screening, four genes were identified, *selA*, *selB*, *selC*, and *selD*, that affected selenium metabolism [13]. *selC* encodes the Sec-specific tRNA ($tRNA^{Sec}$, $tRNA_{UCA}$) [14]. Besides being the longest

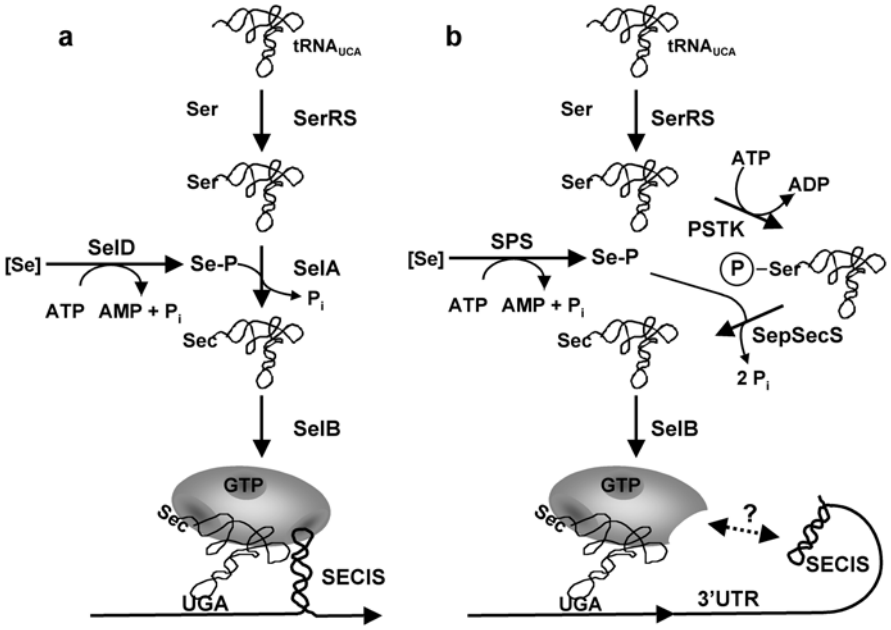

Fig. 5.1 Schematic of selenocysteine biosynthesis and incorporation in *Bacteria* (**a**) and in *Archaea* (**b**). Abbreviations are: *3′ UTR 3′* untranslated region, P_i orthophosphate, *PSTK* seryl-tRNA^{Sec} kinase; *[Se]* reduced selenium-species, *Sec* selenocysteine, *SelA* Sec synthase, *SelB* Sec-specific elongation factor, *SelD* and *SPS* selenophosphate synthetase, *SepSecS O*-phosphoseryl-tRNA^{Sec}:selenocysteine synthase, *Ser* serine, *SerRS* seryl-tRNA synthetase, *Se-P* seleno(mono) phosphate, and the *dashed arrow* indicates an unknown recoding mechanism. The figure is adapted from [77]. See text for details

tRNA in *E. coli*, a number of "invariant" base positions deviate from the consensus sequence of canonical tRNAs (reviewed in [15]). It has an unusually long extra arm and a D stem closed to a six base pair helix minimizing the D loop to four nucleotides, which restricts the types of tertiary interactions within the molecule [16]. However, the most conspicuous difference to canonical elongator tRNAs, is the eight-base-pair aminoacyl-acceptor stem; all other tRNA species have a seven-base-pair stem.

Transfer RNA^{Sec} is aminoacylated with L-serine by canonical seryl-tRNA synthetase (SerRS) (Fig. 5.1a) [14]. The conversion of seryl-tRNA^{Sec} into selenocysteyl-tRNA^{Sec} is catalyzed by Sec synthase (the *selA* gene product), which uses pyridoxal 5′-phosphate (PLP) as a cofactor (reviewed in [15]). The activated selenium donor for this reaction is seleno(mono)phosphate, synthesized from a reduced selenium species by selenophosphate synthetase (SPS), the *selD* gene product [17–19]. Although thiophosphate also serves as substrate in the Sec synthase reaction in vitro leading to cysteyl-tRNA^{Sec}, the catalytic efficiency is much lower [20]. Furthermore, SPS from *E. coli* is highly specific for selenium [20]. Interestingly, mammalian SPS2 (a selenoprotein itself) displays a much higher ambiguity between selenium and sulfur, maybe to "intentionally" generate cysteyl-tRNA^{Sec} in times of selenium starvation [21].

Sec synthase is a ring-shaped homodecamer [22] and binds $tRNA^{Sec}$ in a 1:1 stoichiometry [22, 23]. Furthermore, the $tRNA^{Sec}$•SelA binary complex is bound by SPS resulting in a 1.3 MDa ternary complex, which may serve to channel otherwise toxic selenium species to Sec synthase [24].

Transfer RNA^{Sec} is only recognized by the Sec-specific elongation factor, SelB (the *selB* gene product) [25]. SelB from *E. coli* shares significant homology to the canonical translation elongation factor EF-Tu in its three N-terminal domains [26]; it binds selenocysteyl-$tRNA^{Sec}$ and GTP stoichiometrically and discriminates not only $tRNA^{Sec}$ from the other elongator tRNAs [27], but also distinguishes selenocysteyl-$tRNA^{Sec}$ from uncharged $tRNA^{Sec}$, seryl-$tRNA^{Sec}$ or alanyl-$tRNA^{Sec}$ [28].

A unique property of bacterial SelB is that it interacts with a stem-loop structure of approximately 40 nucleotides, the SECIS (*Sec insertion sequence*) element, immediately 3′ of the UGA codon (the term, SECIS element, was coined for the corresponding eukaryal mRNA element [29]). Binding to the SECIS element is mediated by the L-shaped 17 kDa C-terminal domain IV of SelB, consisting of four winged helix domains [26] not present in EF-Tu (reviewed in [30]). Formation of the quaternary complex between SelB, selenocysteyl-$tRNA^{Sec}$, the SECIS element, and GTP is cooperative, wherein selenocysteyl-$tRNA^{Sec}$ is bound at a much higher affinity in the GTP- than in the GDP-state of SelB [31]. The SECIS element is bound with tenfold higher affinity in the presence of selenocysteyl-$tRNA^{Sec}$ [32]. Furthermore, binding of SECIS not only tethers the tertiary GTP•SelB•tRNA complex to the site of translation, but also serves to activate the molecule. GTP hydrolysis-activity is then stimulated at the ribosome when SECIS is bound [33], which in turn accelerates the release of selenocysteyl-$tRNA^{Sec}$ more than a million-fold [31]. These findings indicate that SECIS-binding induces a conformational switch in the complex, that renders SelB compatible for productive interaction with the ribosome, followed by GTP hydrolysis, and subsequent release of selenocysteyl-$tRNA^{Sec}$ in the proximity of the A site. This causes the affinity of SelB with mRNA to decrease resulting in dissociation of the SelB-SECIS complex. Unlike EF-Tu, SelB has, an approximately 10-fold higher affinity for GTP than for GDP, and a nucleotide exchange factor (EF-Ts) is not required [25].

5.3.2 Selenoprotein Synthesis in Other Bacteria

It is unknown whether all bacteria follow the *E. coli* paradigm during selenoprotein synthesis because no other (non-entero) bacteria have been thoroughly investigated in this respect but presence of the same set of factors involved in Sec biosynthesis and insertion suggests principle conservation of the process. Furthermore, a SECIS consensus, even if weak, can be used to identify selenoprotein genes in bacteria [34]. Still, some bacteria show surprising differences compared to *E. coli*. For example, *Haemophilus influenzae*, a γ-proteobacterium like *E. coli*, synthesizes a Sec-containing selenophosphate synthetase [35].

The likely occurrence of different mechanisms for UGA decoding as Sec among bacteria is illustrated by the fact that selenoprotein genes of Gram-positive bacteria cannot be functionally expressed in *E. coli* [36]. In fact, it is difficult to derive a plausible consensus for a Gram-positive SECIS, even in one organism expressing several selenoprotein genes [37]. On the other hand, the observation that the $tRNA^{Sec}$/SelB pair of *Eubacterium acidaminophilum* effected UGA read-through in *E. coli* of selenoprotein mRNAs from *Desulfomicrobium baculatum*, *Campylobacter jejuni*, and *T. denticola*, respectively, suggests that the SelB/SECIS interaction of Gram-positive bacteria is rather promiscuous [38].

5.4 Selenoprotein Synthesis in *Archaea*

The second prokaryotic domain encompasses the *Archaea*. Due to its accessibility for genetic manipulation and the non-essential nature of its selenoprotein synthesis machinery, *Methanococcus maripaludis* is currently the main model organism for studying selenium metabolism in *Archaea* [39]. It is intriguing to note that six of the eight known archaeal selenoproteins are directly involved in the organisms' primary energy metabolism, methanogenesis [40]. After (draft) sequencing over 500 archaeal genomes, the only *Archaea* containing selenoproteins are still methanogens belonging to the orders *Methanococcales* and *Methanopyrales*.

Archaeal $tRNA^{Sec}$ is more similar to the eukaryal than to the bacterial counterparts and possesses all structural features differentiating it from other elongator tRNAs [15]. No homolog of SelA was found in *Archaea*, and instead, a kinase was detected, which occurs only in selenoprotein-containing archaea and eukaryotes [41]. Biochemical analysis revealed that the protein transfers a phosphate group from ATP to seryl-$tRNA^{Sec}$ generating *O*-phosphoseryl-$tRNA^{Sec}$, which led to its designation as *O*-phosphoseryl-$tRNA^{Sec}$ kinase (PSTK) [41, 42]. The physiological significance of this reaction in vivo was unclear until a PLP-containing protein from *Methanococcus maripaludis*, which is homologous to the soluble liver antigen/liver pancreas protein (SLA/LP) [43, 44], was shown to catalyze the selenophosphate-dependent conversion of *O*-phosphoseryl-$tRNA^{Sec}$ to selenocysteyl-$tRNA^{Sec}$. As a result of these findings, the enzyme was named *O*-phosphoseryl-$tRNA^{Sec}$:selenocysteine synthase (SepSecS) [45]. An elaborate biochemical study, conducted simultaneously, showed that the pathway in mammals is identical, and therein, SepSecS was designated SecS [46]. Although catalyzing very similar reactions, the tetrameric SepSecS and the decameric SelA seem to have arisen via convergent evolution [22, 47].

As the function of bacterial Sec synthase in converting seryl-$tRNA^{Sec}$ to selenocysteyl-$tRNA^{Sec}$ is expanded during evolution and was separated into two reactions, PSTK and SepSecS, in *Archaea* and *Eukarya*, the question arose as to what evolutionary advantage is conferred by employing an "extra" enzyme, PSTK, that was so stably retained in evolution. Currently, there are three plausible explanations: since (1) *O*-phosphoseryl- would provide a better leaving group (phosphate) than seryl- (water) for replacement with selenium, the overall kinetic efficiency may

be improved in the case of PSTK/SepSecS as compared to SelA [45]; since (2) seryl-tRNA^{Sec} is more sensitive to deacylation than other aminoacyl-tRNAs [48], phosphorylation by PSTK could stabilize the aminoacyl-tRNA [41]; since (3) cysteyl-tRNA^{Sec} is formed in vitro with thiophosphate more efficiently from *O*-phosphoseryl-tRNA^{Sec} [21] than from seryl-tRNA^{Sec} [20], the replacement of Sec with Cys could be achieved in the event selenium is scarce. Indeed, severely selenium starved rats incorporate Cys at the position of Sec in thioredoxin reductase, probably in order to salvage at least some enzymatic activity [49]. However, Sec-utilizing archaea like *Methanococcus voltae* or *M. maripaludis* synthesize whole sets of separately encoded isoenzymes containing Cys at the position of Sec, which substitute for the selenoproteins under selenium-deprived conditions [40]. Furthermore, *M. maripaludis* is able to insert Cys or tryptophan at Sec-encoding UGA via a mechanism not involving the Sec machinery [50]. Thus, it appears not necessary for *M. maripaludis* to render Sec codons prone to Cys insertion via cysteyl-tRNA^{Sec}.

As observed in *Bacteria* and *Eukarya*, Sec insertion in *Archaea* is directed by UGA on the mRNA. However, *Archaea* do not contain SECIS elements within the coding region of the selenoprotein mRNAs, but in the 3′ untranslated regions (3′ UTRs) [51, 52]. Notably, the SECIS elements employed in the three domains of life appear to be completely unrelated.

Since SelB is a key component of the bacterial selenoprotein synthesis machinery, it was assumed that the situation would be the same in *Archaea*. Indeed, the archaeal SelB homolog (aSelB) binds guanosine nucleotides and aminoacyl-tRNA^{Sec} as expected [53] and a *M. maripaludis* strain lacking aSelB could not produce selenoproteins [54]. The C-terminal extension of aSelB is rather short and completely unrelated to that of bacterial SelB, where it is responsible for SECIS-binding. Although analysis of the crystal structure suggested that the C-terminal extension of aSelB may bind RNA [5], all our efforts to demonstrate direct aSelB-SECIS interaction in the *M. maripaludis* system were thus far unsuccessful ([53] and C. Sattler, S. Goetz, M. Rother, unpublished). These observations, and the fact that the situation is similar in the eukaryal system, suggest that in *Archaea*, communication between the SECIS element in the 3′ UTR effecting recoding and the site of that recoding (on the ribosome at UGA) is established by one or more as of yet unknown factor(s).

5.5 Selenoproteins of Prokaryotes

Despite the fact that the majority of prokaryotes do not contain selenoproteins, nearly all phylogenetic clades harbor members which do. Their unifying feature appears to be an (facultative) anaerobic life style, which possibly reflects the primordial nature of the Sec-utilizing trait. Unambiguous identification of selenoproteins still relies on experimentally verifying the presence of Sec in the protein, either via mass spectrometry or via incorporation of radioactive selenium. However, bioinformatic approaches proved to be very helpful in identifying potential selenoprotein

genes and, thus, the selenoprotein-containing organisms [55, 56]. Such predictions are based on searching sequenced genomes for UGA codons, either in conjunction with potential SECIS elements [34], or through analyses of sequences adjacent to in-frame UGA codons, because most selenoproteins contain homologs in which Sec is replaced with Cys [57]. Based on bioinformatic analyses, selenoproteins have been classified into more than 50 families [58, 59], 25 of which occur in prokaryotes [60]. Another surprising insight revealed by analyzing large data sets is the potential size of prokaryotic selenoproteomes. While *Mycobacterium* species only encode a single selenoprotein, formate dehydrogenase, *Alkaliphilus oremlandii* OhILA encodes 13, and *Syntrophobacter fumaroxidans* encodes 39 [56, 57, 61].

5.5.1 Predicted Selenoproteins

Numerous "new" prokaryotic selenoproteins were identified by bioinformatic approaches (reviewed in [59]), most of which are oxidoreductases potentially involved in signaling, detoxification, e.g., of reactive oxygen species, and maintaining cellular redox homeostasis (e.g., alkylhydroperoxidase-like protein, arsenate reductase, disulfide-bond oxidoreductase-like protein, glutaredoxin, glutathione peroxidase, glutathione S-transferase, peroxiredoxin, thiol-disulfide isomerase-like protein, and thioredoxin). Intriguing is the presence of homologs of mammalian deiodinases and selenoprotein W (encoded in, for example, *Gemmata obscuriglobus*). The function of these "eukaryal" selenoproteins in these organisms is not known.

5.5.2 Formate Dehydrogenase

Formate dehydrogenase (FDH) is the most widely distributed selenoprotein found in nature and it has been suggested that the genes encoding FDH, together with the genes encoding the system for Sec biosynthesis and incorporation into protein, were extensively transferred laterally [60]. FDH catalyzes the reversible reduction of CO_2 to formate and can be involved in energy metabolism, carbon fixation or pH homeostasis. The diverse cellular roles of FDH are reflected by the considerable differences found in subunit composition, kinetic properties and types of electron acceptors utilized [62]. The natural electron acceptor for Sec-containing FDH can be cytochrome (cyt_b for the *E. coli* FdhN), $NADP^+$ (for the *Moorella thermoacetica* enzyme), ferredoxin (for the *Clostridium pasteurianum* enzyme) or F_{420} (a 2-deazaflavin derivative functionally analogous to NAD^+; in the FDH of methanogenic archaea), where FDH functions in formate oxidation rather than CO_2 reduction. FDH contains Fe/S clusters and a pterin cofactor coordinating either molybdenum or tungsten [63].

5.5.3 Hydrogenase

Sec-containing Ni-/Fe-hydrogenase is the second most common selenoprotein in prokaryotes. Hydrogenases catalyze the reversible reduction of protons and are widely distributed among prokaryotes and lower eukaryotes. The electron carriers used vary depending on the organism. In the active site of Ni/Fe hydrogenase either three Cys plus a Sec (or four Cys) residues coordinate a Ni atom, with two of these Cys also binding a Fe atom [64]. The Sec-containing hydrogenases of methanogenic archaea are either coenzyme F_{420}-reducing or F_{420}-non-reducing. The latter enzyme is tightly associated with the heterodisulfide reductase and electrons derived from H_2 oxidation are transferred via Fe/S clusters and FAD from the hydrogenase to both the heterodisulfide and ferredoxin by flavin-based electron bifurcation [65]. Central to this tight interaction and to electron transfer is the hydrogenase delta subunit VhuD, which contains two Sec residues [66].

5.5.4 Glycine Reductase

The glycine reductase system is almost exclusively found in amino acid fermenting clostridia [67]. It consists of three proteins, P_A, P_B, and P_c. While P_c does not contain Sec, P_B is a substrate-specific selenoprotein. Although the corresponding subunits of all characterized substrate-specific P_B proteins of glycine, betaine, sarcosine and proline reductase, respectively (see below), contain Sec, [67], homologous sequences in which the Sec UGA codon is replaced by a Cys UGU or UGC codon were identified in databases [11]. P_A is the only selenoprotein for which no Cys-containing homolog is known [57]. Sarcosine reductase and betaine reductase share their P_A and P_C components with glycine reductase but contain different substrate-specific, Sec-containing P_B proteins [68].

5.5.5 Proline Reductase

The reduction of D-proline to 5-aminovalerate in clostridia seems to proceed by a different mechanism compared to glycine reduction. Still, D-proline reductase is similar to P_B of glycine reductase and composed of three different subunits, PrdB which contains Sec, and two proteins resulting from processing of proprotein PrdA [69].

5.5.6 Methionine Sulfoxide Reductase

Methionine sulfoxide reductase (Msr) reduces oxidized methionine residues in proteins, which arise by action of reactive oxygen species [70]. MsrA is specific for the *S*-form of methionine sulfoxide, whereas MsrB is specific for the *R*-form. Both

MsrA and MsrB can either be selenoproteins or non-selenoproteins, depending on the organism. Sec-containing MsrA was characterized in *Alkaliphilus oremlandii* OhILA [61]. Biochemical analysis showed that Sec-containing Msr has a vastly higher activity, but also that it is more difficult to reductively regenerate the selenoproteins, than the Cys-containing isoforms [71, 72].

5.5.7 Seleno(mono)phosphate Synthetase

Sec-containing SPS has been found in all three domains of life. The enzyme provides the activated selenium donor during Sec synthesis. What exact role the Sec residue plays during catalysis in these enzymes has not been shown, but it could account for the enzyme's promiscuity towards sulfide in generating thiophosphate. Also, requirement of a Sec-containing enzyme for Sec synthesis may allow for direct integration of environmental signals, like the availability of selenium, into regulating selenoprotein synthesis.

5.5.8 Formyl-Methanofuran Dehydrogenase

Formyl-methanofuran dehydrogenase (FMD) catalyzes the reduction of CO_2 and methanofuran to formyl-methanofuran, which is the initial step in methanogenesis from CO_2 in all methanogenic archaea. FMD contains either Mo or W, and Fe/S clusters and is composed of five subunits, wherein two share considerable similarity with FDH [73].

5.5.9 Heterodisulfide Reductase

Obligate hydrogenotrophic methanogens reduce the heterodisulfide of coenzyme M and coenzyme B by a cytoplasmic multienzyme complex composed of the F_{420}-nonreducing hydrogenase (see above) and a soluble heterodisulfide reductase, an iron-sulfur flavoprotein, which in *Methanococcus* and *Methanopyrus* species also contains Sec. However, the role of Sec in this enzyme is not known.

5.5.10 HesB-Like Selenoprotein

M. maripaludis synthesizes a small, approximately 11 kDa selenoprotein, similar to HesB from *Synechococcus*, that was originally identified through genome analyses [57]. However, it is not an essential protein [74]. Beside its distant relation to IscA, which is involved in Fe/S-cluster assembly, nothing is known about the function of the HesB-like selenoprotein.

5.5.11 Benzoyl-CoA Reductase

Strictly anaerobic sulfate-reducing bacteria capable of aromatic degradation (e.g., *Geobacter* and *Desulfococcus*) synthesize three selenoproteins [75]. As deduced from genomic sequences, one of them, BamF, which is homologous to F_{420}-non-reducing hydrogenase, is potentially a Sec-containing subunit of benzoyl-CoA reductase, BamBCDEFGHI [76]. However, the role of Sec in this complex remains to be elucidated.

5.6 Concluding Remarks

Despite the remarkable depth of knowledge gained about physiological, mechanistic, structural, and evolutionary aspects of selenoprotein synthesis in prokaryotes, considerable gaps in our understanding remain to be closed to fully appreciate selenium biology in these organisms. For example, it is still not known how selenium is specifically transported across the cytoplasmic membrane. Furthermore, some microorganisms, like *M. maripaludis*, regulate gene expression in a selenium-responsive fashion, but the underlying mechanism and how the availability of selenium in the environment is sensed is unknown. In *Archaea*, the recoding of UGA from a nonsense to a Sec codon by a SECIS element in the 3′ UTR appears similar to the mechanism present in eukaryotes. However, factors mediating this recoding in *Archaea* have not yet been identified. Lastly, the cellular roles of the many newly identified selenoproteins will have to be elucidated. Thus, research dedicated to selenium biology in prokaryotes promises exiting new insights into the complex metabolic and regulatory networks within such simple organisms.

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Chapter 6
The Role of Selenium in Human Evolution

Louise White and Sergi Castellano

Abstract Human migration around the world has resulted in habitation of environments that differ widely in their soil selenium (Se) levels. Consequently, populations around the world have widely different dietary intakes of this essential micronutrient. Localized adaptation to dietary differences has occurred in genes that process macronutrients, such as lactose and starch, as well as micronutrients, such as Se, iron and iodine. Recent evidence indicates that local adaptation to dietary Se intake has occurred in genes that incorporate Se into selenoproteins and regulate the use of this micronutrient. The genetic adaptation signal is particularly strong in populations that live in the Se-deficient regions of China, suggesting that Se homeostasis has been important during recent human evolution and that changes in the use and regulation of this element may have helped humans to inhabit environments that contain an inadequate supply of dietary Se.

Keywords Diet • Humans • Local adaptation • Micronutrients • Natural selection • Nutrition • Selenium

6.1 Introduction

Adaptation to local environmental conditions has shaped human genomes throughout history, enabling populations to settle in many diverse environments across the globe [1]. Inadequate nutrition is an important threat to survival and fertility. Because diets around the world differ in both composition and ability to supply essential nutrients, local adaptation of genes that modify nutrient uptake and metabolism has been prevalent in human history [2]. Levels of the essential micronutrient, selenium (Se), vary widely around the world [3], and these differences combined with the impact of Se deficiency on health and fertility [4] suggest that differences in dietary Se intake sustained over many generations may have shaped the evolution of selenoproteins and genes that regulate use of this micronutrient in humans.

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This chapter will explore the current evidence for human adaptation to dietary Se levels. We begin by discussing some instructive examples of positive selection (the rise in frequency, within a population, of alleles that confer an advantage to survival or reproduction) in other nutrition-related genes. We will then outline two factors that are important for local adaptation to Se: geographical variation in Se levels and genetic variation in Se-related genes. We will then present evidence for human local adaptation to dietary Se first on a global scale and then more specifically in China, where Se deficiency diseases have been endemic [5]. Finally, we will discuss some of the genes and single nucleotide polymorphisms (SNPs) that have likely contributed to this process.

6.2 Diet and Natural Selection

Diets around the world vary in their composition, and this variance provides scope for adaptation to local conditions. Thus, nutrient-related genes are frequently identified as targets of natural selection [2]. Indeed, two of the most widely known examples of environmental adaptation in humans concern alleles involved in the metabolism of the macronutrients, lactose and starch.

Lactose is the primary carbohydrate in mammalian milk, and its digestion requires hydrolysis by the lactase enzyme encoded by the *LCT* gene. Most mammals secrete lactase only until weaning and are thus unable to fully digest milk in adulthood [6]. With the advent of dairy herding, human populations in some parts of the world began consuming milk beyond infancy. In response to this dietary change, adaptation to prolong lactase expression into adulthood occurred independently in both Europe and Africa. In Europe, the derived T allele at *LCT* position -13,910 enhances the activity of the *LCT* promoter and allows lactase transcription to continue beyond infancy [7]. This lactase persistence allele is carried by 6–96 % of the European population in a cline from south to north [8]. The *LCT* -13,910 T allele is believed to have been rare until around 4,000 years ago based on its absence from a large group of ancient European DNA samples dated to before 2,450 BCE [9]. This rapid rise in allele frequency at a time when pastoralism became established in Europe suggests that lactase persistence may have been advantageous in that environment, leading to positive selection on the LCT -13,910 T allele [8, 10]. The *LCT* locus also shows signatures characteristic to positive selection, namely a longer haplotype, less microsatellite diversity, and greater population differentiation than expected for a neutrally evolving gene [10]. In Africa, three different alleles (-14,010C, -13,915G, and -13,907G) in the *LCT* locus have been subject to positive selection in pastoralist populations [11, 12].

Starch is a major component of many human diets in post-agricultural societies [13]. One of the two enzymes that function in starch digestion is salivary α-amylase, which is encoded by the *AMY1* gene [14]. This gene exhibits a positive selection

signal (excess population differentiation) in populations that have historically consumed agriculture-based diets [13].

Genes that affect micronutrient metabolism have also been found to have experienced positive selection during human history. Iron is an essential micronutrient that is required for respiration, DNA synthesis, and oxygen transport in the blood [15]. The C282Y mutation of the iron uptake regulatory gene *HFE* increases iron serum and hemoglobin levels in heterozygous individuals as a result of increased intestinal absorption of iron from the diet [16]. Given that iron deficiency is estimated to affect 15–30 % of the global population and often leads to poor health outcomes [17], a mutation such as C282Y that increases iron absorption could be advantageous in circumstances of low-iron diets. Homozygotes for C282Y, however, have an even higher increase in iron levels leading to detrimental iron deposition in the liver and other organs (hereditary hemochromatosis) [18]. Despite this homozygous disadvantage, haplotype analysis indicates that the *HFE* C282Y mutation appears to have been under positive selection in Europe [19]. Population-level iron deficiency has not been correlated with geographical variance in soil iron concentrations, suggesting that the geology of Europe has not been a selective force on the *HFE* gene [20]. One possibility is that an iron accumulation phenotype became important in Europe with the introduction of farming, which led to a dietary shift from a meat-rich, high-iron diet to a cereal-based diet low in iron [21].

The micronutrient iodine is essential for the synthesis of the thyroid hormones triiodothyronine (T3) and thyroxine (T4). These hormones play roles in metabolism, adipose function, cardiac function, and the regulation of liver enzymes and pituitary hormones [22]. The distribution of iodine around the world is uneven, and many regions are deficient [23]. A genome-wide selection scan identified the *IYD* gene that facilitates iodide salvage in the thyroid and the *TRIP4* gene that activates thyroid receptor in the presence of thyroid hormone as being under positive selection in African Pygmy populations [24]. Pygmy and Bantu populations both live in the same iodine-poor environments but the incidence of goiter in Pygmy populations is only 25 % of that in Bantu populations, suggesting that variants in these genes have allowed Pygmy populations to adapt to iodine deficiency [24].

Given that Se is an essential micronutrient that has a wide variance in dietary availability around the world, genes involved in Se metabolism or selenocysteine (Sec) synthesis and insertion are likely to have been similarly impacted by natural selection during human history.

6.3 Worldwide Availability of Se

Human dietary Se intake is mainly determined by the Se content of the soils used for food production [3]. Soil levels depend largely on the Se content of the underlying bedrock [3, 25], although other factors, such as precipitation, may also influence

soil Se content [26]. The global variation in soil Se levels is vast. For example, some regions of India have 0.025 mg/kg, while regions in Ireland have 1,200 mg/kg [3]. Substantial variance can also occur on a more local scale. For instance, China has some of the highest and lowest soil Se levels in the world [3].

This variation in soil Se levels results in a correspondingly wide variation in Se status in different populations around the world [3, 27]. Estimates of one billion Se-deficient individuals globally have been reported [25], and these people may experience increased risk of mortality, immune dysfunction, infertility, and cognitive decline as a result [4]. Recorded average daily Se intakes have ranged from less than 10 μg per day in parts of Malawi to more than 4,900 μg per day in parts of China [28, 29]. This vast difference in Se intakes offers scope for genetic adaptation in populations that live in parts of the world where Se levels are not optimal.

6.4 Genetic Variation in Human Se-Related Genes

Human genetic variation in selenoprotein and other Se-related genes has not been investigated on a global scale until recently. Most reported polymorphism in these genes has been biased towards variants with known or suspected functional effects. These variants are likely to have been discovered due to their intermediate or high frequency in populations, although this does not necessarily imply local adaptation. Detection of natural selection relies on the pattern of polymorphism at non-functional sites linked to the functional allele [30]. Thus, to adequately examine genetic adaptation to environmental factors such as dietary Se, an unbiased set of variants containing both coding and non-coding polymorphisms that have been uniformly ascertained in a global set of populations is needed. Furthermore, such investigation would allow variants found in only one or a few populations as well as low-frequency variants to be identified. These variants are more likely to cause local adaptation and correlate to levels of Se in the diet.

Such data have recently become available as part of the second release of the selenoprotein database SelenoDB [31]. We surveyed genetic variation in selenoproteins and genes involved in the regulation of Se and Sec metabolism in 53 human populations with diverse Se nutritional histories. The populations were from CEPH-HGDP, a resource that consists of populations from Africa, the Middle East, Europe, Asia, Oceania, and America that have not experienced recent migrations, making them suitable for studies of local adaptation [32]. We employed an array capture approach to enrich all coding regions and UTRs in addition to 200 nucleotides (nt) of the flanking introns and 2,000 nt upstream of each gene to include promoter regions before sequencing on the Illumina platform [31]. The variants identified were stringently filtered, leading to uniformly high data quality across all populations. SelenoDB 2.0 currently contains polymorphism data for 928 individuals and approximately 8,000 SNPs in selenoprotein and Se-related genes. Nearly 75 % of these SNPs are newly reported. The database thus represents a valuable resource for future evolutionary, functional, and medical studies of Se-related genes.

6.5 Human Adaptation to Dietary Se

Since migrating out of Africa around 60,000 years ago [33], humans have come to inhabit a wide range of environments. In order to thrive in these diverse environments, these populations have not only employed cultural and technological advances, but have also often experienced genetic adaptation [2, 34]. The positive selection of genetic variants that enable more efficient utilization of dietary components, such as carbohydrates and micronutrients, has been important during human evolution (discussed in Sect. 6.2). Colonization of new environments exposed these populations to various dietary Se levels (discussed in Sect. 6.3). Given the importance of Se to human health and fertility, we hypothesize that human populations that live in environments with insufficient Se experienced adaptive evolution in genes that use or regulate Se.

Several properties observed in present-day genomes provide an indication that positive selection has occurred. If we are interested in local adaptation that occurred as humans migrated around the world to inhabit new environments, then looking at patterns of population differentiation provides a measure well-suited for detection of signals of positive selection that occurred during this time frame [35]. Genes that show unusually high levels of population differentiation are strong candidates for having experienced adaptation to local environmental conditions, as when an ancestral population splits into two populations and each inhabits a different environment, alleles that are not important to adaptation to environmental conditions are likely to remain at similar frequencies in the two populations. Conversely, alleles that are advantageous under particular environmental conditions are likely to rise in frequency in the population in this environment but not in the population in a different environment. Thus, significant allele frequency differences develop between the two populations. Allele frequency differences among populations can be quantified using the F_{ST} statistic [36], which represents the proportion of the overall genetic diversity that is accounted for by allele frequency differences between pairs of populations. F_{ST} values range from 0 (no population differentiation) to 1 (no allele sharing among populations) [37].

As with all tests for genetic adaptation, when using F_{ST}, it is necessary to distinguish the pattern of genetic variability expected under neutrality from that expected under positive selection. To achieve this, loci that are thought to be evolving neutrally, such as pseudogenes, can act as controls. First, F_{ST} values for both candidate genes and pseudogenes for the same pairs of populations are calculated, and then only the candidate gene F_{ST} values that are extreme relative to the neutral pseudogene F_{ST} value distribution for each population pair are used. As a result, the effects of neutral demographic processes, such as population size changes, that can impact F_{ST} are largely removed.

Proper expression of all 25 human selenoproteins depends on the function of at least 19 genes that regulate and process Se in the body, creating a situation in which many genes are dependent on the supply of a single nutrient (Se). Therefore, these genes will likely experience natural selection as a group in an interdependent way

Table 6.1 Candidate genes grouped according to type and biological process[a]

Genes			
Selenoproteins	**Cys-containing paralogs**	**Regulation of Se and Sec[2]**	
Glutathione peroxidase (GPx) 1, 2, 3, 4 and 6	Glutathione peroxidase (GPx) 5, 7, and 8	**Transport and uptake of Se into cells**	**Incorporation of Sec into proteins**
Iodothyronine deiodinase (DIO) 1, 2 and 3	Selenoprotein R (SelR) 2 and 3	Selenoprotein P (SEPP1)	CUGBP, Elav-like family member 1 (CELF1)
15 kDa selenoprotein (Sep15)	Selenoprotein W 2 (SepW2)	Apolipoprotein E receptor 2 (ApoER2)	Elongation factor for Sec (EFSec)
Selenoprotein H (SelH)	–	Megalin	Eukaryotic translation initiation factor 4A3 (EIF4A3)
Selenoprotein I (SelI)	–	**Metabolism of Se**	ELAV like RNA binding protein 1 (ELAVL1)
Selenoprotein K (SelK)	–	Selenocysteine lyase (SCLY)	Ribosomal protein L30 (RPL30)
Selenoprotein M (SelM)	–	Se binding protein 1 (SELENBP1)	SECIS binding protein 2 (SBP2)
Selenoprotein N (SelN)	–	**Biosynthesis of Sec**	Selenophosphate synthetase 1 (SPS1)
Selenoprotein O (SelO)	–	O-phosphoryl tRNASec Kinase (PSTK)	tRNASec 1 associated protein 1 (TRNAU1AP)
Selenoprotein R 1 (SelR1)	–	Selenophosphate synthetase 2 (SPS2)	Exportin 1 (XPO1)
Selenoprotein S (SelS)	–	O-phosphoseryl-$tRNA^{Sec}$ Se transferase (SEPSECS)	–
Selenoprotein T (SelT)	–	Seryl-tRNA synthethase (SARS2)	–
Selenoprotein V (SelV)	–	**tRNA**	–
Selenoprotein W 1 (SepW1)	–	Ser-$tRNA^{Sec}$, copy in chromosome 17	–
Thioredoxin reductase (TXNRD) 1, 2 and 3	–	Ser-$tRNA^{Sec}$, copy in chromosome 19	–
–	–	Ser-$tRNA^{Sec}$, copy in chromosome 22	–

[a]Modified from [38]

[2]SEPP1 and SPS2 are regulatory selenoproteins

(polygenic adaptation). To look for signals of polygenic adaptation in Se-related genes, we divided the human genes in SelenoDB 2.0 into three groups: selenoproteins, cysteine (Cys)-containing paralogs, and regulatory genes (see Table 6.1 and

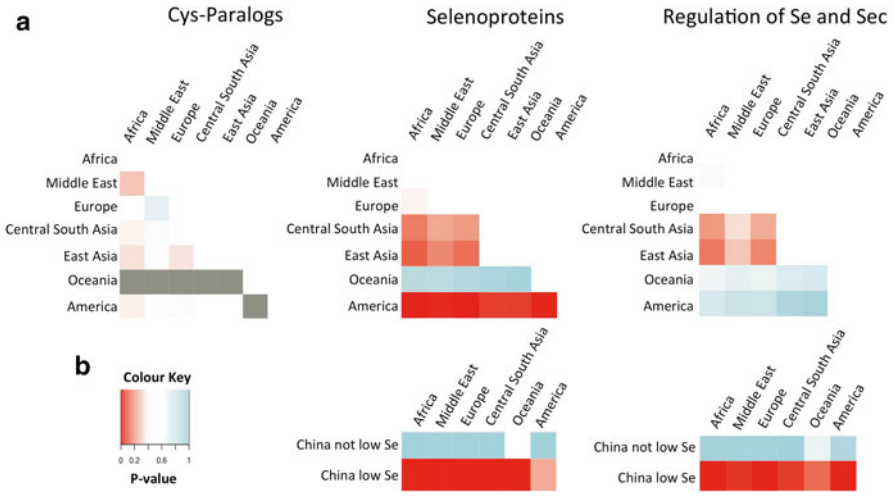

Fig. 6.1 Comparison of F_{ST} values falling in the top 5 % of the distribution of neutral F_{ST} values across populations from different world regions. The relative departure of these values from the neutral expectation was tested such that populations from the left-hand regions that appear in *red* are significantly more differentiated than populations living in the regions at the top. Regions in *gray* do not have F_{ST} in the top 5 %. (**a**) Comparison among regions in the world. (**b**) Comparison between populations in China living in either Se-deficient or Se-adequate regions and other world regions. Figure taken from [38] by permission of Oxford University Press

[38]). We investigated whether these groups of genes had differentiated, in certain regions of the world, beyond the neutral expectation. We focused on genes that bear the strongest signature of local adaptation. That is, we examined genes with F_{ST} values that rank in the top 5 % of the distribution of neutral pseudogene F_{ST} values. We then compared the relative strength of their departure from the neutral expectation among different regions of the world. Both selenoproteins and regulatory genes exhibit significant signatures of local positive selection in certain world regions (Fig. 6.1).

Both selenoproteins and regulatory genes show a polygenic signal of local adaptation in Central-South and East Asia, and selenoproteins exhibit an additional signal in America. Most of the populations from East Asia are from China, a part of the world with large Se-deficient regions [5, 27]. Low soil Se has also been reported in Pakistan, where most of the Central South Asian populations were sampled [32, 39–41]. Therefore, these signals of positive selection likely reflect adaptation to environmental Se levels. The positive selection signal in America, however, is interpreted more cautiously since Se deficiency in humans has not so far been reported in this region [27]. In contrast to the adaptation signals in Asia, the signal in America is largely accounted for by the *DIO2* gene and is also largely localized to the Columbian population rather than being widespread across the region, suggesting that positive selection in America may not reflect adaptation to Se but may be explained by other selective pressures.

The Cys-containing paralogs do not show a significant adaptation signal in any world region. These genes have functions similar to those of selenoproteins and may therefore be hypothesized to play a role in compensating selenoprotein function under low-Se conditions. Sec and Cys, however, differ in their catalytic properties and are not functionally interchangeable [42–44]. Therefore, the Cys-containing genes may be unable to compensate for the function of selenoproteins. Indeed, the lack of significant adaptation signals in these genes likely reflects their independence from Se and suggests that Cys-containing genes do not play a compensatory role in low Se conditions.

6.6 Adaptation to Se Deficiency in China

As previously mentioned, China contains large Se-deficient regions [27], in which diseases linked to Se deficiency, such as Keshan cardiomyopathy and Kashin-Beck osteoarthropathy, were endemic prior to recent Se supplementation [3, 5]. To examine the signal of adaptation in East Asia in more detail, we grouped the populations from China according to residence within the Se-deficient regions (six populations) or Se-adequate regions (eight populations) [38]. We then tested the relative strength of departure from the neutral expectation as before (Fig. 6.1b). These investigations revealed that the signal of local positive selection in selenoproteins and regulatory genes was strongly localized to the populations in the Se-deficient regions, supporting the idea that Se deficiency is driving adaptation in these genes.

6.7 Genes That May Be Important for Adaptation to Se Deficiency

The signatures of local positive selection in Asia result from the synergy of several alleles. Table 6.2 lists some genes and SNPs that contribute most strongly to the signatures of local positive selection in selenoproteins and regulatory genes in East Asia.

DIO2 is the gene that makes the greatest contribution to the signals of positive selection in Asia and is the only gene with a large contribution to the signals in America [38]. DIO2 is an oxidoreductase that catalyzes the conversion of the iodine-dependent thyroid hormone T4 to its active form T3. The activity of this enzyme thus depends on the supply of two micronutrients (Se and iodine), and deficiency of these micronutrients has been linked to Kashin–Beck disease in China [45]. A threonine (Thr) to alanine (Ala) substitution, which ranks among the SNPs with the strongest population differentiation in East Asia (rs225014), has been shown to affect maximal DIO2 enzyme activity [46]. Individuals homozygous for the Ala allele at this locus exhibit significantly lower DIO2 enzyme velocity than

Table 6.2 Genes and SNPs that are major contributors to the signatures of positive selection in East Asia[a]

Gene rank	Gene	SNP Rank within gene	SNP ID in SelenoDB 2.0, dbSNP[2]	Gene region
Selenoproteins				
1	DIO2	1	SNP00000190_2.0	Coding/Intron
		16	SNP00000160_2.0, rs225014	3′ UTR/Coding
2	SelS	1	SNP00005709_2.0	Intron
		15	SNP00005750_2.0, rs34713741	Promoter
3	GPx1	1	SNP00000352_2.0	5′ UTR/ Promoter
		10	SNP00000330_2.0, rs1050450	3′ UTR/Coding
		11	SNP00000377_2.0, rs3811699	Promoter
Regulatory genes				
1	CELF1	1	SNP0005905_2.0	Intron
		2	SNP0005814_2.0	Downstream/ 3′ UTR
2	SPS2	1	SNP00001698_2.0	5′ UTR
		2	SNP00001710_2.0	Promoter
3	SEPSECS	1	SNP00001861_2.0	Coding
		2	SNP00001794_2.0	Intron
4	ELAVL1	1	SNP0006324_2.0	3′ UTR/ Downstream
5	Ser-tRNA^{Sec}	–	–	–

[a] Modified from [38]

[2]The SNP ID in dbSNP is given for those SNPs discussed in the text

those with Ala/Thr or Thr/Thr genotypes [46]. Of the other genes in the *DIO* family, *DIO3* contributes to the selection signals in Asia, but *DIO1* does not.

The gene with the second largest contribution to the selection signals in Asia is SelS, a gene involved in stress response in the endoplasmic reticulum and in mediating inflammation [47]. This gene contains a non-coding G-to-A substitution (rs34713741) that shows high levels of population differentiation in Asia and has been associated with colorectal cancer risk in European and Asian populations [48, 49]. Inflammation is an important component of the innate immune response to pathogens [50], and genes that influence the inflammatory response have been shown to be frequent targets of natural selection [51]. Thus, *SelS* may have experienced local adaptation, not solely in relation to Se, but also in response to pathogens. One pathogen that may be pertinent to this selection is the Coxsackie B virus, which is thought to exhibit increased virulence under conditions of Se deficiency and has been suggested as a potential factor in the etiology of Keshan disease [52].

Another gene with a large contribution to the signal of selection in Asia is *GPx1*, which is the most abundantly expressed selenoprotein gene, and it is expressed in almost all tissues [53]. The GPx1 enzyme functions as an antioxidant to reduce hydrogen peroxide, thereby protecting cells from oxidative damage. Two SNPs in *GPX1*, a proline (Pro) to leucine (Leu) amino acid substitution (rs1050450) and a noncoding A-to-G SNP in the promoter region (rs3811699) exhibit high levels of

population differentiation in East Asia and have been shown to influence transcriptional and enzyme activity [54]. The rs1050450 Pro allele, which is found at higher frequencies in East Asia than in the rest of the world (excluding America), results in 40 % higher GPx1 activity than the Leu allele [54]. This allele has been previously suggested to be under positive selection in Asia [55]. In addition, the A allele at rs3811699 in GPx1 was shown to exhibit 25 % higher transcriptional activity than the G allele [54]. This A allele is also found at higher frequencies in East Asia than anywhere else (except America). This predominance of the higher activity alleles at these loci may reflect adaptation to the low Se levels across much of the region. Association studies have implicated variants in *GPx1* in the pathogenesis of both Keshan disease and Kashin-Beck disease. Individuals that have at least one derived (Leu) allele at rs1050450 have a greater risk of Keshan disease [56]. In addition, GPx1 is expressed abnormally in adults with Kashin-Beck disease, and individuals with at least one derived allele at rs1050450 are at greater risk of developing this disease [57, 58].

Amongst the regulatory genes with the strongest signatures of selection in Asia are Sec biosynthesis genes, notably *SPS2*, *SEPSECS*, and the Sec-specific tRNA on chromosome 17. Variants in these three genes likely alter the overall efficiency of Sec synthesis, and this may explain why these genes have been targeted by natural selection. SPS2 is particularly interesting because, in addition to being a selenoprotein, this gene product also synthesizes selenophosphate used to convert serine aminoacylated onto $tRNA^{Sec}$ to Sec [59, 60]. Since SPS2 is a selenoprotein and therefore is dependent on the supply of its own product, it has been proposed to serve as an auto-regulator of selenoprotein synthesis [61]. One of the "master regulators" of selenoprotein synthesis, SBP2 [62] (the other being $tRNA^{Sec}$), does not show strong signals of positive selection in Asia, but strikingly, two genes that regulate SBP2, *CELF1* and *ELAVL1*, are amongst the most strongly selected genes in Asia. CELF1 and ELAVL1 bind the 3′ end of *SBP2* mRNA and likely regulate its translation by competing with the mRNA degradation machinery for mRNA binding, thereby slowing or preventing the degradation process [62, 63]. By regulating SBP2 expression, variants in these genes could affect the expression of the entire selenoproteome, making these genes prime targets for natural selection.

As the Se transport protein SEPP1 acts as a conduit to Se availability throughout the body, *SEPP1* is a strong candidate to have experienced adaptive evolution during human acclimatization to low Se conditions. Indeed, *SEPP1* is one of the regulatory genes that contributes to the signal of positive selection in Asia. This likely reflects the importance of the hierarchical prioritization of Se supply to certain tissues for the maintenance of cellular function in the absence of sufficient dietary Se [64]. The specific SEPP1 receptor for the brain and testis (ApoER2) contributes a small amount to the selection signal in Asia, whereas Megalin, the SEPP1 receptor for the kidney and other organs, does not contribute. This difference may reflect adaptation to preserve acceptable levels of Se in the brain and testis under conditions of Se deficiency due to the critical role of these organs in survival and repro-

duction, although these tissues are also thought to have a high Se requirement as a result of their high metabolic activity [65]. In mice, the disruption of SEPP1 or ApoER2 function results in reduced fertility and severe neurological impairment [65, 66]. In humans, plasma levels of SEPP1 are decreased under conditions of Se scarcity [67]. Therefore, variants that compensate for this reduction in *SEPP1* expression by increasing the Se transport efficiency of SEPP1 may underlie the modest signal of positive selection in this gene in Asia.

6.8 Concluding Remarks

The micronutrient Se appears to have been important during recent human evolution, and as humans spread around the world, adaptation in the genes that incorporate Se or regulate its use may have helped humans to inhabit environments that are deficient in this element. Se availability is, for the most part, highly localized, so fine-scale sampling of populations from a variety of locations may reveal further examples of genetic adaptation to Se intake levels around the world. The multitude of micronutrients that are essential for human health and yet variable in their worldwide dietary availability means that many more examples of local adaptation in micronutrient-related genes are likely to be uncovered. Indeed, micronutrient deficiencies may have co-existed in some populations throughout human history. For example, Se and iodine tend to be in short supply concomitantly [68]. The finding that deiodinase genes, which depend on both Se and iodine for their function, bear some of the strongest signals of local adaptation among selenoprotein genes supports this notion. The use of quantitative trait loci, which correlate genetic variants with phenotypic measurements (*e.g.*, tissue micronutrient levels), compliments population genomics as an approach to identify genes involved in adaptation to micronutrients [69].

While the SNPs contributing most to the signals of positive selection in selenoprotein and regulatory genes in Asia and America have been identified [38], little is known about their functional consequences. Future functional studies are likely to elucidate some of the mechanisms behind the identified signals of local adaptation. Furthermore, these SNPs may help explain differences in the worldwide prevalence and severity of Se-related diseases. One major limitation of the current work on adaptation to Se levels is the absence of comparable quantitative data on soil, plant, or dietary Se levels across the world. This absence of data hinders the correlation of human Se status with genetic data. Therefore, further insight into the biology of micronutrients and the evolution of their use by humans is likely to come from linking alleles at functional sites with precise measurements of micronutrient levels throughout the world.

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Chapter 7
The Chemistry of Selenocysteine in Proteins

Rebecca N. Dardashti*, Linoy Dery*, Reem Mousa*, Shahar Dery*, Post S. Reddy*, and Norman Metanis

Abstract While the existence of the rare 21st proteinogenic amino acid, selenocysteine (Sec, U) in cellular proteins has been known for over 40 years, recent advances in peptide chemistry have supported its importance not only in the biological function of natural selenoproteins, but also in synthetic systems. Besides its obvious applications in the synthesis of selenoproteins, fundamental differences between the chemistry of cysteine's thiol and Sec's selenol moieties have added a surprising number of technologies to the protein chemist's toolbox. In this chapter, we discuss Sec's impact on chemical protein synthesis, folding of challenging disulfide-rich proteins, and the chemistry of the little-understood selenoproteins, SEP15 and SELM. Additional important aspects on Sec will be the subjects of other chapters in this book.

Keywords Chemical protein synthesis • Deselenization • Desulfurization • Native chemical ligation • Oxidative protein folding • Selenocysteine • Selenoproteins

7.1 Selenocysteine in Chemical Protein Synthesis

The 21st naturally encoded amino acid selenocysteine (Sec, U), the Se-containing analog of cysteine (Cys, C), is present in the three domains of life, Bacteria, Archaea and Eukarya, in the form of selenoproteins. These proteins exploit the unique chemical properties offered by Sec, which is typically located in the protein's active site. While Sec and Cys are isosteric, key differences in their chemistries allow Sec to perform unique activities within the cell. Compared to sulfur, Se is a better nucleophile and electrophile in nucleophilic exchange reactions [1, 2] due to its higher polarizability. The lower pK_a of its selenol compared to the thiol of Cys (5.5 vs. 8.7)

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[3] means that Sec is mostly deprotonated at physiological pH (selenolate), which further enhances its nucleophilicity. In addition, the considerably lower reduction potential of Sec [4, 5] as compared to Cys means that selenols are easily oxidized upon air exposure and are more resistant to reduction [6, 7].

The chemical similarities between Sec and Cys have been applied, most notably, in chemical protein synthesis. Because different mechanisms of Sec incorporation in bacteria and mammals [8] are a source of inefficient recombinant expression of selenoproteins, chemical protein synthesis (or semi-synthesis) has become a robust and versatile method for accessing selenoproteins. This method is based extensively on two technologies: solid-phase peptide synthesis (SPPS) [9] and chemical ligation of unprotected peptides, most commonly native chemical ligation (NCL) [10, 11]. Regarded as one of the main tools used in the synthesis of moderately-sized proteins (up to ~300 amino acids) [12, 13], classical NCL is initiated when the C-terminal thioester of a peptide undergoes transthioesterifcation when attacked by an N-terminal Cys of another peptide. A subsequent S→N acyl shift affords a native peptide bond at the ligation site (Fig. 7.1) [10]. In 2001, improved access to selenoproteins was enabled when three separate research groups reported the expansion of NCL to Sec in addition to Cys (Fig. 7.1) [14–16]. While one study suggested the low pK_a of selenol could be exploited as a chemoselective ligation at acidic pH [14], others observed slow Sec ligations due to the sensitivity of selenols to air oxidation and stability of the diselenide bonds in solution [15, 16]. This was later supported with evidence that a free selenol was necessary to initiate ligation [17]. Sec-mediated ligation has also been applied to expressed protein ligation (EPL) [18, 19], in which an expressed segment with a C-terminal intein is converted to a C-terminal thioester, finally undergoing NCL with a synthetic peptide. Such an approach was used to prepare a variant of the copper binding protein azurin, in which the Cys ligand of copper was replaced with Sec (Cys112Sec) [20].

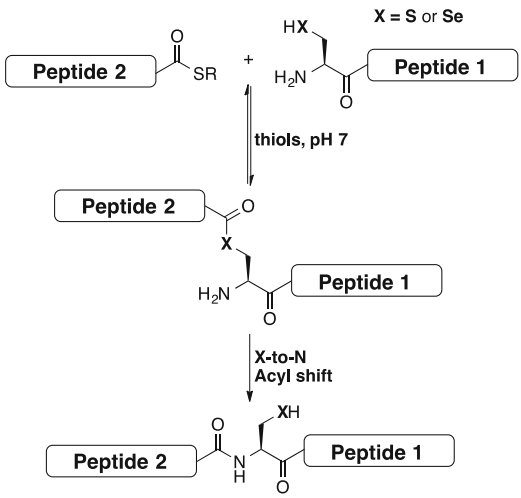

Fig. 7.1 Native chemical ligation (NCL) at Cys or Sec

Due to the rarity of naturally-occurring selenoproteins and both chemical and monetary hurdles in accessing selenols during SPPS [21], few researchers in the field investigated Sec in chemical protein synthesis for some time. The breakthrough that refueled interest in the use of Sec in chemical protein synthesis was the development of selective deselenization of Sec to Ala in the presence of unprotected Cys residues [17]. Deselenization's precursor, desulfurization, expanded available ligation sites in peptide synthesis by allowing ligation at the rare amino acid Cys; subsequent thiol removal provides the native Ala, which is a much more common residue [22]. As originally suggested [22], this approach was later expanded to other ligation sites, (Phe [23], Val [24, 25], Lys [26, 27], Leu [28, 29], Pro [30, 31], Thr [32], Arg [33], Asp [34], and Trp [35]), provided a synthetic "thiolyated" amino acid was accessible.

However, both metal-based desulfurization [22] and radical desulfurization removed all unprotected Cys residues in the sequence (Fig. 7.2a) [36]. Only Cys residues that were orthogonally protected with groups appropriate for the specific method could be preserved [37, 38]. Incorporations of thiol protecting groups on amino acid side-chains and subsequent deprotection inevitably led to lower yields in synthesis [39].

In contrast to these desulfurization procedures, deselenization is a chemo- and enantioselective reaction (Fig. 7.2a). It enabled conversion of Sec to Ala via incuba-

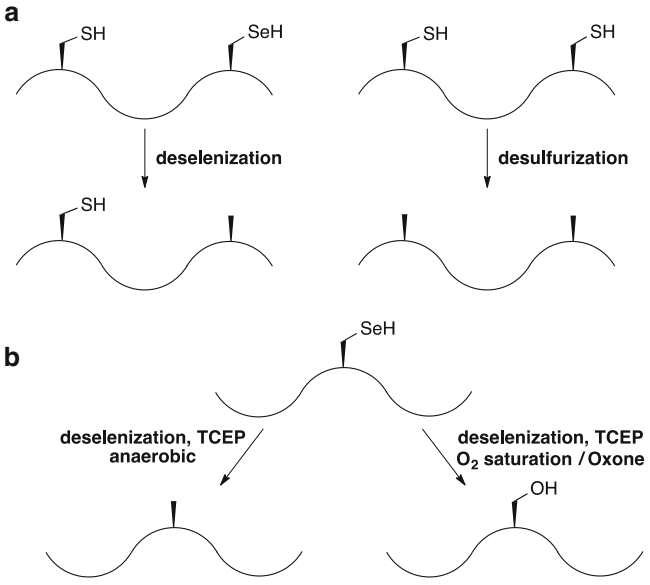

Fig. 7.2 Deselenization *vs*. desulfurization reactions. (**a**) The deselenization of Sec produces Ala, while keeping unprotected Cys residues unaffected, whereas desulfurization leads to conversion of all unprotected Cys residues to Ala. (**b**) While deselenization under anaerobic conditions produces Ala, under oxygen saturation or in the presence of the mild-oxidant oxone, Ser is selectively produced

tion in buffer with *tris*(2-carboxyethyl)phosphine (TCEP) alone, leaving any unprotected Cys residues in the sequence unaffected [17]. As suggested in the original work [17], unique amino acids with β-selenols [40, 41] have also been synthesized to expand ligation sites to other amino acids beside Ala.

In describing selective deselenization, the authors highlighted Se's ability to form a radical species as a key source of unique reactivity, and noted that no radical initiator was needed for the reaction to proceed completely [17]. Further evidence for a radical mechanism was presented in later studies, which showed that in radical promoting conditions, such as heat and UV irradiation, as well as in the presence of radical initiators, the reaction rate increases [42]. However, the presence of the common radical scavenger, sodium ascorbate, decreased, and even halted, the rate of the reaction [42].

Recent studies also indicated that selective deselenization may be applied to expand ligation reactions to Ser [42, 43]. First described as an unwanted side-reaction of the deselenization reaction [17], conversion of Sec to Ser was eliminated under anaerobic conditions [42]. This indicated that the Ser-product formed via a similar radical mechanism in the presence of molecular oxygen. This was supported in a study of the deselenization of β-selenol-phenylalanine [40], wherein the selenol-to-alcohol conversion was enhanced due to the stability of the benzylic radical formed during deselenization. To support this hypothesis, saturation with O_2 [42] or addition of the mild oxidant oxone [43] under deselenization conditions led exclusively to formation of the Ser product. This approach was chemoselective in the presence of reactive amino acid side chains and in the synthesis of the glycoproteins, MUC5AC and MUC4 [43]. Notably, as Ser is one of the most common amino acids in proteins, this constitutes a major step forward in the field of chemical protein synthesis.

To apply selective deselenization in the synthesis of longer proteins (>150 AA), which require the ligation of multiple segments, selenazolidine (Sez) [44] has recently been used as a masked precursor for N-terminal Sec. Sez, the seleno-analog of thiazolidine (Thz) [45], has proven especially useful in the synthesis of proteins with non-strategically placed Cys residues. A key element of this approach is the facile and smooth conversion of the Sez to Sec using $MeONH_{2\ \text{at low pH}}$. Selective deselenization after NCL affords the natural Ala at the ligation site [44].

Sez was utilized in the total chemical synthesis of a 125-residue protein, human phosphohistidine phosphatase 1 (PHPT1) and a protein variant containing an unnatural His analog, β-thienyl-*L*-alanine (Thi), PHPT1 (His53Thi) (Fig. 7.3) [44]. PHPT1's three Cys residues are located toward the C-terminus (Cys69,71 and 73) and, as such, are not strategically placed for multiple ligation steps. Due to both the protein's size and sequence, PHPT1 served as a very appropriate model to show Sez's utility in future syntheses [44].

Replacing sulfur with Se in NCL can also be achieved by replacing the C-terminal peptide thioester with a selenoester. A facile ligation at the Pro–Cys junction, which is typically extremely slow, was executed using a preformed prolyl selenoester peptide. Through a comparative study, α-selenoesters were found to be at least two orders of magnitude more reactive acyl donors than peptide α-thioesters [46].

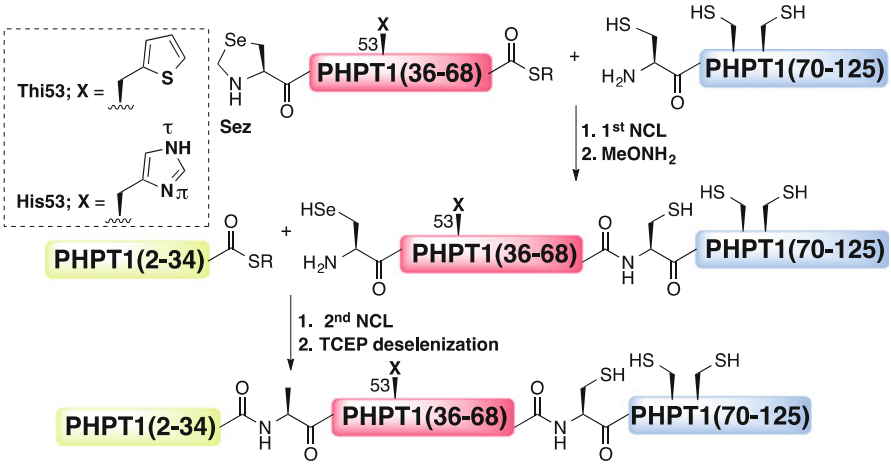

Fig. 7.3 Chemical protein synthesis utilizing Sez as a masked Sec for proteins with non-strategically placed Cys residues. This approach was used recently in the chemical synthesis of two analogs of the protein PHPT1

Recently, a rapid and additive-free ligation between a C-terminal peptide selenoester and N-terminal Sec-peptide was demonstrated. This ligation was completed in minutes, even at sterically hindered ligation junctions [47].

As described here, the use of Se in chemical protein synthesis has increased as it provides access not only to selenoproteins, but also to a variety of previously difficult-to-access sequences. We envision that these synthetic tools will enable easy synthesis of selenoproteins and will be of great utility in protein chemistry in general.

7.2 Selenocysteine in Oxidative Protein Folding

Sec incorporation, which has been utilized in chemical protein synthesis to prepare selenoproteins and access long, difficult sequences, has also been applied in the world of protein folding. Sec's low redox potential [4, 5] and pK_a [3], as well as its increased nucelophilicity and electrophilicity [1, 2], can enhance thiol-disulfide-like exchange reactions that are essential for protein folding.

Proper folding is critical for protein function. Under suitable in vitro conditions, proteins fold spontaneously into their three-dimensional native states, as all the information required for this process is contained in the primary amino acid sequence [48]. However, for many Cys-rich proteins, the number of possible disulfides increases substantially and, accordingly, the folding process becomes more complicated, with an increased risk of non-native disulfide bond formations (scrambled isomers) and/or the formation of "trapped" intermediates [49–51]. In addition, many Cys-rich proteins also have an increased risk of aggregation, due to intermo-

lecular disulfide bond formation. Replacing native Cys residues in the protein with Sec has been demonstrated as a promising strategy to simplify oxidative folding of Cys-rich peptides in vitro without changing their native conformations or biological activities.

The use of Cys-to-Sec substitution in protein folding was first applied to study the 21-residue endothelin-1 (ET-1) [52], a peptide with potent vasoconstrictor activity and two disulfide bonds. An analog was prepared via SPPS in which the residues of one native disulfide pair were replaced with Sec residues. Notably, the preferred formation of the diselenide bond reduced the likelihood of formation of scrambled isomers. The function and structure of the folded seleno-analog, [Sec^{3},Sec^{11},Nle^{7}] ET-1, was almost indistinguishable from that of wild type ET-1.

After establishing Sec's utility in ET-1 folding without affecting its bioactivity, apamin, an 18-residue toxin with four Cys residues, was studied. Although its native structure was known, its folding pathway was ambiguous. Accordingly, three apamin analogs were synthesized [53, 54] with strategic Cys-to-Sec substitutions mimicking the three possibilities of the first disulfide bond formation: two crossed (globule), parallel (ribbon), or consecutive (bead). Through oxidative folding, the diselenide intermediate was trapped and, using CD and NMR analysis, the isomer most likely to lead to the native structure, was predicted. Thus, through Cys-to-Sec substitution, a greater understanding of the folding pathway of apamin was reached [52].

Sec incorporation has also been applied to proteins with folded structures that are notoriously complex. In 2006, this technique was applied by synthesizing α-selenoconotoxins as an alternative to naturally occurring conotoxins with therapeutic properties. The synthetic seleno-analogs were more stable than wild type, implying that they could be useful in designing stable scaffolds for peptide-based drugs [55]. Sec incorporation was also used to investigate the conotoxin ω-GVIA [56, 57], whose native structure contains three disulfides, and, as a result, yields of the natively folded protein tended to be low. To investigate the effect of Cys-to-Sec substitution on ω-GVIA's folding, Bulaj synthesized three analogs, each of which had one native disulfide replaced with a diselenide [56]. C8U/C19U ω-GVIA folded with the greatest efficiency, and substituting a diselenide for a native disulfide in GVIA had no detectable effect on function while enabling a marked acceleration in the kinetics of folding and an increase in yield of the properly folded analogs [56].

This strategy has been extended to other disulfide-rich peptides with medical applications, such as μO-conotoxin MrVIB, which targets voltage-gated sodium channels (VGSCs). μO-conotoxin MrVIB availability is limited due not only to its scarcity in natural sources, but also due to its hydrophobic nature and tendency to aggregate during isolation and folding. Similar to previous studies, synthetically prepared seleno-analogs of MrVIB with native diselenides [58] showed a decrease in the number of isomers formed with incorrect crosslinks and an increased yield of the natively folded analogs. As an added benefit, a study of seleno-MrVIB analogs' biological activity showed that in TTX-R sodium channels, C2U/C20U MrVIB exhibited slightly higher potency and greatly enhanced selectivity when compared to the wild type MrVIB [58].

Cys-to-Sec substitution was also applied to the folding of bovine pancreatic trypsin inhibitor (BPTI), a 58-residue protein with three disulfide bonds (5–55, 14–38, 30–51). BPTI's folding occurs via a bifurcated pathway whose intermediates contain only native disulfide bonds, and is considered a model for many other protein folding pathways [50, 51]. In contrast to previous Cys-to-Sec studies, the effect of substituting a non-native disulfide with a diselenide was applied in the hopes of altering the population of intermediates. During folding, C5U/C14U BPTI indeed bypassed two long-lived intermediates present in folding of wild type BPTI, N*(5–55;14–38) and N′(14–38;30–5) and, as a result, the rate of folding was greatly enhanced [66]. Further studies [67] showed that replacing only one Cys with Sec, rather than a disulfide pair, successfully accelerated folding. However, the intermediate N*(5–55;14–38) was still present during folding. Finally, replacing a native disulfide 14–38 crosslink with diselenide was also successful in accelerating folding. However, the solvent-exposed selenols caused the protein to undergo aggregation and lowered the overall yield.

In addition to employing Sec substitution as an intramolecular catalyst for protein folding, small molecule diselenides have been used as intermolecular catalysts for oxidative protein folding. Because they are easily re-oxidized after reduction, diselenides have long been known to enable thiol/disulfide exchanges, even when present in catalytic amounts [59]. As such, a variety of small-molecule diselenides have been developed and used in the folding of challenging proteins both in vitro and in vivo. The most studied molecule, selenoglutathione (GSeSeG) (Fig. 7.4), is the seleno-analog of natural glutathione, which acts as a redox buffer in vivo and in vitro [6, 7, 60, 61]. Folding studies with GSeSeG have been performed with a variety of biologically relevant proteins such as BPTI, hirudin, lysozyme, human epidermal growth factor and interferon α-2a. This technology has also been applied in the folding of more challenging proteins such as bovine serum albumin, which

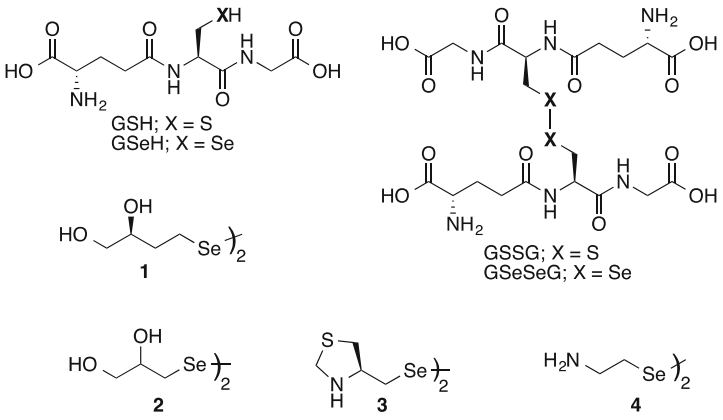

Fig. 7.4 Reduced (GSeH) and oxidized (GSeSeG) selenoglutathione, the selenium analogs of common folding redox buffer GSH and GSSG. Other, smaller-size diselenides, 1–4, have been recently tested as additives for oxidative protein folding

contains 17 disulfide bonds, and the antigen-binding (Fab) fragment of the antibody MAK33 [61]. In all cases, the diselenide molecules facilitated the folding process of these proteins and resulted in higher yields, perhaps through rescuing trapped intermediates [62]. Recently, a new class of small molecule diselenides, either commercially available or readily prepared (Fig. 7.4), were found to be as effective as GSeSeG in promoting oxidative protein folding [7, 63]. As difficult-to-fold proteins are revisited or discovered, the chemistry of selenols in the form of Sec residues and small seleno-molecules will continue to contribute to the field of protein folding.

7.3 SEP15 and SELM in In Vivo Protein Folding

Sec is not only utilized to optimize protein folding in vitro, but is also suspected to play a role in protein folding pathways in vivo. Of the 25 known genes encoding for human selenoproteins [64], seven are located in the endoplasmic reticulum (ER): the 15 kDa selenoprotein (SEP15), selenoprotein M (SELM), SELS, SELK, SELN, SELT and DIO2 [65]. Because protein folding and disulfide bond formation occur in the ER, it has been proposed that these selenoproteins, or at least some of them, are involved in the formation, isomerization (shuffling) or reduction of incorrect disulfide bonds of proteins undergoing folding pathways in the cell. Two of the better-studied selenoproteins in the ER are SEP15 and SELM. These proteins share 31 % sequence identity and both have structures that are highly similar to the thioredoxin fold [66]. However, their exact biological functions are still unknown.

SEP15 was first described in human T-cells and its tissue distribution suggests it is most common in prostate and liver [67, 68]. This 162 residue protein contains Sec in a conserved CXXC-like motif found in the thiol-disulfide family of oxidoreductases. Interestingly, the motif in SEP15, CXU, is one amino acid shorter than similar motifs in comparable proteins. While SEP15 is found in the ER, it does not itself contain an ER retention sequence. A major breakthrough in uncovering its probable function was published in 2001, where SEP15 was found to form a tight 1:1 complex (K_D of 20 nM) with the ER protein UDP-glucose:glycoprotein glucosyltransferase (GT) [69]. GT is responsible for specific glycosylation of misfolded proteins in the ER [70], which are then folded by the calnexin/calreticulin glycoprotein folding system [69]. SEP15's Cys-rich domain at its N-terminus, a conserved domain from plants to humans, is an essential element of the SEP15:GT complex [71]. Therefore, SEP15's presence in the ER can be attributed to its interaction with GT.

Based on the above findings, it was proposed that SEP15 plays a role in the detection and refolding of glycoproteins with erroneously formed disulfide bonds. Through utilization of its CXU motif as a reductant or oxidant, refolding of the misfolded glycoprotein marked by GT is initiated [71]. It has also been suggested that SEP15 could be related to the regulation of the enzymatic activity of GT [71].

The distantly related, homologous selenoprotein, SELM, was discovered after SEP15, but even less is known about its activity [72]. Similar to SEP15, SELM

contains the redox-active motif CXXU. While it is expressed as a 145-residue protein, the mature SELM consists of 122 amino acids after its N-terminal 23-amino acid signal peptide is cleaved in the cell [72]. In contrast to SEP15, SELM is found mostly in the brain [72], contains an ER retention sequence at the C-terminal end [72], and does not interact with GT [71].

Additional evidence for thioredoxin-like activity for SEP15 and SELM was found in the NMR structures of wild type SEP15 from *Drosophila melanogaster* and the U48C mutant of SELM from *Mus musculus* [73]. The study revealed that the two proteins share the characteristic α/β-fold of the thioredoxin superfamily. Furthermore, the active site Sec of SEP15 and SELM were in locations similar to that of catalytic Cys in thioredoxin [73].

The exact roles of SELM and SEP15 are still unknown (see Chap. 19), but possible functions have been suggested. Localization in the ER, which is an oxidizing environment responsible for disulfide bond formation, and the presence of a similar redox-motif to thiol-disulfide oxidoreductases indicate their potential thiol-disulfide oxidoreductase activity [74]. Additionally, the identity of the X amino acids in the CXXC-like motif can determine both the redox potential of the enzyme the function of the enzyme as thiol-disulfide reductase, oxidase, or isomerase [75].

While the role of Se in the active site of SEP15 and SELM is still a mystery, evidence of the impact of Sec in a similar context was presented in a study of the thiol-disulfide oxidoreductase, glutaredoxin 3 (Grx3) [5]. Through synthesis and analysis of the wild type and three seleno-analogs of Grx3, it was shown that Cys-to-Sec substitution in the active site motif CXXC influenced the redox potential dramatically, leading to an average difference of ~73 mV and an almost two orders of magnitude higher rate of thiol–disulfide exchange reactions [5]. The latter result was supported by studies of a Grx1 seleno-analog [76]. These findings provide suggestive implications toward the function of selenoproteins with similar redox motifs.

Determining the redox potential of selenoproteins in general, and that of SEP15 and SELM in particular, will be a fascinating step forward in our understanding of their cellular functions. Future studies on these ER-resident selenoproteins should be directed to provide supporting evidence for their proposed function in protein folding.

7.4 Concluding Remarks

The impact of Sec chemistry in the world of protein synthesis has been wide and deep. Sec incorporation via solid-phase peptide synthesis has opened the door to the study of synthetic selenoproteins, while a fuller understanding of the different chemistries of selenols and thiols has enabled the discovery and optimization of selective deselenization in the presence of unprotected Cys residues. The additional development of selenazolidine as a masked Sec precursor allowed for the synthesis

of yet longer proteins with non-strategically placed Cys residues. Its incorporation into standard chemical biology techniques can provide access to proteins, both with and without Se, that were previously challenging to synthesize.

In addition, Sec incorporation has afforded a more thorough study of protein folding and activity. Therapeutic peptides, whose Cys-rich sequences' complicated folding pathways and structures led to notoriously low yields during synthetic production, have seen dramatic improvement of yield and efficiency through Cys-to-Sec substitution methods. Small diselenide molecules with straightforward synthetic schemes have also been shown to enhance protein folding, even when present in catalytic amounts. These findings provide insights not only to Cys-rich peptides in vitro, but also promise to elucidate the role of Sec-containing peptides in vivo, such as the natural ER-resident selenoproteins SELM and SEP15. As the world of selenopeptide chemistry continues to advance, we expect it to promote great strides in the world of chemical biology.

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Chapter 8
Evolution of Selenophosphate Synthetase

Marco Mariotti, Didac Santesmasses, and Roderic Guigó

Abstract Selenophosphate synthetase (SPS or SelD) provides the active selenium (Se) donor for the synthesis of selenocysteine (Sec), the 21st amino acid in the genetic code. In this chapter we summarize the distribution, phylogeny and function of all SPS genes across the tree of life. SPS is a selenoprotein itself in many prokaryotes (SelD) and eukaryotes (SPS2). As most other selenoproteins, SPS has orthologs with cysteine (Cys) in place of Sec. Although absent in many lineages, selenoproteins and SPS occur in bacteria, archaea and eukaryotes. In prokaryotes, SPS supports additional forms of Se utilization besides Sec, most notably the use of selenouridine in tRNAs. The study of selenophosphate synthetases, while serving a map of Se utilization across all sequenced organisms, also highlighted examples of functional diversification within this family. Within archaea, a few *Crenarchaeota* species exhibit a *SelD*-like gene. This is derived from *SPS*, but probably carries a different function, since it never co-occurs with other Se utilization genes. Within eukaryotes, many metazoan genomes, including humans, carry a paralog called *SPS1* in addition to *SPS2*, which replaces the Sec/Cys site with some other amino acid (i.e., threonine, arginine, glycine, or leucine). Strikingly, *SPS1* genes were generated through distinct gene duplication events of *SPS2* in several metazoan clades (e.g., vertebrates and insects). Their function is still unknown, but it appears to be

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different from that of SPS2. Despite independently originated in parallel lineages, *SPS1* genes were shown to share a common function. Thus, this function was likely already present in the parental *SPS2* gene, driving the gene duplication events by sub-functionalization.

Keywords Evolution • Rescue • SelD • Selenocysteine • Selenium utilization • Selenophosphate synthetase • Selenoprotein • SPS • Sub-functionalization

8.1 Introduction

The main biological functions of selenium (Se) in human health are exerted by the non-standard amino acid selenocysteine (Sec), inserted co-translationally into selenoproteins [1]. Selenoproteins are found in organisms across the tree of life. Sec is encoded by in-frame UGA codons present in selenoprotein mRNAs and is incorporated through a dedicated machinery upon recognition of RNA structures acting as cis-signals (SECIS elements, for *Sec i*nsertion *s*equences). Sec synthesis occurs on the cognate tRNA ($tRNA^{Sec}$), in a multi-step process that requires monoselenophosphate. Although showing obvious homology, the mechanisms of Sec biosynthesis and insertion exhibit important differences between bacteria, archaea and eukaryotes (reviewed in [2–5]). Selenophosphate synthetase (SPS, or SelD in prokaryotes) is responsible for providing the active Se donor for the synthesis of Sec, activating selenide to generate selenophosphate at the expense of ATP [6]. *SPS* genes are conserved in all known Sec-encoding genomes, with a remarkable ~30 % sequence identity between *Escherichia coli* and *Homo sapiens* ([7] and see Fig. 8.1).

The three-dimensional structure of SPS proteins [8–11] is highly conserved from bacteria to eukaryotes. SPS acts as a dimer and ATP is bound at the subunit interface, with the binding mediated by magnesium atoms. SPS hydrolyzes ATP subsequently to ADP and then AMP. A mobile N-terminal segment binds the substrate and holds it throughout the whole reaction. The residue responsible for this is thought to be a cysteine (Cys) located in a glycine-rich loop on the N-terminal domain. This same Cys residue is replaced by Sec in many organisms [7, 12], making SPS the only Sec machinery protein that is itself also a selenoprotein. As explained later in this chapter, several metazoans evolved a second, paralogous gene (*SPS1*), carrying other amino acids at this position. SPS1 proteins appear to have a function distinct from selenophosphate synthesis. In all *bona fide* selenophosphate synthetase proteins (i.e., SelD, SPS2), this position is instead conserved with either Sec or Cys. Given their broad phylogenetic distribution, Sec utilization and thus SPS are arguably ancestral traits, already present in the last universal common ancestor. Selenophosphate synthetase, as both a constitutive part of Sec machinery and ancestral selenoprotein family, can be used as a proxy to follow the evolution of Sec utilization across the tree of life.

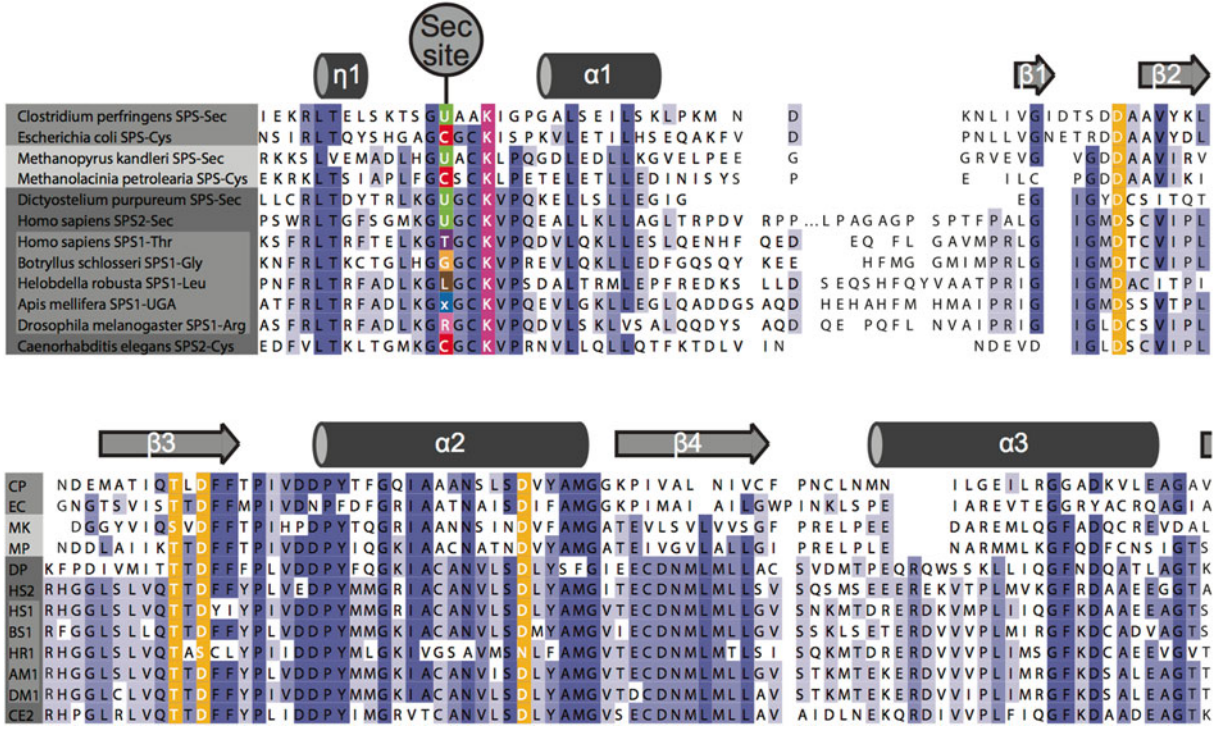

Fig. 8.1 Alignment of SPS protein sequences. This figure contains representative sequences from bacteria, archaea and eukaryotes, including various metazoan *SPS1* genes. *Blue* is used to represent the degree of conservation at each position. Above, the secondary structure inferred from the solved crystal structure in [10] is displayed. Additionally, a few columns of the alignment are highlighted: the Sec site is colored differently after each residue found here, following the color scheme of Figs. 8.2 and 8.3; the nearby essential lysine is in magenta; the residues involved in metal-mediated ATP interactions are in *orange*. Sequences were trimmed at N and C termini, and a few additional positions were removed to improve visualization (e.g., a short fragment was removed from the bacterial sequences after helix α9, which contained the β-sheet and helix named β10 and α10 in [10])

β5
β6
n2
β8
β7
α4
n3
α5
α6
β8
α7
β9
n4
α8
α9
CP
EC
MK
MP
DP
HS2
HS1
BS1
HR1
AM1
DM1
CE2

Fig. 8.1 (continued)

8.2 SPS Supports Diverse Se Utilization Traits in Prokaryotes

8.2.1 Bacteria

Those bacteria that utilize Se are characterized by multiple pathways (see Fig. 8.2). SPS proteins, found in 26–38 % of sequenced bacteria [7, 13], are considered a fundamental genetic indicator of any of the Se utilization traits. In literature, all SPS-dependent pathways (Sec, SeU, etc.) are referred to as "Se utilization traits" [13–17]. We will follow this nomenclature, but the reader should be aware that this excludes other more "passive" Se pathways in which there is no activation to selenophosphate (e.g., Se detoxification in plants). In the last several years, numerous studies exploited the presence of SPS in combination with other gene markers to profile the various forms of Se utilization across genomes [7, 13–17].

The most common form of Se utilization is the insertion of Sec in selenoproteins (18–25 % of sequenced bacteria [7, 13]). Besides *SPS*, Sec-containing organisms possess also the rest of the Sec machinery genes (*tRNA*^{Sec} *or SelC*, *SelA*, *SelB*), as well as one or more selenoprotein genes. The second most common form of Se utilization (16–22 % of sequenced bacteria [7, 13]) is the use of 5-methylaminome thyl-2-selenouridine (SeU) at the anticodon wobble position of certain tRNAs [18]. SeU is synthesized by 2-selenouridine synthase (YbbB), which together with SPS acts as marker of SeU usage in genomes. A third Se utilization trait was recently discovered through the analysis of genomes with an "orphan" *SPS* gene, i.e., not coupled with any other marker of known Se utilization [16, 19]. It appears that Se is used by these organisms as a cofactor to certain molybdenum-containing hydroxylases [20]. Two genes, *YqeB and YqeC*, seem to be required in this process, and thus can be used in conjunction with *SPS* as genetic markers for this form of Se utilization [13, 17]. An estimated eight percent of bacteria possess this trait. It is entirely possible that other forms of Se utilization exist besides these three, and they may be uncovered as more and more sequences become available. As a matter of fact, a recent study showed that, even taking into account the markers for Sec, SeU and Se-cofactor, there are a few species that possess an orphan SPS [13]. These findings, if confirmed, would point to the existence of additional forms of Se utilization.

The known Se utilization traits significantly overlap: many bacterial species possess more than one trait. *SPS* genes are found in all genomes with at least one of these traits. Only in Sec-encoding bacteria, SPS is sometimes present as a selenoprotein. In all other cases, SPS contains Cys at the Sec homologous site. When it contains Sec, the *SPS* gene includes a SECIS element typical of bacteria, which is a small hairpin-loop structure overlapping the Sec-coding UGA.

In many bacterial lineages, SPS is found fused with other genes, a frequent feature of bacterial proteins. Two types of SPS fusions are particularly common with: i) a NADH-dehydrogenase-like domain; and ii) a NifS-like domain (Cys sulfinate

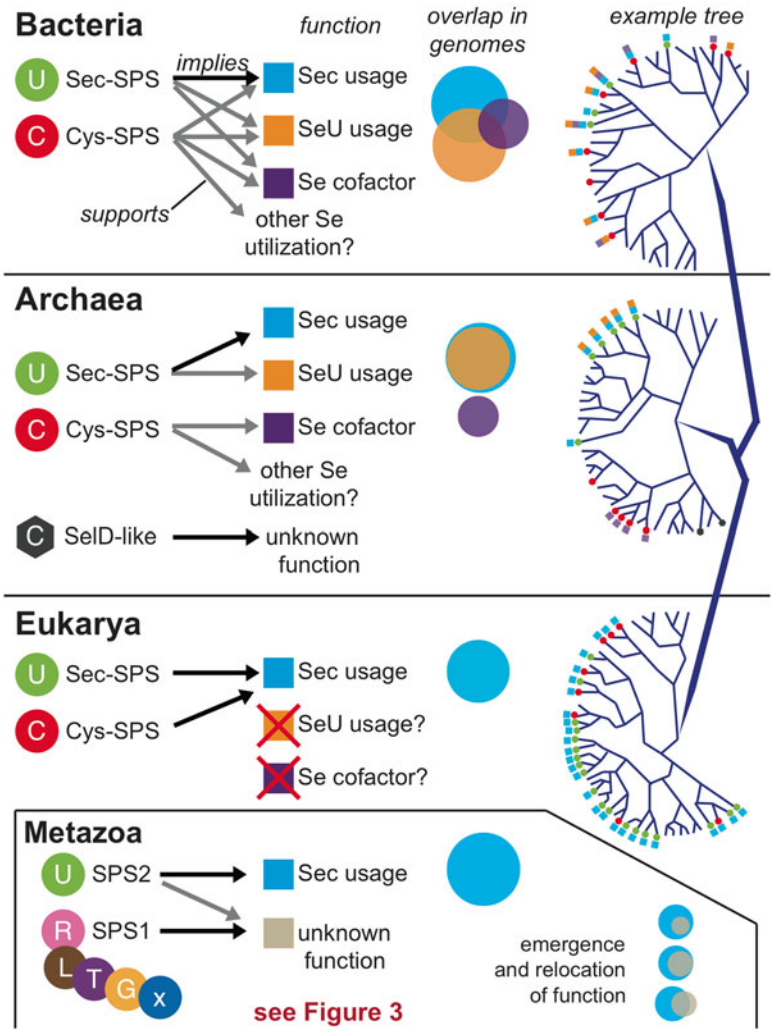

Fig. 8.2 SPS and Se utilization in bacteria, archaea and eukaryotes. The schema summarizes the genome distribution and function of SPS proteins. Each *SPS* gene is represented by a *colored circle* enclosing one letter, which indicates the amino acid found at the Sec site (e.g., *C*: Cys; *U*: Sec). SPS supports diverse ways of Se utilization in prokaryotes. In bacterial genomes, these traits show a complex mosaic pattern of overlap. The same traits are found in archaea, but their distribution appears limited to specific lineages. In eukaryotes, Sec/Cys *SPS* genes are always accompanied by the rest of Sec machinery, suggesting that Sec usage is the only eukaryotic Se utilization trait. Some archaea possess a protein family derived from SPS, called SelD-like [17] (*grey hexagon*). Although functionally uncharacterized, there are indications that SelD-like may be involved in a process other than selenophosphate synthesis. In metazoans, *SPS1* genes (see Fig. 8.3) are known to carry a function different from selenophosphate synthesis, which is still unknown

desulfinase) [7, 16]. In all of these cases, SPS is found at the C-terminal side of the fused polyprotein. Also, in all SPS fusions, SPS does not carry Sec, with a single known exception (SPS-NifS of *Geobacter sp. FRC-32*).

8.2.2 *Archaea*

Se utilization appears to be less common in archaea than it is in bacteria, and is strictly limited to specific lineages, in contrast to the mosaic pattern in bacteria (see Fig. 8.2). SPS proteins are found in only 12 % of sequenced archaea [17]. To date, Sec utilization has been observed only in the orders of *Methanococcales* (15 genome sequences available) and *Methanopyrales* (with *Methanopyrus kandleri* being its only sequenced species). All archaea in these two orders possess a specific set of selenoproteins involved in hydrogenotrophic methanogenesis, plus a Sec-containing SPS protein [4, 7, 13, 17]. *SPS* genes contain an archaeal SECIS element in the 3'UTR, as expected for archaeal selenoproteins [4]. All *Methanococcales* possess a peculiar bipartite form of YbbB and utilize SeU in tRNAs [21], while *M. kandleri* lacks YbbB and therefore does not use SeU. In fact, SeU utilization appears to be absent from all archaeal lineages other than *Methanococcales* investigated thus far. *SPS* genes are found also in some archaeal species belonging to the order of *Halobacteriales* [13, 17]. The co-occurrence of the genes *YqeB* and *YqeC* in these genomes suggests that these organisms use Se as a cofactor. In all of these species, SPS is present in a Cys form. Another archaeal genome, *Methanolacinia petrolearia* (previously known as *Methanoplanus petrolearius*), belonging to the order of *Methanomicrobiales*, also possesses a Cys form of SPS, but this is not coupled to any other Se utilization marker [13, 17]. This orphan SPS may hint at yet another undiscovered form of Se utilization in archaea.

A recent study [17] reported a novel protein family related to SPS, named SelD-like. These proteins are similar in sequence to *bona fide* SPS, but they form a separate phylogenetic cluster. SPS and SelD-like most likely share a common ancestor, and possibly a common catalytic mechanism. The SPS Sec/Cys site is conserved in SelD-like with Cys. Furthermore, conservation of critical residues suggests that most likely SelD-like proteins bind certain metal atoms (e.g., magnesium) as well as ATP or its analogues, similarly to SPS. *SelD*-like genes were found uniquely in certain species within the *Crenarchaeota* phylum, and more specifically in the orders of *Thermoproteales* and *Sulfolobales*. No Se utilization marker is found in these genomes, or in any other sequenced *Crenarchaeota*. In *Thermoproteales*, SelD-like is fused to an acylphosphatase-like protein, located at the C-terminus. Although acylphosphatase genes are found throughout the tree of life, fusions with SPS were never observed. To date, the biological function of SelD-like proteins remains unknown, but based on gene co-occurrence, it was speculated that it may be involved in sulfur metabolism [17]. It cannot be excluded, however, that SelD-like proteins are just divergent selenophosphate synthetases implicated in yet another form of Se utilization.

8.3 SPS in Eukaryotes

In eukaryotes, *SPS* genes are always accompanied by the rest of the Sec machinery and by selenoprotein genes in the same genome (see Fig. 8.2). The only exceptions are certain *SPS1* genes, which are explained below. This suggests that, in eukaryotes, the insertion of Sec in selenoproteins may be the only Se utilization trait supported by SPS. The gene, *YbbB*, is in fact generally missing from eukaryotic genomes, with a few exceptions attributed to gene transfer from bacteria (unpublished data). Most likely, Sec utilization was directly transmitted through the line of descent from bacteria to archaea to eukaryotes, while the other Se utilization traits were not. Although several lineages are devoid of selenoproteins (e.g., fungi, land plants), Sec utilization is spread across the eukaryotic phylogenetic tree, and it is highly represented in most metazoans [22, 23]. As in prokaryotes, eukaryotic Sec-encoding genomes contain a *SPS* gene carrying either Sec or Cys at its homologous site. Since SPS is a selenoprotein in Sec-utilizing archaea, and since a Cys to Sec conversion has necessarily to be considered an event with very low probability, the most parsimonious explanation is that the last common ancestor of eukaryotes had a Sec-containing SPS.

The reconstructed tree of *SPS* genes from the three domains of life broadly follows their known phylogenetic relationships, which supports the scenario of direct inheritance of the Sec trait. There are few exceptions, however, as observed in a number of protist organisms, such as green algae, most of alveolates and certain amoebas, exhibit a bacterial-like SPS protein. These were likely acquired by horizontal gene transfer in relatively recent times [7]. Many of these transferred genes are fusions of SPS with other proteins. Giving support to the horizontal gene transfer hypothesis, the most common SPS fusions in protists are with a NADH-dehydrogenase-like domain, and with a NifS-like domain, as found in bacteria. Two additional cases of extended SPS were observed, which are unique to protists. First, the heterolobosean amoeba, *Naegleria gruberi*, has SPS fused to a methyltransferase domain [24]. Strikingly, the genome of this species also contains a second *SPS* gene, fused with a NifS-like domain. Second, all *Plasmodia* species have a single *SPS* gene with an additional large domain at its N-terminus (>500 residues). This domain shows no homology to any known proteins, and its function remains unknown.

8.3.1 Emergence of SPS1 Genes in Metazoa

Almost every available eukaryotic genome outside metazoa possesses either a single *SPS* gene or none at all, and, when present, it contains Sec or Cys. In contrast, many metazoans, including *Homo sapiens* and *Drosophila melanogaster*, possess two distinct genes, *SPS1* and *SPS2*. The *SPS2* gene carries Sec at the usual site, and it was shown to synthesize selenophosphate from selenide and ATP, constituting the true

functional homologue of SPS in non-metazoans [25–27]. Instead, SPS1 proteins carry neither Sec nor Cys at this homologous site, whereas human SPS1 has threonine (SPS1-Thr) and fruit fly SPS1 carries arginine (SPS1-Arg). Diverse lines of evidence suggest that SPS1 proteins do not catalyze the canonical SPS function [26–29], although their molecular function has not been characterized thus far.

Notably, *SPS1-Thr* and *SPS1-Arg* are not phylogenetic homologues. Instead, they were generated through two distinct gene duplication events in different lineages. Accordingly, other similar cases have been observed. To date, we detected a total of four gene duplications across parallel lineages in metazoans [7] (see Fig. 8.3). In each case, the duplication involved the ancestral Sec-containing *SPS2* gene, and generated a novel *SPS* gene carrying some amino acid different from Sec or Cys at the homologous site. For convenience, we used the term SPS2 to designate all *bona fide SPS* genes, carrying Sec or Cys, and we use SPS1 to designate the other metazoan genes with any other amino acid. Despite their phylogenetically independent origin, various *SPS1* genes can partially rescue the phenotypic effect of *Drosophila* SPS1 knockout. This indicates that the diverse SPS1 proteins share a common function, yet distinct from that of SPS2 [7]. Thus, *SPS1* genes in different metazoan lineages seem to be functional homologues, despite the lack of direct phylogenetic orthology. We hypothesized that this may be explained by a process of parallel sub-functionalization. In this scenario, the ancestral metazoan *SPS2* gene had acquired a second function, on top of its canonical selenophosphate synthesis activity. Later in metazoan evolution, there was selective pressure to separate the two functions to two distinct genes. This resulted in the observed pattern of gene duplications across parallel lineages, with *SPS1* genes emerging to assume this second function, while *SPS2* retained just the original, canonical activity. Upon duplication, *SPS2* exhibited in each case a decrease of selective pressure on protein sequence, which we interpret as a signature of sub-functionalization. In contrast, the newly generated SPS1 proteins show a tight level of conservation in every case. The metazoan *SPS2* genes that never duplicated, which are expected to possess both SPS1 and SPS2 functions, consistently show a high level of selective pressure. The various metazoan *SPS* duplications, although analogous in outcome, occurred through diverse molecular mechanisms, as detailed below (see Fig. 8.3).

The *SPS1-Thr* gene is found only in vertebrates and is present in all available genomes in this lineage, with the species in the *Cyclostomata* group (jawless vertebrates, such as lampreys) being the sole exception. *SPS1-Thr* most likely originated through one of reported rounds of genome duplication at the root of vertebrates (Fig 8.3, duplication #1). *SPS1-Thr* maintained the overall gene structure of its parental *SPS2*, which features several introns in conserved positions. The *SPS2* gene later went through additional evolutionary events in mammals [30]. It appears that placentals replaced the original *SPS2* gene (*SPS2a*) with one of its retrotransposed copies (*SPS2b*). As a result, the only extant *SPS2* gene in placentals (including human and mouse) has no introns. Certain non-placental mammals, such as the marsupials, *Macropus eugenii* and *Monodelphis domestica*, still retain the two *SPS2* copies in their genome; i.e., *SPS2a* with the ancestral gene structure, and intronless *SPS2b*. It is not known whether both copies are functional in these organisms.

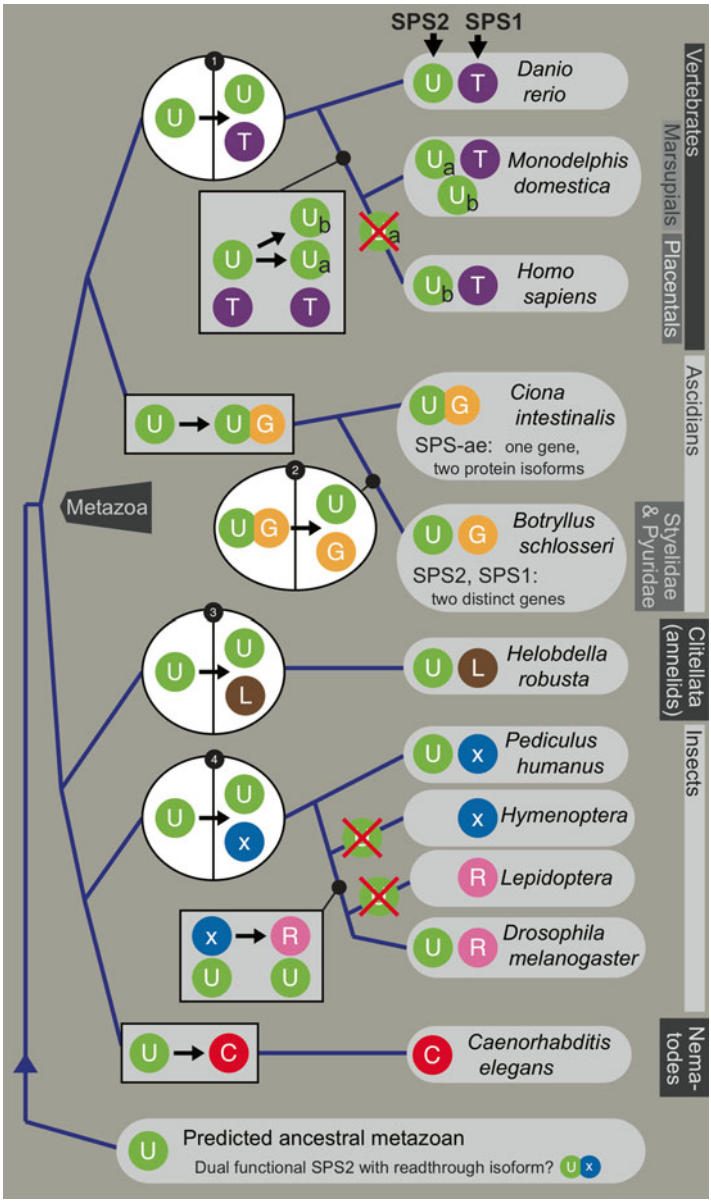

Fig. 8.3 Emergence of metazoan *SPS1* genes by parallel gene duplications of *SPS2*. The schema summarizes the reconstructed phylogeny of SPS proteins described in [7]. *SPS* genes are represented as in Fig. 8.2. *SPS2* genes carry Sec (U) or Cys (C, uniquely in nematodes), while *SPS1* genes carry other amino acids at this site (*T*: threonine, *G*: glycine, *L*: leucine, *R*: arginine; *x*: unknown residue). Gene duplications are highlighted in the *bipartite white circles*

Another *SPS1* gene emerged within tunicates (Fig 8.3, duplication #2). Here, the ascidians in the *Styelidae* and *Pyuridae* sister families exhibit a *SPS1* gene with a glycine residue aligned to the Sec site (*SPS1-Gly*). This gene presents the ancestral metazoan gene structure, featuring several introns. The same organisms also possess a *SPS2* gene with Sec, which, in contrast, has no introns. The analysis of other tunicate genomes allowed reconstructing how this situation came about [7]. While non-ascidian tunicates, such as *Oikopleura dioica*, display a single "standard" *SPS2* gene with the ancestral intron structure, the same gene has a peculiarity in the model species *Ciona intestinalis* and other ascidians. This gene (*SPS-ae*) produces two different transcript isoforms that differ in their 5′ end, resulting in two distinct protein isoforms, a Sec-containing SPS2-like isoform, and a SPS1-like isoform with glycine. Whereas this dual gene configuration is found in most ascidians, at the root of *Styelidae* and *Pyuridae*, the *SPS2*-like transcript retrotransposed to the genome, generating a novel intronless *SPS2* gene. Hence, the *SPS-ae* gene then specialized into the *SPS1*-like isoform only, becoming *SPS1-Gly*.

Another *SPS1* gene emerged within annelids, in the class of *Clitellata* (Fig 8.3, duplication #3). This gene carries a leucine residue aligned to the homologous Sec position (*SPS1-Leu*). Since *SPS1-Leu* possesses the ancestral *SPS2* gene structure, it must have been generated by duplication of *SPS2* through a conservative mechanism, such as homologous recombination.

Lastly, another *SPS1* gene emerged in insects (Fig 8.3, duplication #4). The phylogenetic history of this gene is particularly complex, and it is complicated by the phenomenon of Sec extinctions in this lineage [31]. Insects, in fact, went through a progressive depletion of selenoprotein genes, culminating with the complete loss of the Sec trait in multiple lineages (see [32]). Several events of Sec extinction occurred independently across parallel insect lineages. Some of them affected entire taxonomic orders (e.g., *Hymenoptera*, *Lepidoptera*), while others happened more recently and affected only certain species (e.g., *Drosophila willistoni* and *Acyrthosiphon pisum* [33]). In each selenoprotein-less insect, *SPS2* was lost together with other parts of the Sec machinery, while *SPS1* was maintained. We traced the origin of the insect *SPS1* gene prior to all Sec extinctions, as far back as the last common ancestor of all insects. In our reconstruction [7], the insect *SPS1* gene first emerged by duplication of *SPS2* in a rather bizarre form, as it maintained its in-frame UGA codon (translated as Sec in SPS2), but it lost its SECIS element. We believe that this gene (*SPS1-UGA*) is translated by a read-through mechanism that does not involve insertion of Sec or Cys, though we still do not know which amino acid is used at this site. The *SPS1-UGA* gene can be observed in the genomes of all extant *Hymenoptera*, which cannot synthesize Sec, as is also found in some paraneopterans, such as *Pediculus humanus* and *Rhodnius prolixus*. These latter organisms instead encode SPS2 and other selenoproteins. After its genesis, the UGA codon in *SPS1-UGA* was then converted to an arginine codon, yielding *SPS1-Arg*. This event occurred independently in at least two lineages in *A. pisum* and in the entire order of *Diptera* that includes *D. melanogaster*.

In terms of *SPS* presence, we can summarize metazoan genomes as either: (1) carrying one *SPS2* gene with Sec UGA and SECIS, which is presumably

dual-functional; (2) carrying two distinct *SPS2* and *SPS1* genes following duplication or two transcript isoforms (e.g., see *Ciona*); and (3) carrying only a *SPS1* gene following duplication and then *SPS2* gene loss (e.g., see insects).

Only the nematode lineage escapes all these categories. In this phylum, most organisms have a single *SPS* gene with Cys. This gene appears to be the direct descendant of metazoan *SPS2* that underwent a Sec to Cys conversion. However, nematodes, similar to insects, went through a progressive selenoproteome depletion, with certain parasitic nematodes enduring a complete Sec loss [34]. In selenoprotein-less nematodes, SPS2 was also lost, making them the only known metazoans devoid of any SPS proteins. In the sub-functionalization scenario, the nematode SPS2 is expected to have lost the SPS1 function at some point.

The evolution of *SPS* genes may provide additional hints about how the SPS1 function came into existence. We observed that SPS1 proteins perform a novel function, distinct but derived from the original SPS2 function. The most relevant difference between SPS2 and SPS1 is the replacement of the Sec/Cys site by some other amino acid. In SPS2, Sec is inserted in response to a UGA codon through a recoding mechanism. If such a mechanism is not fully specific to Sec, and random amino acids are sometimes inserted instead, then a UGA containing *SPS2* gene would produce a SPS1-like protein. We think that such a non-Sec recoding mechanism may have been the key to the origin of SPS1 function, and then to its selected maintenance in the dual-function ancestral metazoan SPS2. Indeed, *SPS2* genes contain conserved stem structures overlapping the Sec-UGA, previously reported in several selenoprotein genes and named SRE (Sec redefinition elements). The SRE of human selenoprotein N was shown to promote a non-Sec read-through activity in the absence of a SECIS element downstream and of its whole genomic context [35, 36]. Plausibly, the SRE in *SPS2* genes is expected to possess a similar activity. We believe that in the ancestral metazoa, SRE in *SPS2* was selected to support a minor non-Sec read-through activity, while at the same time maintaining the production of its main Sec-containing isoform. The dual-function state of this gene may be explained by the presence of these two protein isoforms; i.e., the Sec isoform carrying the canonical SPS2 selenophosphate synthesis activity, and the non-Sec isoform carrying the SPS1 function. In support of this view, the insect *SPS1-UGA* genes all contain stable structures similar to SRE elements, which we named hymenopteran read-through element (HRE). We think that HRE derived from SRE elements, which specialized in some yet uncharacterized form of non-Sec UGA recoding upon gene duplication and sub-functionalization.

Today, the molecular function of *SPS1* genes remains unknown. Structural comparisons suggest that SPS1 proteins catalyze a phosphorylation reaction similar to SPS2, but likely acting on a substrate different from selenide. Various indirect evidences have linked SPS1 to diverse biological pathways. Human SPS1 was reported to interact with Sec synthase [37] and has been proposed to function in Sec recycling due to rescue experiments in *E. coli* growing on L-Sec [38]. In fruit flies, *SPS1* knockout mutants were reported to have defects in selenoprotein expression [39]. However, conservation of SPS1 function in selenoprotein-less insects suggests that it is unrelated to Sec synthesis [29]. Based on differential gene expression analyses

performed upon SPS1 knockdown, *Drosophila* SPS1 has been proposed to be involved in vitamin B6 metabolism [40]. Previously, a role in redox homeostasis was proposed, based on the observation that heterozygous fruit flies with a compromised SPS1 copy are more sensitive than wild type flies to oxidative stress [41]. Hopefully, the enzymatic function of SPS1 proteins will be resolved in the future, clarifying the many aspects of their evolution that today remain elusive and necessarily subject to speculation.

8.4 Concluding Remarks

Selenophosphate synthetases (SelD/SPS) are required for the synthesis of Sec, and thus are present in every organism utilizing this amino acid. SPS are peculiar in that, besides participating in the Sec pathway, they are themselves often selenoproteins. The study of *SPS* genes allowed delineating the Se utilization traits across genomes. Meanwhile, it also uncovered classes of SPS proteins that apparently evolved novel functions, distinct from selenophosphate synthesis and likely unrelated to Sec synthesis, yet still to be defined (i.e., SPS1 in metazoans, SelD-like in *Crenarchaeota*). We can see the SPS family as a case in point of how comparative genomics can help in unraveling the complex nature of genes and their functions. Yet, this often results in further questions. What is the molecular and biological function of SPS1? What residue is used at the Sec homologous site of *SPS1-UGA* in Hymenopterans, and why is it inserted by a read-through stop codon? Are there additional means of natural Se utilization supported by SPS in prokaryotes and eukaryotes? What is the role of gene fusions in bacterial and protist *SPS* genes? What is the function of archaeal SelD-like proteins? These are just few of the questions, which we hope to see answered in the upcoming years of SPS research.

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Chapter 9
Structure and Mechanism of Selenocysteine Synthases

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Abstract Selenocysteine synthases, SelA in bacteria and SepSecS in archaea and eukaryotes, catalyze the terminal reaction of selenocysteine synthesis during which either serine or phosphoserine is converted into selenocysteine while being attached to $tRNA^{Sec}$. The reaction mechanism is based on the PLP co-factor and requires selenophosphate as the selenium donor. Although adopting different structures, SelA and SepSecS employ similar general principles for substrate recognition and chemical catalysis. Five SelA homodimers assemble into a ring structure that harbors ten tRNA-binding and catalytic sites. Each SelA subunit binds to the D-arm and T loop of $tRNA^{Sec}$. By contrast, two SepSecS homodimers arrange into a tetramer that binds to the acceptor and variable arms of $tRNA^{Sec}$. In both instances, at least two enzyme homodimers are needed to bind and act on one $tRNA^{Sec}$ molecule. A 'non-catalytic' dimer employs its N-terminal domain to dock the tRNA to the enzyme, whereas residues of the C-terminal domain of the 'catalytic' dimer orient the aminoacyl group into the active site for catalysis to occur. Herein, the mechanisms and structures of selenocysteine synthases are summarized.

Keywords PLP • SelA • Selenocysteine • Selenocysteine synthase • SepSecS • $tRNA^{Sec}$

9.1 Introduction

Selenium is the only essential dietary micronutrient that is found as a constitutive component in specific proteins and enzymes in all domains of life. Its precise location in selenoproteins is encoded in the DNA. The major physiological form through which selenium exerts its biological roles is selenocysteine, the 21st proteinogenic

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amino acid. Since its discovery in proteins [1–3], questions were raised about the importance and function of selenocysteine, and whether it could be substituted by isosteric amino acids, cysteine and serine. A typical selenoproteome is of limited size [4–6], but its members are important for the health, development, and organism survival. Although small in size [4], the human selenoproteome is vital for the maintenance of the cellular redox potential, protection of the membrane and DNA from oxidative damage, removal of reactive oxygen species, and regulation of the thyroid hormone homeostasis and metabolic rate (reviewed in [7–9]). Moreover, substitution of selenocysteine with cysteine or serine either completely diminishes or significantly reduces catalytic prowess of selenoenzymes [10–18]. Given that mutations affecting selenoprotein gene translation cause various pathologies including cancer (reviewed in [7–9, 19–23]) and that the mouse $tRNA^{Sec}$ knockout mutant is embryonically lethal [24], it is reasonable to suggest that the efficient synthesis and insertion of selenocysteine are fundamental biological processes. It is thus not surprising that significant efforts were invested in determining identities of macromolecular components, the sequence of events, and the mechanism(s) of discrete steps governing selenocysteine synthesis across kingdoms.

Early studies indicated that selenium is an essential component of archaeal hydrogenase, bacterial glycine reductase [3], nicotinic acid hydroxylase [25], formate dehydrogenase [1], xanthine dehydrogenase [26], and avian and mammalian glutathione peroxidases [27–29]. The assumption was that selenium was incorporated into proteins by an unknown post-translational mechanism. However, surprising observations that an in-frame UGA (*opal*) stop codon in the bacterial formate dehydrogenase and mouse glutathione peroxidase signals insertion of selenocysteine [30, 31], ignited an avalanche of extraordinary and unforeseen discoveries. Genetic and biochemical studies in bacteria have identified *selA*, *selB*, *selC*, and *selD* as essential genes for selenoprotein synthesis. The monocistronic *selC* gene [32] was shown to encode $tRNA^{Sec}$, which is serylated by the cytosolic SerRS, 'reads' the UGA codon, and supports insertion of selenocysteine with the aid of specialized elongation factor SelB [33]. $tRNA^{Sec}$ was subsequently shown to adopt a novel fold, 8/5 in prokaryotes and 9/4 in eukaryotes, characterized by 13 base pairs in the acceptor-TΨC arm, and extended D- and variable arms [34–38]. This is in contrast to a canonical 7/5 fold and a 12-base pairs long acceptor-TΨC arm found in all other elongator tRNAs. These results validated a proposition that selenocysteine is synthesized from serine [39] on its tRNA [40] in a reaction that requires SelD [40] and SelA [32, 41–44]. Concurrent studies on the bacterial and eukaryotic orthologs have established that SelD is selenophosphate synthetase, the enzyme that converts selenide into selenophosphate in the presence of ATP [45, 46]. On the other hand, it was suggested that SelA is a selenocysteine synthase that promotes conversion of Ser-$tRNA^{Sec}$ into Sec-$tRNA^{Sec}$ [41, 43]. Hence, a novel two-step indirect aminoacylation pathway for selenocysteine synthesis was proposed. In the first step, SerRS attaches L-serine onto $tRNA^{Sec}$ at the expense of one ATP molecule, and in the second, SelA promotes the serine-to-selenocysteine conversion while utilizing selenophosphate [41, 45].

Whereas the bacterial cycle of selenocysteine was delineated in the early 1990s, understanding of the analogous process in archaea and eukaryotes was significantly lagging. It took almost 20 years to ascertain that an unusual phosphoserine tRNA from mammalian brain, liver [47, 48] mammary glands [49], and avian liver [48], and the bovine liver *opal* suppressor serine tRNA [50, 51] represent, in fact, eukaryotic $tRNA^{Sec}$ [52]. After purification of a specific kinase that phosphorylates the *opal* suppressor $Ser\text{-}tRNA^{Sec}$ [53] it was proposed that the eukaryotic $Sec\text{-}tRNA^{Sec}$ is synthesized from $Ser\text{-}tRNA^{Sec}$ *via* a $Sep\text{-}tRNA^{Sec}$ intermediate [54–56]. This proposal was thereafter reversed and it was suggested that the eukaryotic and bacterial processes follow the same reaction mechanism [57, 58]. Around the same time, a report that autoantibodies from patients suffering from autoimmune hepatitis [59–61] precipitated a stable complex between a 48-kDa protein and $tRNA^{Sec}$ went almost unnoticed because the precipitated protein factor was considered to be involved in co-translational incorporation of selenocysteine [62]. The protein factor, known as soluble liver and pancreas antigen (SLA/LP) [59–61], was later sequenced, cloned and purified [63, 64]. The bioinformatics analyses have revealed that SLA/LP is well conserved in archaeal and eukaryotic organisms that contained selenoproteins and it is likely a PLP-dependent enzyme [65, 66]. Carlson *et al.* then identified murine *O*-phosphoseryl-$tRNA^{Sec}$ kinase (PSTK), which promotes the phosphoryl group transfer from ATP onto $Ser\text{-}tRNA^{Sec}$ and not onto $Ser\text{-}tRNA^{Ser}$ [67]. The discovery of PSTK not only lent credence to the original proposal that synthesis of selenocysteine in higher organisms proceeds *via* the phosphoseryl intermediate, but it also explained why phosphoserine tRNA and the kinase activity were detected in the first place [47–49]. In spite of this, the evidence that a specific enzyme can form selenocysteine from the tRNA carrying phosphoserine was missing. That such enzymatic activity may exist was suggested by an unrelated study on the tRNA-dependent synthesis of cysteine in certain archaea. Sauerwald *et al*. identified an archaeal enzyme, SepCysS, which converted $Sep\text{-}tRNA^{Cys}$ into $Cys\text{-}tRNA^{Cys}$ [68]. The enzyme was the Fold Type I PLP dependent enzyme, just like SelA. This prompted an intense search for an enzyme that could utilize $Sep\text{-}tRNA^{Sec}$ as a substrate. Two research groups independently identified murine and archaeal *O*-phosphoseryl-$tRNA^{Sec}$:selenocysteinyl-$tRNA^{Sec}$ (SepSecS; SepS) that supported selenocysteine and selenoprotein synthesis [69, 70]. The enzyme promoted phosphoseryl-to-selenocysteinyl conversion in the presence of selenophosphate while retaining stringent specificity towards $Sep\text{-}tRNA^{Sec}$. Importantly, murine and archaeal SepSecS were shown to be homologs of human SLA/LP identified in the early 1990s, which, in turn, explained why SLA/LP formed a stable complex with $Sep\text{-}tRNA^{Sec}$. Thus, all results suggested that the selenocysteine cycle in higher organisms is composed of three reactions. As in bacteria, SerRS first attaches serine onto $tRNA^{Sec}$. Subsequently, PSTK converts $Ser\text{-}tRNA^{Sec}$ into $Sep\text{-}tRNA^{Sec}$, and in the terminal reaction, SepSecS substitutes selenol for phosphoryl yielding $Sec\text{-}tRNA^{Sec}$. Although yielding the same reaction product, SelA and SepSecS use different tRNA-based substrates, implying that their catalytic mechanisms and structures are not completely conserved. In this chapter, the current understanding of selenocysteine synthases is presented.

9.2 The Structure and Architecture of the Bacterial SelA

Following genetic and biochemical studies that identified SelA as the bacterial selenocysteine synthase, significant strides were made to determine the structure and architecture of both holo SelA and the SelA:tRNA^{Sec} binary complex. The CryoEM studies on *Escherichia coli* and *Moorella thermoacetica* SelA established that ten enzyme subunits, each containing one PLP molecule, arrange into a ~500-kDa five-fold symmetric structure [71, 72]. However, the exact stoichiometry of the SelA:tRNA^{Sec} complex remained controversial [72, 73]. Most recently, Itoh *et al.* provided a wealth of structural and biochemical information on this system by studying *Aquifex aeolicus* SelA and *Thermoanaerobacter tencongensis* tRNA^{Sec} [35, 74]. The crystal structure of holo SelA revealed that the enzyme is a homo-decamer in which ten subunits are arranged into a pentamer of dimers (Fig. 9.1). Each SelA subunit is composed of three domains: an N-terminal domain (NTD; residues 1-66), a core domain (residues 90-338) and a C-terminal domain (CTD; residues 339-452), which fold into a structure typical of Fold Type I PLP-dependent enzymes (Fig. 9.1a). A short linker (residues 67-89) connects NTD with the core domain. The enzyme belongs to a distinct taxon that is likely to form an independent group related to the cystathionine γ-synthase family [74]. Structurally, two SelA monomers form an intimate dimer at which interface two active sites are formed. Each active site contains PLP covalently attached to a conserved Lys^{285}

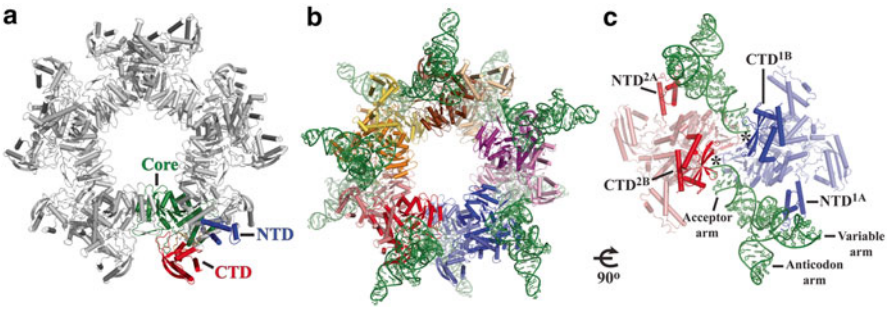

Fig. 9.1 Bacterial SelA forms a ring structure that binds ten tRNA^{Sec} molecules. (**a**) A ribbon diagram of holo SelA (PDBID: 3W1J), viewed above the ring plane, reveals a pentameric ring-like structure. Domains of one SelA monomer are colored: NTD is *blue*, the core domain is *green*, and CTD is *red*; other SelA subunits are *grey*. (**b**) The SelA:tRNA^{Sec} complex (PDBID: 3W1K) shows that ten tRNAs bind to the SelA decamer. tRNA is *green*, and SelA dimers are *red* and *pink*, *orange* and *olive*, *brown* and *beige*, *purple* and *light purple*, and *blue* and *light blue*. (**c**) Two intimate dimers (*pink* and *light blue*) bind two tRNAs. NTD of monomer 1 (NTD1A; *blue*) binds to the D-arm and T loop, and CTD of monomer 2 (CTD2B; *red*) interacts with the CCA-end. This is repeated on the other side of the dimer where NTD2A (*red*) binds to the D-arm and T loop of the second tRNA, and the acceptor arm binds to CTD1B (*blue*). *Asterisks* designate locations of the catalytic grooves

residue (see Sect. 9.4). The mobile NTD protrudes from the plane of the ring structure and interacts with the core domain of the neighboring intimate dimer (Fig. 9.1). On the other hand, CTD is oriented differently from that in other Fold Type I PLP enzymes, and this presumably enables formation of the pentameric SelA ring. The ring harbors large interdimer clefts that play an important function in tRNA binding and catalysis.

The crystal structure of the SelA:$tRNA^{Sec}$ complex provided the first hints at how SelA recognizes $tRNA^{Sec}$ (Fig. 9.1b). The binary complex crystal contained ten $tRNA^{Sec}$ molecules bound to the SelA decamer, thus suggesting 1:1 stoichiometry. $tRNA^{Sec}$ interacts with the enzyme through its extended D- and acceptor-TΨC arms, whereas the variable and anticodon arms do not participate in binding (Fig. 9.1c). Two intimate dimers bind one tRNA molecule in an arrangement in which one SelA dimer (e.g., subunits A, B or $SelA^{AB}$) binds the tRNA body, while the other one (e.g., subunits C, D or $SelA^{CD}$) positions and orients the aminoacylated CCA-end into the catalytic pocket (Fig. 9.1c). This observation implies that only in a decameric arrangement SelA forms a productive complex with $tRNA^{Sec}$. Indeed, mutations that disrupted the decamer structure inhibited the SelA activity, but retained ~90 % of its tRNA-binding capacity [74].

Perhaps the most important structural element for tRNA recognition is NTD of SelA, which binds the D-arm and TΨC loop of $tRNA^{Sec}$. The deletion of NTD abolishes binding of SelA to both Ser-$tRNA^{Sec}$ and unacylated $tRNA^{Sec}$ [35]. Moreover, this segment is needed to distinguish $tRNA^{Sec}$ from other tRNAs including homologous $tRNA^{Ser}$. Particularly, the NTD interacts with the fifth and sixth base pairs G14:U21 and C15:G20a of the D-arm, and with the triple base pair in the D loop. Because these base pairs are present in $tRNA^{Sec}$ only, it is postulated that they may be specificity elements for all enzymes involved in selenocysteine synthesis. The length of the acceptor-TΨC arm of $tRNA^{Sec}$ (eight nucleotides *vs*. seven nucleotides found in canonical tRNAs), which is important for binding the $tRNA^{Sec}$-specific elongation factor, is not critical for recognition by SelA due to conformational mobility of the NTD. Further, an intimate dimer that binds to the D-arm and T loop does not interact with the CCA-end and thus does not act on that particular $tRNA^{Sec}$ (Fig. 9.1c). Instead, this dimer positions $tRNA^{Sec}$ so that the acceptor arm can interact with the C-terminal domain of the neighboring intimate dimer. The tip of the acceptor arm binds to a large interdimer cleft where residues 423 and 424 of SelA interact with the first base pair G1:C72 and G73, the discriminator base. Mutational analyses have confirmed that recognition of the acceptor arm tip is important for selenocysteine synthesis [35]. Furthermore, structural modeling suggested that the binding pocket for A76 is composed of residues from subunits A and C, Asn^{218A} and Phe^{224C}, which are in proximity only in the pentameric SelA. Hence, a model of tRNA recognition by SelA emerged in which one SelA dimer deploys NTD to dock $tRNA^{Sec}$ onto the decamer, while the neighboring dimer uses CTD to interact and position the seryl-CCA into its catalytic groove. This pattern repeats on both sides of the SelA homodecamer yielding a large ribonucleoprotein assembly capable of simultaneously acting on ten tRNA substrates.

9.3 The Structure of the Archaeal and Eukaryotic SepSecS

The X-ray crystallography studies on the archaeal and murine holo SepSecS [75, 76], and the human SepSecS:tRNA^{Sec} binary complex [37] revealed the structure of selenocysteine synthase in higher organisms. SepSecS is a tetramer composed of a dimer of intimate dimers (Fig. 9.2a). The intimate dimer interface encloses two active sites each carrying one PLP molecule covalently linked to a conserved Lys^{284} (see Sect. 9.4). SepSecS is composed of three domains: an extended NTD (residues 1–130), a core domain (residues 131–315), and a CTD (residues 360-501) (Fig. 9.2a). A linker composed of long α-helices (residues 316–359) joins the core domain and CTD. With the exception of the extreme C-terminus, the overall structure of SepSecS is well conserved. The CTD of the archaeal SepSecS is shorter than the mammalian counterpart; it lacks a segment (residues 473–493) identified as the antigenic region in patients suffering from chronic autoimmune hepatitis (reviewed in [20]).

SepSecS forms its own branch in the phylogenetic tree of Fold Type I PLP-dependent enzymes that may be related to the sugar aminotransferase family [74, 76]. SepSecS is a primordial enzyme, probably present in the last common ancestor. A certain level of structural homology with the dimeric SepCysS and selenocysteine lyase is evident in the core and C-terminal domains. However, both of these homologs lack the NTD, an element pivotal for oligomerization of SepSecS. In particular, N-terminal helices α1, α2, and α4 from each subunit interact with one another and establish a hydrophobic core of the tetramer. The importance of these interactions

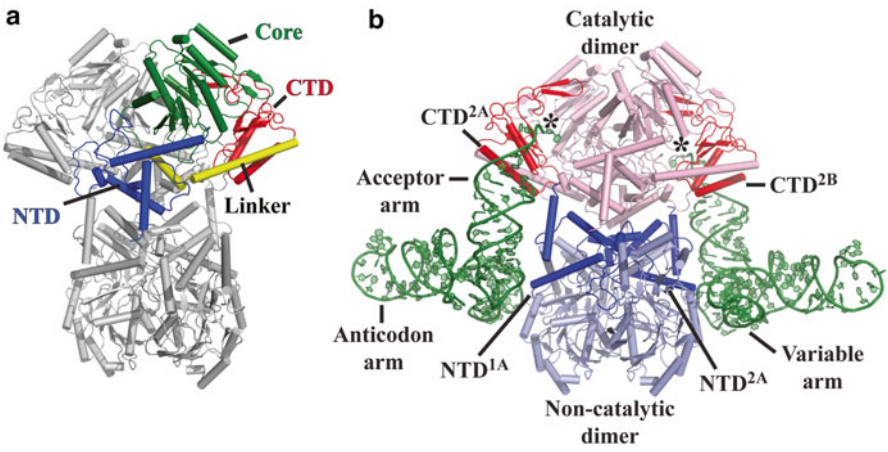

Fig. 9.2 SepSecS homotetramer binds up to two tRNA^{Sec} molecules. (**a**) Two SepSecS homodimers arrange into a tetramer. Domains of one subunit are colored: NTD is *blue*, the core domain is *green*, linker is *yellow*, and CTD is *red*. The rest of the tetramer is *grey*. (b) The binary complex structure (PDBID: 3HL2) shows that NTD (NTD1A, NTD2A) of the non-catalytic dimer (*light blue*) binds to the acceptor arm of tRNA, and that CTD (CTD2A, CTD2B) of the catalytic dimer (*pink*) binds to the tip of the acceptor arm. *Asterisks* mark the catalytic grooves (see Fig. 9.3 for more detail)

for tetramerization was shown by studies in which the deletion of helix α1 yielded a dimeric enzyme incapable of supporting selenocysteine synthesis [76]. Thus, the appended NTD of SepSecS and the uniquely oriented CTD of SelA [35] are the major oligomerization elements in selenocysteine synthases.

The crystal structure of SepSecS:tRNA^{Sec} revealed the architecture of the binary complex, the mechanism of tRNA recognition, and provided hints about the complex stoichiometry and reaction mechanism [37]. The structure showed that two tRNA^{Sec} molecules are bound to the SepSecS tetramer in a cross-dimer fashion (Fig. 9.2b), suggesting that only the tetrameric SepSecS can bind and presumably act on Sep-tRNA^{Sec}. However, SepSecS binds to the opposite side of tRNA when compared to SelA. The N-terminal helix α1 of the non-catalytic dimer binds to the long acceptor-TΨC arm, which is the major recognition element, and a segment of its core domain interacts with the long variable arm [37]. The tip of the arm binds to the C-terminal helices α14 and α15 of the catalytic dimer. Three conserved arginine residues in helices α14 (Arg^{398}) and α15 (Arg^{453}, Arg^{456}) interact with the G1:C72 base pair and the G73 discriminator nucleotide [37]. Recognition of the G73 discriminator is important for selenocysteine synthesis [37]. In addition, while there is no evidence that SepSecS undergoes a conformational change upon tRNA binding, the superimpositioning of the complexed [37] and unbound tRNA^{Sec}[38] revealed that the acceptor-, TΨC- and variable arms rotate around the vertical axis projecting through the anticodon arm upon complex formation [77]. This may be pivotal for orienting the phosphoseryl-CCA towards the catalytic groove.

Although SepSecS harbors four tRNA-binding sites, the binary complex crystal contained only two tRNAs bound to the tetramer. The same complex stoichiometry was observed when mimics of Sep-tRNA^{Sec} were used in binding assays [78]. The results of fluorescence-quenching binding assays and small angle X-ray scattering analyses confirmed that SepSecS preferentially binds either one or two tRNA^{Sec} molecules at a time [79]. This suggests that SepSecS employs a half-sites activity, but it remains to be seen if this functional asymmetry is of physiological significance and if it is regulated.

9.4 Divergent Active Sites of Selenocysteine Synthases and a Conserved Catalytic Mechanism

Both SelA and SepSecS harbor active sites formed at the interface of intimate dimers. Before atomic resolution structures were available, detailed enzymatic studies on SelA and SepSecS were pursued with the aim to delineate the possible catalytic mechanism. The SelA-catalyzed substitution of selenol for hydroxyl proceeds through an anhydroalanyl intermediate covalently attached to PLP [41]. In presence of SelD, reduced selenium, ATP and sodium borohydride, SelA converted Ser-tRNA^{Sec} into Ala-tRNA^{Sec} with a fraction of pyruvate being released into solution. The reaction was stoichiometric with 2 mol of enzyme acting on 1 mol of

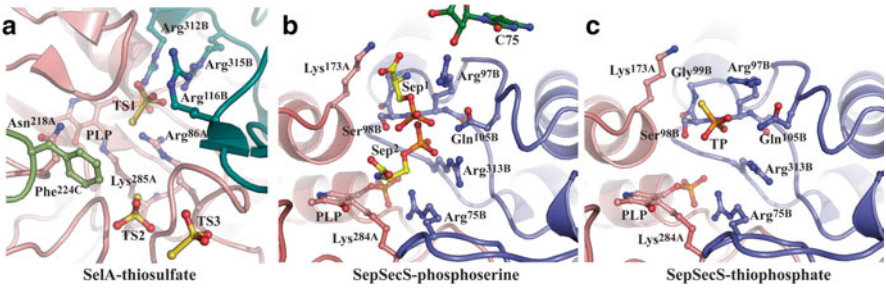

Fig. 9.3 Catalytic sites in SelA and SepSecS and binding pockets for phosphoserine and selenophosphate. (**a**) The active site in SelA is composed of residues from subunits A (*teal*) and B (*pink*) of the intimate dimer, and at least one residue from the neighboring dimer subunit C. Thiosulfate ion TS1 (*gold sticks*) designates the binding pocket for selenophosphate, and TS2 and TS3 mark the A76-binding pocket. (**b**) The active site in SepSecS is composed of residues only from subunits of the intimate dimer (*pink* and *blue*). Free phosphoserine (Sep1, Sep2; *gold sticks*) binds in two orientations, none of which are optimal for catalysis. (**c**) Thiophosphate (TP; *gold sticks*) binds to the P loop (Ser98-Gln105), like TS1 in SelA. Catalytic residues in SelA (**a**) and SepSecS (**b**) are shown as sticks

Ser-tRNA^{Sec}. Studies on the archaeal [69], murine [70] and human [37] systems confirmed that PLP is required for phosphoserine-to-selenocysteine conversion, but the anhydroalanyl intermediate remained elusive. This discrepancy was ascribed to the instability of reaction intermediates bound to SepSecS. Recent structural and mutational studies provided a wealth of information about the active sites of SelA and SepSecS, and possible catalytic mechanisms.

The crystal structures of SelA in complex with thiosulfate (TS) [35], holo SepSecS [75, 76] and the human SepSecS:tRNA^{Sec} complex [37] revealed the three-dimensional structure of the active sites and suggested residues pivotal for catalysis. In the SelA structure, TS1 binds to the selenophosphate-binding pocket composed of Arg86A, Arg312B and Arg315B (Fig. 9.3a). The pocket sits atop PLP, which is coupled *via* Schiff base to Lys285A. When a string of arginine side chains was mutated into alanines, the catalytic activity of SelA was markedly reduced. The side chains of Arg119B and Asp284A, which interact with Arg86A and Arg312B, might be important for catalysis (Fig. 9.3a). Intriguingly, TS2 and TS3 bind near Asn218A and Phe224J, which are spatially proximal to the TS1-binding pocket. Because mutations of these two residues significantly diminished SelA activity, it was proposed that TS2 and TS3 mimic the binding of A76. As a corollary, this proposal suggested that only as a pentameric ring could SelA bind Ser-tRNA^{Sec}, which was later shown to be correct [74].

Further, the crystal structures of SepSecS provided a comparable level of structural information. We shall briefly discuss only the human enzyme, but with a note that the same conclusions most likely apply to the archaeal and other eukaryotic orthologs. The SepSecS subunit of the catalytic dimer that interacts with the CCA-end (monomer A) provides PLP linked to Lys284A, Gln172A, and Lys173A (Fig. 9.3b). The other subunit (monomer B) provides the P-loop (Ser98B and

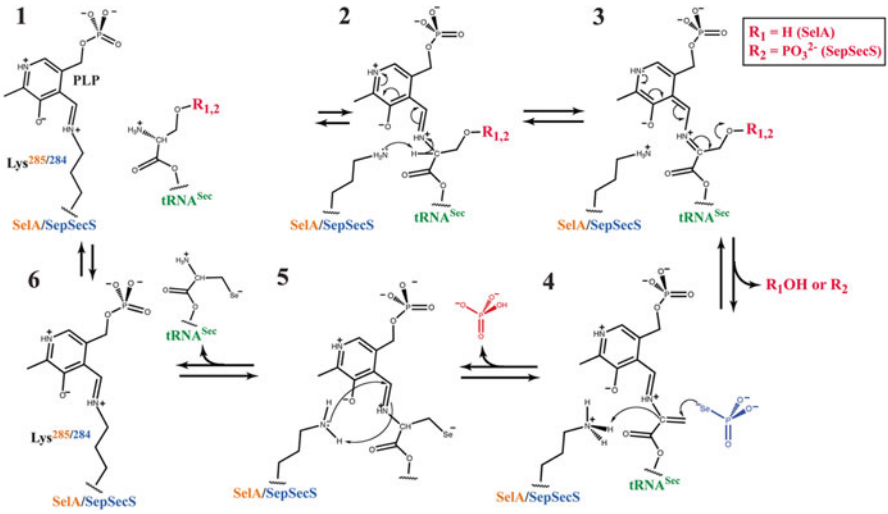

Fig. 9.4 Proposed PLP-dependent mechanism of the terminal reaction of selenocysteine synthesis catalyzed by SelA and SepSecS. For details on the mechanism see Sect. 9.4 of the main text and Fig. 4 in Refs. [37] and [35]. The leaving groups in aminoacyl groups are highlighted in *red*: R_1 (SelA) is H and R_2 (SepSecS) is PO_3^{2-}

Arg97B), which binds thiophosphate, and Arg75B, Gln105B, and Arg313B that are important for catalysis (Fig. 9.3b, c). Free phosphoserine binds to the active site of the catalytic dimer only, but in two orientations neither of which are optimal for catalysis. The covalent attachment of phosphoserine to $tRNA^{Sec}$ is thus essential for its proper placement into the active site. Because thiophosphate binds to the similar site in the non-catalytic dimer, it was suggested that selenophosphate binds to SepSecS only after phosphate is eliminated from Sep-$tRNA^{Sec}$.

Taken together, a unified PLP-dependent mechanism for the terminal reaction of selenocysteine synthesis was proposed (Fig. 9.4) [37, 41, 75, 76]. The reaction begins with binding of Ser-$tRNA^{Sec}$ and Sep-$tRNA^{Sec}$ to SelA and SepSecS, respectively. The amino group of the seryl/phosphoseryl moiety attacks the Schiff base thus yielding the external aldimine. The liberated Lys285/284 side chain abstracts the Cα proton from serine/phosphoserine. The electron delocalization by the pyridine ring leads to β-elimination of water and phosphate from Ser- and Sep-$tRNA^{Sec}$, respectively, and the anhydroalanyl-$tRNA^{Sec}$ intermediate is formed. Upon water/phosphate release, selenophosphate binds to the P-loop. The concomitant attack of water on selenophosphate and of the nucleophilic selenium onto the anhydroalanyl moiety yields an oxidized form of Sec-$tRNA^{Sec}$ while releasing a second phosphate equivalent. Alternatively, selenophosphate attacks anhydroalanine and forms phosphoselenyl-$tRNA^{Sec}$, which subsequently breaks down to Sec-$tRNA^{Sec}$ after water attack. Lastly, Lys284/285 re-establishes the internal aldimine, and Sec-$tRNA^{Sec}$ is released from the enzyme. It is suggested that, besides PLP, non-homologous arginine residues play a critical role in catalysis. However, further structural and

enzymatic studies are needed to both define precise role(s) for these residues and provide a complete picture about the remarkable mechanism of selenocysteine synthases.

9.5 Future Directions

Despite the remarkable progress, important questions about selenocysteine synthases and their biological roles remain unanswered. In particular, SelA and SepSecS were crystallized with the unacylated $tRNA^{Sec}$ and interactions of the seryl and phosphoseryl groups with the active sites were not visualized. Also, anhydroalanyl-$tRNA^{Sec}$ bound to SepSecS has not been captured, leaving the exact mechanism in the dark. Given that SelA and SepSecS are downstream of SelD and SPS2, it would be important to assess if they regulate the overall selenium homeostasis. Furthermore, it would be important to establish if the half-sites activity of SepSecS is allosterically regulated. Because SelA and SepSecS bind $tRNA^{Sec}$ from the opposite sides when compared to SerRS and PSTK, respectively, it is plausible that multi-enzyme 'selenosomes' facilitate selenocysteine synthesis. Lastly, given recent clinical reports [80–83], studies on the role of SepSecS and selenoproteins in the development and maintenance of the healthy human brain are warranted.

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Chapter 10
Mechanism, Structure, and Biological Role of Selenocysteine Lyase

Hisaaki Mihara, Ryuta Tobe, and Nobuyoshi Esaki

Abstract Selenocysteine lyase is a pyridoxal 5′-phosphate-dependent enzyme catalyzing the degradation of L-selenocysteine to L-alanine and elemental selenium. It is unique in that it acts exclusively on L-selenocysteine but not on its sulfur counterpart, L-cysteine. The enzyme is proposed to function not only in the recycling of selenium via degradation of L-selenocysteine derived from selenoproteins, but also in energy metabolism linked to obesity and metabolic syndrome. Crystallographic studies have shed light on the catalytic mechanism that allows the enzyme to distinguish between L-selenocysteine and L-cysteine, which possibly contributes in uncovering the physiological role of selenocysteine lyase in mammals.

Keywords Catalytic mechanism • Selenium metabolism • Selenium recycling • Selenopersulfide • Selenoprotein biosynthesis

10.1 Mammalian Selenium Metabolism

The chemical forms of selenium in diets are mostly either selenomethionine, selenocysteine, selenite or selenate. Among these, selenomethionine is the predominant form, because about 90 % of the total selenium in plants exists as protein selenomethionine residues that have randomly and frequently replaced native methionine residues [1]. Although inorganic selenium compounds are less abundant than selenomethionine, they can easily be taken up as a source of selenium for selenoprotein biosynthesis [2]. Selenate needs to be reduced to selenite prior to its utilization as a selenium source, through a mechanism similar to the nitrate reduction process. Alternatively, selenate can be reduced to selenite by adenosine 5′-phosphosulfate

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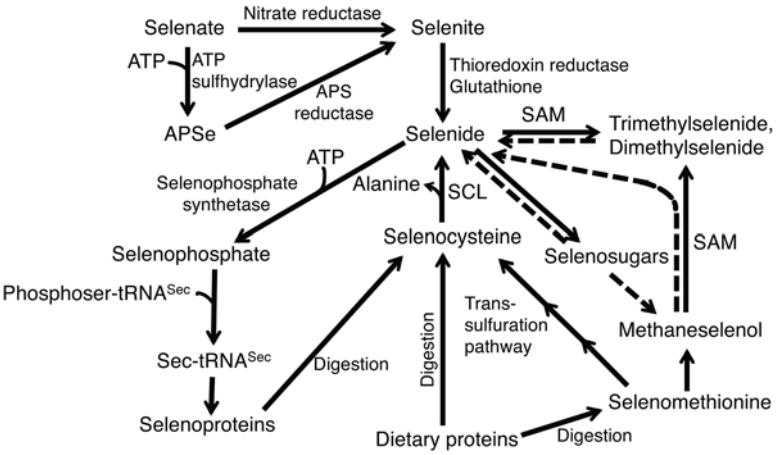

Fig. 10.1 Selenium metabolism and incorporation of selenium into selenoproteins. *ATP* adenosine 5′-phosphate, *APS* adenosine 5′-phsphosulfate, *APSe* adenosine 5′-phosphoselenate, *SAM* *S*-adenosylmethionine, *SCLY* selenocysteine lyase

reductases similar to the reduction of sulfate to sulfite. Selenite is then reduced to selenide by glutathione or thioredoxin reductase.

As shown in Fig. 10.1, selenide is the key compound for selenoprotein biosynthesis. A selenocysteine residue is encoded by the UGA codon in selenoprotein mRNA, which is decoded by selenocysteyl-tRNA$^{[Ser]Sec}$ [3, 4]. The selenocysteine moiety on selenocysteyl-tRNA$^{[Ser]Sec}$ is biosynthesized using selenophosphate, which is produced from selenide and ATP by selenophosphate synthetase [5, 6]. However, the metabolic pathway providing selenide for selenophosphate synthetase remains unclear. Excess selenide is excreted in urine as trimethylselenide or dimethylselenide after methylation [7]. Selenosugar (1β-methylseleno-*N*-acetyl-D-galactosamine) is another excreted form of selenide that can also be utilized as a selenium source for selenoprotein biosynthesis [8, 9]. However, the bioavailability of selenosugars remains unclear, one hypothesis being that selenide or methaneselenol could be released from these.

Selenomethionine is the major dietary source of selenium for humans, and is contained in proteins from both plant and animal sources [10]. Selenocysteine can also be a selenium source from animal proteins. Free selenomethionine is easily available through the digestion of dietary proteins and is further metabolized to selenocysteine via a selenium version of the transsulfuration pathway [11]. In addition, selenomethionine is degraded by cystathionine γ-lyase to produce methaneselenol, which may be used as a selenium source for selenoprotein biosynthesis [12]. Methaneselenol is further methylated to dimethylselenide and trimethylselenide, which might also be utilized as a selenium source after demethylation [10]. However, little is known about the chemistry of their demethylation. Selenocysteine is also produced through digestion of internal or external (namely dietary) proteins, whereas free selenocysteine is rarely found in cells according to speciation studies [13]. Nevertheless, free L-selenocysteine is the specific substrate of selenocysteine

lyase (SCLY), which decomposes selenocysteine into selenide and alanine [14]. Subsequently, selenium can be recycled via SCLY for the synthesis of new selenoproteins (Fig. 10.1). The free form of selenide is less commonly present in organisms than the protein-bound form [15]. Selenium is liberated from selenocysteine by SCLY and covalently bound to the particular cysteine residue of the enzyme as described below. Therefore, SCLY itself is regarded as one of such selenium-binding proteins.

10.2 Identification of SCLY in Mammals and Bacteria

A study on selenocysteine biosynthesis in rat liver homogenates [11] revealed the existence of SCLY (EC 4.4.1.16) catalyzing the degradation of selenocysteine to form alanine and selenide. SCLY was then purified to homogeneity from pig liver and characterized [14]. The enzyme from pig is a homodimer with a subunit M_r of 48,000, each containing one molecule of pyridoxal 5′-phosphate (PLP) as a coenzyme. It shows a typical PLP-enzyme, UV-visible spectrum with maximum absorption at 420 nm. L-Selenocysteine is stoichiometrically converted to H_2Se and L-alanine in the presence of excess dithiothreitol (DTT), with a specific activity of 37 μmol/min/mg at an optimal pH of 9.0. On the other hand, if DTT is not added in excess to the reaction mixture, an elemental form of selenium, Se^0, is formed from L-selenocysteine generating a red precipitate. Thus, the intrinsic product of the enzymatic reaction is Se^0 and not H_2Se as Se^0 is spontaneously reduced to H_2Se when excess DTT is added to the standard reaction mixture. The K_m value for L-selenocysteine is 0.83 mM, which is comparable to the K_i for L-cysteine, a competitive inhibitor of SCLY. The cellular concentration of free L-selenocysteine is probably much lower than the K_m value whereas free L-cysteine is generally found at concentrations similar to the K_i value. Therefore, the cellular SCLY reaction probably happens extremely slowly, suggesting that an unknown mechanism might exist to specifically deliver L-selenocysteine to SCLY in cells.

SCLY activity occurs in the homogenates of various mammalian tissues such as liver, kidney, pancreas, adrenal gland, heart, lung, testis, brain, thymus, spleen, and muscles of rat, dog, mouse, pig, and other mammals [14]. Liver and kidney homogenates showed particularly high activities compared to others. This conforms with the results of Western blot analysis of proteins extracted from mouse tissues in which Scly was abundantly present in the liver, kidney, and testis [16]. On the contrary, little Scly activity was found in blood and fat tissues.

The first cDNA cloning of mammalian *Scly* was reported in 2000 for the mouse *Scly* gene [16]. The cDNA for mouse Scly is 2172 bp in length, with an open reading frame encoding a polypeptide chain of 432 amino acid residues (M_r=47,201). The recombinant mouse SCLY overproduced in *Escherichia coli* is a homodimer with a subunit M_r of 47,000. cDNA cloning has also been performed for the rat gene [17]. Steady-state kinetic analysis of the recombinant rat SCLY revealed that the V_{max} and K_m values for L-selenocysteine were 26 μmol/min/mg and 5.5 mM, respectively.

Prokaryotes also have a similar enzyme, which was purified from *Citrobacter freundii* [18, 19]. The bacterial enzyme is markedly different from the mammalian enzyme with respect to its physicochemical properties and amino acid composition. Contrary to the homodimeric pig liver SLCY enzyme with a subunit M_r of 48,000, the bacterial enzyme is monomeric with a M_r of 64,000 [19]. Nevertheless, the enzyme is very similar to the mammalian enzyme regarding its enzymatic properties, that is, catalyzing the degradation of L-selenocysteine into L-alanine and selenium, but being inert against L-cysteine. The apparent K_m for L-selenocysteine is 0.95 mM, and the enzyme shows maximal activity at pH 7.0. L-Cysteine behaves as a competitive inhibitor of the enzyme with a K_i of 0.65 mM. SCLY activity is also found in some bacterial strains such as *Alcaligenes viscolactis* and *Pseudomonas alkanolytica* [18]. However, none of the bacterial *Scly* genes have been identified or cloned to date, and their biological roles remain unknown.

10.3 Orthologous Genes in Various Organisms

A number of genes sharing sequence homology with *Scly* have been deposited in the nucleotide sequence databases. These include orthologous genes in all vertebrate genomes that have been sequenced to date, such as in mammals (human, chimpanzee, rhesus monkey, rat, dog, giant panda, cow, pig, horse, opossum, and platypus), birds (chicken and zebra finch), amphibians (African clawed frog and Western clawed frog), and fishes (zebrafish, Japanese puffer fish, and green spotted puffer). Interestingly, the unicellular choanoflagellate, *Monosiga brevicollis*, which is among the closest unicellular relatives of animals, also has a gene with moderate (>36 %) sequence similarity with *SCLY*. Apart from *NifS*-type genes (described below), no other genes significantly homologous to *Scly* were found in the genomic sequences of plants, fungi, bacteria, and archaea, suggesting that SCLY activities detected in some bacterial and archaeal strains are probably related to side reactions generated by NifS-type cysteine desulfurases (CDS) and/or catalytic actions provoked by unidentified monomeric bacterial SCLY, significantly different from mammalian SCLY.

10.4 Homology to NifS-type Cysteine Desulfurases

Mammalian SCLY is distantly related to NifS-type CDS in the primary structure with overall sequence identity of less than 30 % [16, 20]. CDS catalyzes the desulfurization of L-cysteine to provide sulfur for iron-sulfur clusters, thiamine, molybdopterin, and thionucleotides in tRNA [21]. The CDS enzymes family generally uses both cysteine and selenocysteine as substrates, but with different catalytic rates [22–26]. However, the physiological significance of selenocysteine degradation by CDS has not been clarified. Unidentified genes with weak similarity to both *SCLY*

and CDS genes have been detected in the genomes of diatoms, green algae, and euglenozoans such as *Trypanosoma brucei* and *Leishmania infantum* [20]. However, their catalytic actions on selenocysteine and cysteine remain unknown.

10.5 Structure and Open-Close Conformational Change

Crystal structures of SCLYs from rats [17] and humans [27] have been solved with accession numbers 3A9X (rat Scly (rScly) in a native form), 3A9Y (rScly in complex with L-cysteine), 3A9Z (rScly in complex with selenopropionate), 3GZC (human SCLY (hSCLY) in a native form), and 3GZD (hSCLY in another native form). The protein folds of both SCLYs are similar to those of *E. coli* IscS [28], *Thermotoga maritima* NifS-like protein [29], *Synechocystis* sp. PCC 6803 SufS [30], *E. coli* CsdB/SufS [31–33], *Synechocystis* cysteine C-S lyase [34], and *Pseudomonas fluorescens* kynureninase [35]. In fact, they all belong to the same Fold type I family of PLP-dependent enzymes [36, 37]. CDSs are known to be divided into two major families: Group I, including IscS and NifS-like proteins, and Group II, including SufS and CsdB proteins [21, 23, 38]. SCLY shows higher similarity with Group I CDS than with the others [16].

SCLY has an important cysteine residue, Cys375 of rScly, which is completely conserved among mammalian SCLYs. Cys375 of rScly lies within a flexible extended lobe (Ser374-Ile392) and is located near the cofactor PLP, forming a Schiff base with Lys247 at the active site [17]. A mutant SCLY (C375A), in which Cys375 is replaced by alanine, exhibits no activity on selenocysteine, suggesting that Cys375 is essential for the catalytic process. Electrospray ionization-mass spectrometry of rScly incubated with L-selenocysteine revealed the binding of one selenium atom to Cys375 per molecule: a cysteine selenopersulfide intermediate (Scly-S-Se$^-$) was formed at Cys375 [17]. Note that no such molecular species appeared when the C375A mutant enzyme was used. The X-ray structure of the rScly•selenopropionate complex indicated that the thiol group of Cys375 interacts with the selenolate part of selenopropionate, although it is depleted of the amino group and differs from selenocysteine. Therefore, Cys375 probably plays an important role in catalysis of bringing selenocysteine to a key position that facilitates the formation of a Schiff base with PLP. Moreover, the small domain (denoted by the flexible lobe in Fig. 10.2) moves towards the active site, which enables the creation of an ordered structure (otherwise disordered in the unliganded form) allowing the encapsulation of selenopropionate within the active site cavity [17]. This conformational change was also observed in the X-ray structure of the rScly•L-cysteine complexes. This open-closed conformational change enables the active site to be completely covered (as if protected with a lid) and protect the bound selenium from the solvent. The disorder-order transition of the flexible lobe probably plays a pivotal role in the catalytic event, as Cys375 is then oriented properly for the correct interaction with the selenol of the substrate. In addition, the oxygen-sensitive selenopersulfide formed in the cavity is effectively shielded from the solvent. A similar open-closed conformational change was reported in the crystal structure of hSCLY [27].

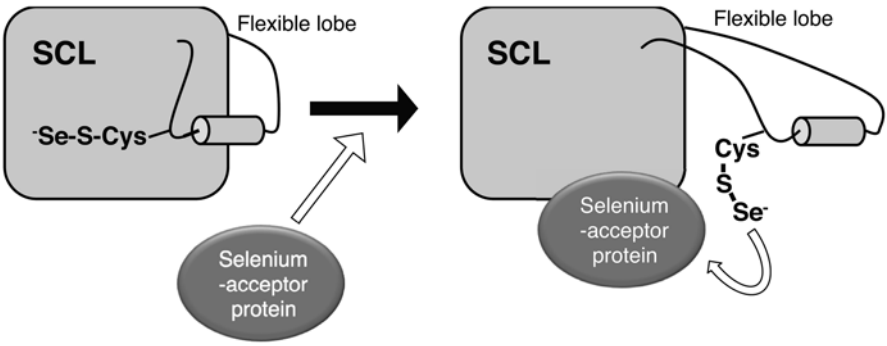

Fig. 10.2 Possible mechanism of selenium delivery to a selenium-acceptor protein. Selenopersulfide formed on the active site cysteine residue (corresponding to Cys375 of rat SCLY) is located on the flexible lobe. Selenopersulfide selenium may be directly transferred to a specific selenium-acceptor protein via a conformational change of the flexible lobe associated with protein-protein interaction

10.6 Catalytic Mechanism

The reaction mechanism of SCLY has been independently studied by two groups [17, 27, 39]. The models presented for rScly [17] and hSCLY [27, 39] are similar except for a slight difference. The small domain rotation and the ordering of the flexible lobe occur with the binding of selenocysteine at the active site of SCLY as described above. Thereby, its γ–Se atom can interact with the thiol group of Cys375 (Fig. 10.3a). The amino group of L-selenocysteine should be deprotonated in order to form the external aldimine with PLP, thus enabling the release of the ε-amino group of Lys247 in a deprotonated form. This form is essential to act as a base to allow the removal of the α-hydrogen from the substrate L-selenocysteine. The protonated Lys247 that is formed can then provide a proton to the C4′ of the quinonoid intermediate, generating the substrate-ketimine intermediate. The substrate-ketimine subsequently transfers selenium to Cys375 to form cysteine selenopersulfide (Cys375–S–Se$^-$) and the alanine-enamine intermediate, which is eventually converted to alanine, while bringing the enzyme back to its initial state. It remains unclear whether selenium is directly released from Cys375-S-Se$^-$ or is trapped by a selenium-transferring protein and subsequently released by a reductant in the

Fig. 10.3 (continued) resulting in the production of the external aldimine to initiate the catalytic reaction. (**b**) When L-cysteine enters the active site, the Sγ atom of the bound cysteine, unlike the α amino group, creates a covalent bond with the C4′ atom of PLP in order to form a reversible non-productive adduct. (**c**) Protonated Cys388, which is stabilized by Asp146, is able to eliminate selenium from the selenocysteine-ketimine intermediate. (**d**) Protonated Cys388 cannot interact with the protonated Sγ atom of the cysteine-ketimine intermediate, resulting in the termination of the reaction. (**e**) When Asp146 is replaced by Lys, the deprotonated Cys388, which is stabilized by Lys146, can eliminate the Sγ atom of the cysteine-ketimine intermediate

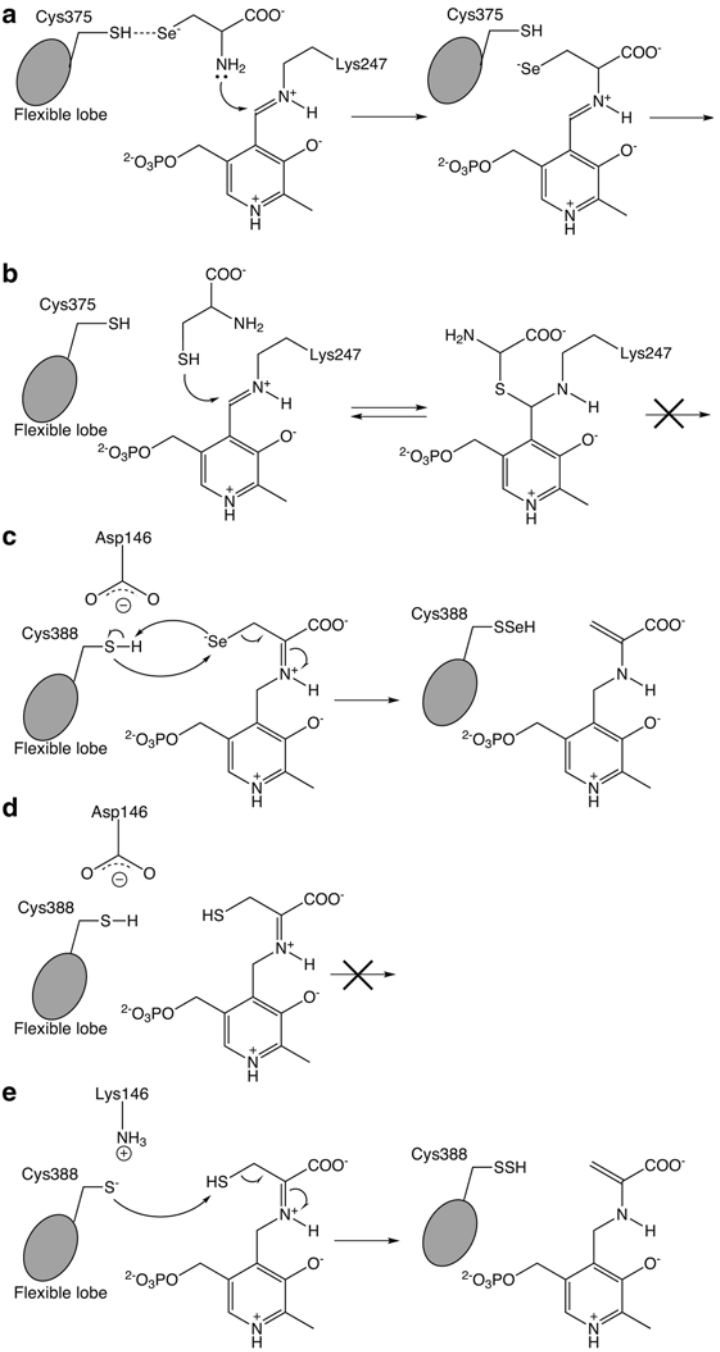

Fig. 10.3 Proposed mechanisms for the discrimination between selenocysteine and cysteine by rat SCLY (**a** and **b**) and human SCLY (**c**–**e**). (**a**) Interaction between the active site Cys375 (in protonated form) and the substrate selenolate allows selenocysteine to form the Schiff base with PLP,

reaction system. If selenopersulfide selenium, which is more sensitive to oxygen than persulfide sulfur, is released directly from an intermediate and diluted by the bulk solvent, its delivery to a specific acceptor molecule would be inefficient. The selenium atom of the selenopersulfide is probably transferred to a selenium-acceptor protein yet unidentified, presumably via specific protein-protein interactions. Selenophosphate synthetase is one of the most promising candidates for such a selenium acceptor role. The following studies support this thought: CDSs from *E. coli* [40] and *Methanococcus vannielii* [41] as well as mouse SCLY (Mihara et al., unpublished results) effectively provided selenophosphate synthetase with selenium from L-selenocysteine in order to produce selenophosphate. Another study using co-immunoprecipitation with a reticulocyte lysate system also indicated that SCLY associates with either selenophosphate synthetase isozymes, SEPHS1 and SEPHS2 [42]. Further studies with a yeast 2-hybrid screening system have shown that SCLY interacts with various proteins from mouse cDNA libraries related to spermatogenesis, protein synthesis, cell viability, and apoptosis as well as major urinary proteins [42]. These studies may provide clues to identify selenium-acceptor proteins from SCLY and to understand their physiological significance.

10.7 Discrimination Between Selenium and Sulfur

Sulfur and selenium atoms resemble each other, and most enzymes acting on sulfur compounds cannot distinguish between these atoms within substrate molecules [11, 43]. However, SCLY is markedly different from CDSs because the latter act indiscriminately on both selenocysteine and cysteine. The strict specificity of SCLY towards selenocysteine can be understood carrying out ultraviolet-visible spectral and crystallographic studies of the C375A mutant of rSCLY [17]. While inactive, the C375A mutant shows the same spectral change when forming a complex with either L-selenocysteine or L-cysteine. In the absence of Cys375, L-selenocysteine cannot form a Schiff base with PLP, and its amino group interacts with the 3′-OH group of PLP. The nitrogen atom of the lysyl residue forming a Schiff base is then deprotonated as in the wild type enzyme complexed with L-cysteine (Fig. 10.3b). Cysteine is distinct from selenocysteine because its thiol group is mostly in a protonated form due to its higher pK_a value (around 8.5). Consequently, the thiol group of L-cysteine interacts with the nitrogen atom of the lysyl residue, forming a Schiff base and thus generating a non-productive complex (Fig. 10.3b). On the other hand, when the wild type SCLY forms a complex with L-selenocysteine (Fig. 10.3a), the thiol group of Cys375 not only acts as the acceptor for the selenium atom liberated from selenocysteine, but also interacts with the deprotonated selenol of selenocysteine (Fig. 10.3a). Thus, the amino group of L-selenocysteine is free to react with the C4′-carbon of the internal Schiff base, leading to the formation of an external aldimine, an essential productive complex as described in Sect. 10.6. A similar mechanism for the discrimination between selenocysteine and cysteine is proposed from the analysis of hSCLY: the protonated form of the active site Cys388 (corresponding

to Cys375 of rScly) is a key factor in conferring specificity of the enzyme towards selenocysteine [39] (Fig. 10.3c–e). Asp146 of hSCLY, which is conserved among all known mammalian SCLYs, is thought to stabilize the deprotonated state of the active site, Cys388, because conversion of Asp146 to lysine, a common feature among CDSs, generated hSCLY with CDS activity [27, 39].

10.8 Biological Role

Selenoprotein P (Sepp1) is unique in that it has multiple selenocysteine residues in a unique two-domain structure. The N-terminal domain only displays one selenocysteine while the C-terminal domain contains many selenocysteine residues, nine in human SEPP1, for example [10]. SEPP1 is the major plasma selenoprotein and is predominantly synthesized in liver before being transported to various organs by the lipoprotein receptor-related proteins megalin and ApoER2 (see Chap. 22). SEPP1 retains local selenium by preventing renal excretion and also acts as a reversible storage device for selenium in brain. Therefore, the deletion of the *Sepp1* gene in mice causes severe neuronal abnormality [44]. Gene silencing studies showed that SCLY is required for the utilization of selenium derived from SEPP1 during selenoprotein biosynthesis in HeLa cells [45]. Nevertheless, *Scly*-knockout mice showed no apparent behavioral or phenotype changes, unlike *Sepp1*-knockout mice [46]. Therefore, SCLY can be readily replaced by other enzymes such as NifS-like CDSs, even though present in normal selenium metabolism. However, *Scly*-knockout mice fed with low-selenium diet displayed a subtle learning deficit and showed a significantly reduced expression of selenoproteins in brain [46]. Moreover, the simultaneous deletion of *Sepp1* and *Scly* genes in mice generated a worsened phenotype compared with single *Sepp1*-knockout mice [47]. Double-knockout mice needed supraphysiological selenium supplementation to survive, even though this addition does not cure the neuronal abnormality. In addition, selenoprotein levels in the brain are reduced in double knockout mice [47]. Therefore, SCLY must play a hitherto unknown role in supporting SEPP1 metabolism, which cannot be fully compensated by other enzymes such as NifS-like CDSs, to maintain selenium homeostasis in brain.

SEPP1 also plays a role in testis and kidney, where SCLY is highly expressed [10]. SEPP1 is transported through the receptor ApoER2, which is abundantly found in Sertoli cells [48]. However, SCLY is not expressed in these cells, and it is not clear whether SCLY has functions as a support for SEPP1 metabolism in testis. Furthermore, it is evident that SCLY is not only present in nuclei of cultured HeLa cells, but also resides in nuclei of mouse organs such as kidney and testis even though the enzyme does not display any clear nuclear localizing signals [45]. The components of the selenoprotein biosynthesis machinery such as $tRNA^{[Ser]Sec}$, SEPHS2, and SECISBP2 are known to be found in the nucleus. In addition, mature selenocysteyl-$tRNA^{[Ser]Sec}$ is protected against nucleocytoplasmic shuttling [49]. Selenoprotein mRNAs are also prevented from nonsense-mediated decay through assemblage with the selenocysteine incorporation complex [50]. Therefore, it may

be reasonable to suggest that SCLY also participates in the selenoprotein biosynthesis machinery complex if it acts in selenium metabolism.

Recent studies have shown that *Scly*-knockout in mice causes diet-induced obesity and enhances the development of metabolic syndrome as compared to wild type mice, even with adequate dietary selenium intake [51, 52]. Interestingly, only the expression of serum SEPP1 was increased by the knockout of *Scly* [52]. Obesity may be due to increased expression of SEPP1, although further studies are required to clarify the physiological significance of this phenomenon. Furthermore, *Scly*-knockout mice showed higher pyruvate levels in liver, higher expression of pyruvate carboxylase and pyruvate dehydrogenase, and increased activity of citrate synthase. These features are all related to the tricarboxylic acid cycle and fatty acid biosynthesis [52]. The yeast two-hybrid system studies showed interactions of SCLY with various proteins related to the energy metabolism such as NADH dehydrogenase [53], as well as other enzymes participating in carbohydrate and lipid metabolism such as aldehyde reductase [42]. Thus, SCLY may have some interesting, but yet unknown, functions in energy metabolism. In the future, further studies will be required to shed light on the biological functions of SCLY.

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Part II
Selenoproteins, Their Occurrence and Function

Selenoproteins are involved in diverse processes, with redox reactions being a common theme. The number of selenoproteins identified in various organisms varies from 1 to more than 50, and some life forms lack these proteins. The chapters in this section focus on selenoprotein occurrence, cellular functions, and their fascinating evolution.

Chapter 11
Eukaryotic Selenoproteomes

Vadim N. Gladyshev

Abstract Progress in high-throughput sequencing and development of computational tools for identification of SECIS elements, selenoprotein genes and selenocysteine machinery allows recognition of organisms that are dependent, or not dependent, on selenium (Se) and identification of selenoproteins responsible for this trait. Full sets of selenoproteins in organisms, designated selenoproteomes, have been characterized for humans, which have 25 selenoprotein genes, as well as for most other organisms with sequenced genomes. This chapter offers an overview of eukaryotic selenoproteins at the level of individual proteins, protein families and entire selenoproteomes. Comparative genomic and ionomic analyses offer exciting avenues for studying selenoprotein function and evolution, provide insights into the biological functions of the trace element, Se, and allow addressing other important biological questions.

Keywords Genomics • Model organism • Selenium utilization • Selenoprotein • Selenoproteome

11.1 Introduction

Selenium (Se) is utilized by organisms in three different ways. First, this element is present in the form of 2-selenouridine in the wobble position of certain bacterial tRNAs [1]. Second, it occurs in some bacterial Se-containing molybdoproteins as a labile cofactor that contains a Se-Mo bond that is directly involved in catalysis [2–4]. Third, Se is present in proteins in the form of selenocysteine (Sec), the 21st amino acid in the genetic code [5, 6]. Sec is encoded by UGA and has been found in each of the three domains of life (i.e., bacteria, archaea and eukaryotes). It is now clear that the essential roles of Se in biology, as well as its beneficial functions in human health, are due to its presence in proteins in the form of Sec. Interestingly, unlike 20 common amino acids in proteins (which can be used throughout the protein sequence), Sec is utilized only when it is essential for protein function.

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Accordingly, it is often a key functional group in proteins. Moreover, Sec is specifically used for redox catalysis. As such, information on identity and functions of selenoproteins is often a key to biological and biomedical roles of Se, and it can be used to address a variety of other biomedical issues.

11.2 Computational Tools for Selenoprotein Identification

All selenoprotein genes have two characteristic features: (1) an UGA codon that designates Sec; and (2) a Sec insertion sequence (SECIS) element. The UGA codon does not provide sufficient information to identify Sec positions. However, SECIS elements can be used for this purpose as these structures are highly specific for selenoprotein genes, have conserved sequence elements and possess a sufficiently complex secondary structure. Therefore, previous bioinformatics analyses mainly focused on SECIS elements, and selenoprotein discovery was based on the following general strategy: (1) finding candidate SECIS elements; (2) analyzing upstream regions to identify coding regions of selenoproteins; and (3) testing these candidates for insertion of Sec by metabolically labeling cells with ^{75}Se. The first selenoproteins identified using this approach were mammalian selenoproteins R (now known as methionine-*R*-sulfoxide reductase 1 or MsrB1), N and T [9, 10]. These searches were initially restricted to small nucleotide sequence databases and later adapted to entire genomes [11–13]. To aid in these analyses, groups of closely related genomes are analyzed in order to identify conserved SECIS elements that belong to selenoprotein orthologs in these organisms [14]. Over the years, the technique was refined, and currently an arsenal of tools is available for identification of SECIS elements and selenoprotein genes, most notably, SECISearch3 [7], Selenoprofiles [8] and Seblastian [7].

In addition to the approach that identifies SECIS elements, a method was developed that searches for UGA codons flanked by conserved coding sequences [13–16]. This approach is based on the finding that the majority of selenoprotein genes have orthologs, in which Sec is replaced with cysteine (Cys). For example, an ortholog of mammalian selenoprotein MsrB1 is a Cys-containing MsrB in yeast and plants. This method is used as follows: protein databases, e.g., protein sequences from NCBI, are searched against NCBI nucleotide sequences with TBLASTN to identify nucleotide sequences which, when translated in one of six reading frames, align with Cys-containing protein sequences from the protein database, such that Cys residues correspond to UGA in the nucleotide sequences and the resulting Sec/Cys pairs are flanked by conserved sequences. Such a Sec/Cys homology approach is completely independent of the searches for SECIS elements. As both Sec/Cys and SECIS-based algorithms identify very similar sets of selenoprotein genes in organisms, both methods show excellent performance and can identify the majority, and often all, selenoprotein genes in sequenced genomes.

Figure 11.1 summarizes known eukaryotic selenoprotein families. We further provide a brief overview of selenoproteins that have been functionally characterized. Readers are referred to individual chapters within the book for additional information on various selenoproteins.

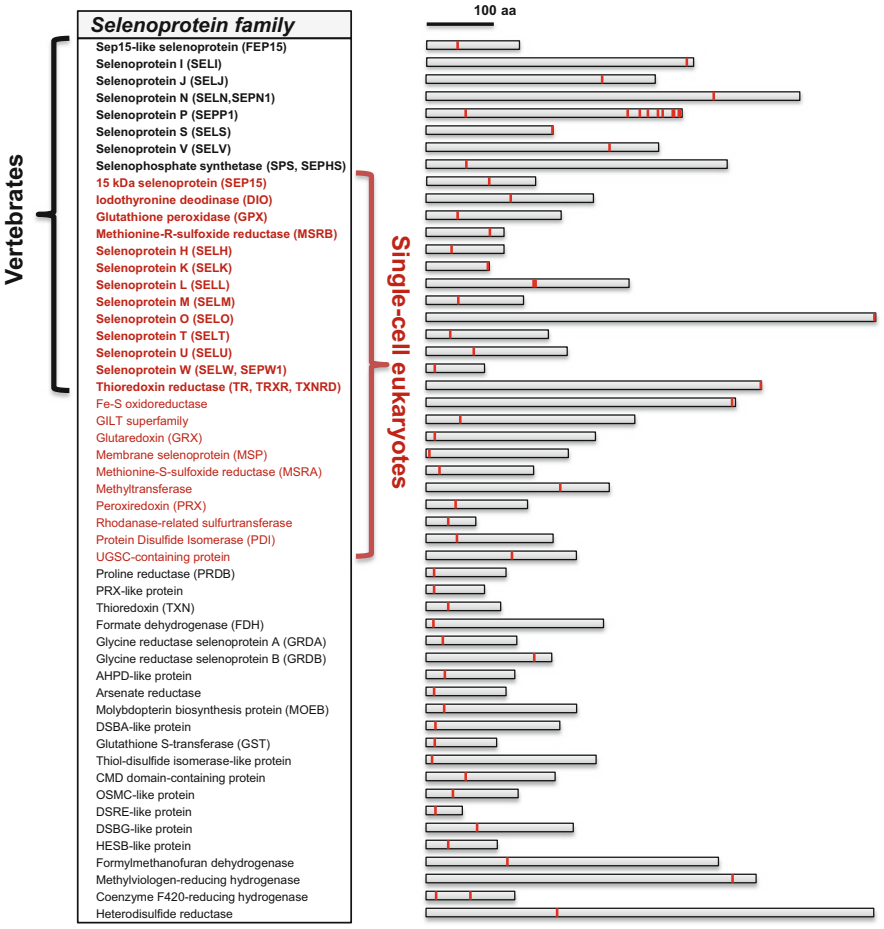

Fig. 11.1 Selenoprotein families. Known selenoprotein families are shown. Selenoproteins that occur in vertebrates and unicellular eukaryotes are indicated and shown in *bold* and *red*, respectively. Additional selenoproteins that only occur in prokaryotes are shown below eukaryotic selenoproteins. On the right, relative sizes of selenoproteins are shown (relative to a 100 amino acid scale) and the location of Sec within protein sequence is shown by a *red mark*

11.3 Mammalian Selenoproteins

11.3.1 Glutathione Peroxidases

There are eight glutathione peroxidases (GPX1-GPX8) in mammals, five of which are selenoproteins (GPX1, GPX2, GPX3, GPX4 and GPX6). GPX1-GPX4 are selenoproteins in all mammals, whereas GPX6 is a selenoprotein in many species, but in some it is a Cys-containing protein [14]. GPX1 was the first animal selenoprotein identified [17]. It is also the most abundant mammalian selenoprotein that exhibits

a particularly high expression in the liver and kidney. This antioxidant enzyme catalyzes glutathione-dependent hydroperoxide reduction. GPX4 has received much attention recently due to its essential function (e.g., its knockout in mice leads to early embryonic lethality), and its role in ferroptosis and regulation of phospholipid hydroperoxide levels [18]. Interestingly, a mitochondrial form of this protein serves a structural role in mature sperm and participates in disulfide bond formation during spermiogenesis [19]. Whereas GPX1 and GPX4 are expressed in all cells, GPX2 expression is largely restricted to the gastrointestinal tract, and GPX3 is primarily made in the kidney and secreted into the blood stream. GPX3 localizes to the basement membrane of the proximal tubules in kidney [20]. However, it remains unclear how it can function in the extracellular milieu in the absence of sufficient levels of thiol reductants. Besides mammals, selenoprotein GPX homologs were found in most animals as well as in various single-celled eukaryotes and even in bacteria. However, the ancestral form of these enzymes is a Cys-containing protein, and it is thought that Cys was replaced with Sec during evolution, making these enzymes better catalysts.

11.3.2 Thyroid Hormone Deiodinases

Mammals have three deiodinase genes (DIO1, DIO2 and DIO3), which activate and/or inactivate thyroid hormones by reductive deiodination. Deiodinases also occur in other vertebrates, and their homologs (some of them are Cys-containing proteins) were even detected in unicellular eukaryotes and bacteria, although their function must be different in these organisms. Like GPXs and the majority of other selenoproteins, deiodinases are thioredoxin-fold proteins.

11.3.3 Thioredoxin Reductases

All three mammalian thioredoxin reductases (TXNRDs) are selenoproteins, hence the entire thioredoxin (TXN) system in mammals is dependent on Se. In these proteins, Sec is present in the C-terminal penultimate position, preceded by Cys and followed by glycine. These enzymes evolved from glutathione reductases by including a C-terminal Sec-containing extension that serves as an intraprotein substrate for the N-terminal active center of pyridine nucleotide disulfide oxidoreductase family members [21–24]. TXNRD1 is a cytosolic and nuclear protein. Its main function is to maintain TXN1 in the reduced state by reducing a disulfide in this protein in an NADPH-dependent manner. It exhibits broad substrate specificity, especially with regard to low molecular weight compounds [25] and occurs in the form of multiple isoforms generated by alternative transcription initiation and splicing [26–28]. Another member of the mammalian TXNRD family is thioredoxin/glutathione reductase (TGR, TXNRD3). This enzyme has an additional N-terminal

glutaredoxin domain [22], which is implicated in the formation/isomerization of disulfide bonds during sperm maturation [29]. TGR can catalyze reactions specific for both TXN and glutathione systems. A third member of the family, TXNRD2, is a mitochondrial protein, which maintains mitochondrial TXN2 as well as glutaredoxin 2 in the reduced state. TXNRD1 and TXNRD2 are essential for embryonic development in mice [30, 31].

11.3.4 Methionine-R-Sulfoxide Reductase 1 (MSRB1)

This protein was initially designated as selenoprotein R [9] and selenoprotein X [10], but after it was found to catalyze stereospecific reduction of methionine-*R*-sulfoxide residues in proteins, it was renamed MSRB1 [32]. Mammals have two additional MSRBs (MSRB2 and MSRB3), which contain catalytic Cys in place of Sec and reside in mitochondria and endoplasmic reticulum, respectively [33]. At least in the liver and kidney of mammals, MSRB1 has the highest activity of all MSRBs, so the protein reductive repair function is dependent on Se in mammals. MSRB1 is located in the cytosol and nucleus [33]. *Msrb1* knockout mice are viable, but are characterized by oxidative stress [34]. An important recent discovery is the role of MSRB1 in regulation of actin polymerization by reversible methionine-*R*-sulfoxidation [35].

11.3.5 15 kDa Selenoprotein (SEP15)

SEP15 is a conserved eukaryotic selenoprotein that occurs in most animals as well as in some unicellular eukaryotes, such as algae. It resides in the endoplasmic reticulum where it binds UDP-glucose:glycoprotein glucosyltransferase, a sensor of protein folding [36]. SEP15 is composed of an N-terminal ER signal peptide, a Cys-rich domain responsible for binding UDP-glucose:glycoprotein glucosyltransferase, and a C-terminal domain characterized by the thioredoxin-like fold. SEP15 may in part be responsible for the effect of Se in cancer [37, 38]. *Sep15* knockout mice are viable, but develop cataracts [39].

11.3.6 Selenophosphate Synthetase 2 (SEPHS2)

By analogy to bacterial selenophosphate synthetase SelD [40], SEPHS2 (also known as SPS2) was thought to synthesize selenophosphate, a Se donor compound. It is essential for selenoprotein biosynthesis in mammals. Mammals and some other metazoans have an SEPHS2 paralog, SEPHS1, whose function remains unknown [41], but it is not related to Sec biosynthesis [42].

11.3.7 Selenoprotein P (SEPP1)

SEPP1 is the only selenoprotein with multiple Sec residues in mammals [43], e.g., there are ten Sec residues in human and mouse SEPP1, and seven in the naked mole rat protein [44]. However, the number of Sec residues in SEPP1 homologs varies greatly (7–16 in mammals) [44]. SEPP1 is the major plasma selenoprotein, which is synthesized primarily in the liver and delivers Se to certain other organs and tissues [45, 46]. The *Sepp1* knockout mouse has been a particularly useful model in examining Se metabolism in mammals [47].

11.3.8 Selenoproteins W (SelW, SEPW1) and V (SELV)

SelW is the smallest mammalian selenoprotein [48]. Although it was one of the first identified, its function remains unknown. SEPW1 homologs were identified in lower eukaryotes and even bacteria, but these findings did not help identify the function of SEPW1 [16]. A SEPW1 paralog, SELV, is a larger protein due to an additional N-terminal sequence of unknown function [14]. This protein is expressed exclusively in testes. Its function is also not known.

11.3.9 Selenoproteins T (SELT), M (SELM) and H (SELH)

Functions of these mammalian selenoproteins are not known. They are listed here together because they belong to a group of thioredoxin-like fold proteins (together with SEP15, SEPW1 and SELV). SELT is among the first selenoproteins identified through bioinformatics [9]. SELM is a distant homolog of SEP15 and, like SEP15, it resides in the endoplasmic reticulum [49]. SELH was first identified as BthD in fruit flies [12, 14]. It resides in the nucleus. Several studies have found that knockdown of these proteins leads to oxidative stress suggesting roles, at least partially, as antioxidants.

11.3.10 Selenoproteins O (SELO) and I (SELI)

SELO is a widely distributed protein with homologs in animals, bacteria, yeast and plants, but the functions of any member of this protein family are not known [14]. Only vertebrate homologs of SELO have Sec, which is located in the C-terminal penultimate position, and the protein is located in mitochondria [50]. SELI is a recently evolved selenoprotein specific to vertebrates [14]. This membrane selenoprotein has no known function.

11.3.11 Selenoprotein K (SELK) and S (SELS)

SELK and SELS are unusual among selenoproteins in that they do not have a pronounced secondary structure [14]. These small selenoproteins contain a single transmembrane helix in the N-terminal sequence that targets them to the ER membrane. Studies revealed the role of SELS and SELK in retrotranslocation of misfolded proteins from the ER to the cytosol, where these proteins are further degraded [51]. Both proteins bind Derlins, which are ER membrane-resident proteins, and associate with multiprotein complexes [52, 53]. In addition, SELS was implicated in inflammation and the immune response, and SELK in protein palmitoylation [54]. A *Selk* knockout mouse model is viable [55].

11.3.12 Selenoprotein N (SEPN1)

One of the first selenoprotein discovered through bioinformatics approaches [10], SEPN1 remains a selenoprotein of unknown function. This protein was implicated in the role of Se in muscle function through biochemical and genetic analyses, as well as through analyses of knockout mice [56], and was found to serve as a cofactor for the ryanodine receptor [57]. Mutations in SEPN1 are associated with a hereditary muscular dystrophy.

11.4 Additional Selenoproteins in Eukaryotes

The following selenoproteins that are absent in mammals were identified in eukaryotes: methionine-S-sulfoxide reductase (MSRA), protein disulfide isomerase (PDI), selenoproteins U (SELU), L (SELL), J (SELJ), FEP15, MCS, and plasmodial selenoproteins Sel1, Sel2, Sel3 and Sel4, and a selenoprotein SelTryp from *Trypanosoma*. MSRA is a widely distributed protein family, whose function is to repair methionine residues in proteins. Like MSRB, it catalyzes a stereospecific reduction of methionine sulfoxides, but is specific for methionine-*S*-sulfoxides. MSRA was initially found in the green algae, *Chlamydomonas* [58], but later was also identified in other eukaryotes as well as in some bacteria. PDI is also very narrowly distributed in eukaryotes [59], in contrast to Cys-containing PDIs, which are essential for the formation of disulfide bonds in the ER of eukaryotic cells. SELU [60], SELJ [61], FEP15 [62], and SELL [63] were only found in fish and/or invertebrates. The four *Plasmodium* selenoproteins (Sel1-Sel4) showed no detectable homology to any other protein [64]. However, Sel1 and Sel4 have Sec in the C-terminal regions and may be related to SELK and SELS.

11.5 Selenoprotein Functions

Eukaryotic selenoproteins for which functions are known are oxidoreductases. In these proteins, Sec is the catalytic residue that is employed because it is superior to Cys in this function [40, 65–68]. In selenoproteins, Sec reversibly changes its redox state during catalysis. Functions of many selenoproteins, particularly those found in vertebrates, are not known. However, by analogy to proteins with known functions, it may be expected that the majority of these uncharacterized selenoproteins are also oxidoreductases.

Eukaryotic selenoproteins may be loosely clustered into two groups based on the location of Sec. The most abundant selenoprotein group includes proteins containing Sec in the N-terminal region or in the middle of the protein. Many of these selenoproteins exhibit thioredoxin or thioredoxin-like folds, but some proteins (e.g., SEPHS2, MSRA) show different folds. In these proteins, Sec is the catalytic group, which often works in concert with a resolving Cys. In the second group, Sec is located in the C-terminal sequences. These proteins include selenoproteins K, S, O, I and TXNRDs. Except for TXNRDs, the function of Sec in selenoproteins in this group is not known.

Non-catalytic functions of Sec, while rare, have also been described. Known examples include Sec residues in the C-terminal region of SEPP1, which function in transporting Se from liver to other organs, and recently evolved Sec residues in the *Metridium senile* MsrB homolog, wherein the function of these Sec residues is not known [69].

11.6 Selenoproteomes

Availability of tools for efficient identification of selenoprotein genes led to the recognition of all, or almost all, selenoproteins in many organisms [5, 6]. Information on the full sets of selenoproteins in organisms (selenoproteomes) offers an opportunity to address diverse questions relevant to the biology of Se, e.g., by linking individual selenoproteins or selenoprotein groups and specific effects of dietary Se [70]. In this regard, Se differs from other trace elements where new proteins are still discovered biochemically, and often by accident, and where full sets of proteins dependent on a particular element is difficult to ascertain.

Among metazoan selenoproteomes, an interesting case is represented by *C. elegans* and *C. briggsae*, which have only a single UGA codon in their genomes that codes for Sec [71]. This codon occurs in the *TxnRd1* gene, and phylogenetic analyses suggested that other selenoprotein genes were lost in nematodes during evolution. Thus, the entire Sec machinery is maintained in these organisms to insert a single Sec residue. Selenoproteinless animals have also been identified, most of which are arthropods [72, 73]. Information about such animals (or other organisms that lost selenoproteins, such as yeast and higher plants) helps explain the changing requirements for Se during evolution. Interestingly, selenoproteinless insects lost

the entire Sec insertion machinery, but preserved SEPHS1, suggesting that this protein is not involved in Sec biosynthesis [42, 72].

A recent study identified 59 selenoproteins in the harmful alga, *Aureococcus anophagefferens* [74, 75]. This organism has the largest and the most diverse selenoproteome identified to date, including known eukaryotic selenoproteins, selenoproteins previously only detected in bacteria, and novel selenoproteins. Similar to smaller selenoproteomes, the *A. anophagefferens* selenoproteome was dominated by the thioredoxin fold proteins, and oxidoreductase functions could be assigned to the majority of detected selenoproteins. Se was required for the growth of *A. anophagefferens* as cultures grew maximally at nanomolar Se concentrations. Moreover, in a coastal ecosystem, dissolved Se was elevated before and after *A. anophagefferens* blooms, but reduced by >95 % during the peak of blooms. Consistent with this pattern, enrichment of seawater with selenite before and after a bloom did not affect the growth of *A. anophagefferens*, but enrichment during the peak of the bloom significantly increased population growth rates. Thus, Se inventories, which can be anthropogenically enriched, can support proliferation of harmful algal blooms through synthesis of a large arsenal of selenoproteins.

Selenoproteome analyses also are capable of uncovering trends in the use of Sec [76], although some limitations of this approach have been described [77]. An analysis of selenoproteomes of several model eukaryotes detected 26–29 selenoprotein genes in two species of *Ostreococcus*, five in the social amoebae, *Dictyostelium discoideum*, and 16 in the diatom, *Thalassiosira pseudonana*, including several new selenoproteins [76]. Further analyses identified massive, independent selenoprotein losses in land plants, fungi, nematodes, insects and some protists. Comparative analyses of selenoprotein-rich and -deficient organisms revealed that aquatic organisms generally have large selenoproteomes, whereas several groups of terrestrial organisms reduced their selenoproteomes through loss of selenoprotein genes and replacement of Sec with Cys. These observations suggested that many selenoproteins originated at the base of the eukaryotic domain and showed that the environment may play a role in selenoproteome evolution. In particular, aquatic organisms apparently retained and sometimes expanded their selenoproteomes, whereas the selenoproteomes of some terrestrial organisms were reduced or completely lost. It is an interesting possibility that aquatic life supports Se utilization, whereas terrestrial habitats lead to the reduced use of this trace element [76].

In a separate study involving vertebrates, reconstruction of evolutionary changes in the Se transport domain of SEPP1 revealed a decrease in the Sec content specifically in the mammalian lineage via replacement of Sec with Cys [44]. Compared to mammals, fish showed higher Sec content of SEPP1, larger selenoproteomes, elevated SEPP1 gene expression, and higher levels of tissue Se. In addition, mammals replaced Sec with Cys in several proteins and lost several selenoproteins altogether, whereas such events were not found in fish. These data suggested that evolution from fish to mammals was accompanied by a decreased use of Sec and that analyses of SEPP1, selenoproteomes and Sec/Cys transitions provide a genetic marker of utilization of this trace element in vertebrates. The evolved reduced reliance on Se raises questions regarding the need to maximize selenoprotein expression by Se

dietary supplements in situations where pathology is not imminent, which is a currently accepted practice.

A more recent study characterized the selenoproteomes of 44 sequenced vertebrates, and detected 45 selenoprotein subfamilies [78]. Twenty-eight of them were found in mammals, and 41 in bony fishes. The study defined the ancestral vertebrate (28 proteins) and mammalian (25 proteins) selenoproteomes, and described how they evolved along lineages through gene duplication (20 events), gene loss (10 events) and replacement of Sec with Cys (12 events). It was shown that an intronless *SEPHS2* gene evolved in early mammals and replaced functionally the original multiexon gene in placental mammals, whereas both genes remain in marsupials. Mammalian TXNRD1 and TGR evolved from an ancestral glutaredoxin-domain containing enzyme, still present in fish. Selenoprotein V and GPX6 evolved specifically in placental mammals from duplications of SEPW1 and GPX3, respectively, and GPX6 lost Sec several times independently. Bony fishes were characterized by duplications of several selenoprotein families (GPX1, GPX3, GPX4, DIO3, MSRB1, SELJ, SELO, SELT, SELlU1, and SEPW2). The study also identified new isoforms for several selenoproteins and described unusually conserved selenoprotein pseudogenes.

11.7 Applications of Selenoproteome Analyses to Biology

11.7.1 Genetic Code Supports Targeted Insertion of Two Amino Acids by One Codon

Strict one-to-one correspondence between codons and amino acids was thought to be an essential feature of the genetic code. However, a recent selenoproteome analysis of the ciliate *Euplotes crassus* revealed that one codon can code for two different amino acids with the choice of the inserted amino acid determined by a specific 3′-untranslated region and location of the dual-function codon within the mRNA [79]. It was found that the codon UGA specified insertion of Sec and Cys in *E. crassus*, that the dual use of this codon could occur even within the same protein, and that the structural arrangements of *Euplotes* mRNA preserved the location-dependent dual function of UGA when expressed in mammalian cells. Thus, the genetic code supports the use of one codon to code for multiple amino acids. This finding challenged one of the foundations of the code, i.e., that only one genetic codeword is used for one amino acid in an organism.

11.7.2 High-Throughput Identification of Catalytic Redox-Active Cysteine Residues

Cys residues often play critical roles in proteins; however, identification of their specific functions has been limited to case-by-case experimental approaches. A recent study developed a procedure for high-throughput identification of catalytic

redox-active Cys in proteins by searching for sporadic Sec/Cys pairs in sequence databases [80]. This method was independent of protein family, structure, and taxon. It was used to selectively detect the majority of known proteins with redox-active Cys and to make additional predictions, one of which was verified. Rapid accumulation of sequence information from genomic and metagenomic projects, coupled with selenoproteome analyses, should allow detection of many additional oxidoreductase families as well as identification of redox-active Cys in these proteins.

11.8 Analyses of Ionomes

Mechanisms that regulate Se and other trace elements in human cells were recently characterized by a genome-wide high-throughput siRNA/ionomics screen [81]. Se levels were controlled through the Sec machinery and expression of abundant selenoproteins. On the other hand, copper balance was affected by lipid metabolism and required machinery involved in protein trafficking and post-translational modifications, whereas the iron levels were influenced by iron import and expression of the iron/heme-containing enzymes.

A separate study examined Se and 17 other elements in the brain, heart, kidney, and liver of 26 mammalian species and reported the elemental composition of these organs, the patterns of utilization across the species, and their correlation with body mass and longevity [82]. Across the organs, distinct distribution patterns for abundant elements, transition metals, and toxic elements were observed. In this analysis, Se clustered with cadmium and arsenic, suggesting that Se toxicity is the property that is particularly important for organisms. Some elements showed lineage-specific patterns, including reduced Se utilization in African mole rats *Heterocephalus glaber*, and positive correlation between the number of Sec residues in SEPP1 and Se levels in liver and kidney across mammals. Interestingly, species lifespan correlated negatively with Se, suggesting that low utilization of Se may help achieve longevity. This study provided insights into the variation of Se levels in mammals according to organ physiology, lineage specialization, and longevity.

11.9 Concluding Remarks

Largely due to the remarkable progress in genomics, we now know which organisms utilize Se, and which do not, and which selenoproteins and Sec machinery mediate the Se utilization trait. Approximately 100 selenoprotein families are currently known, including 25 selenoprotein genes encoded in the human genome. This information allows researchers to study various aspects of Se biology and selenoprotein functions and address questions, not even imaginable until only recently, such as cross-species, cross-population and geographical distribution of Se and selenoprotein utilization and expansion of the genetic code. In selenoproteins with

known functions, Sec is a key functional group that carries out redox catalysis. Further studies on selenoproteins and selenoproteomes should help explain known biological and biomedical effects of Se and identify new biological processes and pathways dependent on this trace element.

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Chapter 12
Prokaryotic Selenoproteins and Selenoproteomes

Yan Zhang

Abstract The essential micronutrient selenium is known to be used in a variety of biological processes in both prokaryotes and eukaryotes. The major biological form of selenium is selenocysteine, which is co-translationally inserted into selenoproteins. In the past decade, bioinformatics tools have been successfully developed to identify all, or almost all, selenoprotein genes in sequenced genomes. This chapter provides general information about currently known prokaryotic selenoprotein families and their major functions. In addition, recent comparative analyses of selenocysteine utilization and selenoproteomes across large groups of species offer important insights into evolutionary trends of different selenoprotein families and key factors that may influence selenoprotein evolution in prokaryotes.

Keywords Bioinformatics • Comparative genomics • Prokaryotes • Selenocysteine • Selenoprotein • Selenoproteome

12.1 Introduction

Selenium (Se) is an important micronutrient in many organisms from bacteria to humans. This micronutrient is known primarily for its functions in redox homeostasis involved in numerous cellular processes [1, 2]. It mainly occurs in proteins in the form of selenocysteine (Sec, U the 21st amino acid in the genetic code [3, 4]. Sec is co-translationally incorporated into selenoproteins by recoding the UGA codon from termination signal to Sec function [5–7]. Moreover, Sec is usually present in the active site of selenoproteins, being essential for their catalytic activity [7, 8].

The biosynthesis of Sec and its specific insertion into proteins involve a complex machinery that has been most thoroughly elucidated in selected groups of prokaryotes and eukaryotes [7, 9–11]. In bacteria, this process requires an in-frame UGA

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codon, a *cis*-acting mRNA structure called Sec insertion sequence (SECIS) element, which is located in the coding region immediately downstream of the Sec-encoding UGA codon, and several *trans*-acting factors dedicated to Sec incorporation [3, 9, 10]. Generally, the SECIS element binds to a specific elongation factor SelB and then forms a complex with Sec-tRNA$^{[Ser]Sec}$, a unique tRNA whose anticodon matches the UGA codon. Transfer RNA$^{[Ser]Sec}$ is initially aminoacylated with serine by seryl-tRNA synthetase and then converted to selenocysteyl-tRNA$^{[Ser]Sec}$ by Sec synthase (SelA). SelA uses selenophosphate, the product of selenophosphate synthetase (SelD), as the Se donor. In eukaryotes and archaea, completely different SECIS elements have evolved in the 3′-untranslated region (3′-UTR) of selenoprotein mRNA, and additional steps and enzymes (such as eukaryotic/archaeal Sec synthase and O-phosphoseryl-tRNA$^{[Ser]Sec}$ kinase) are needed for the incorporation of Sec into protein [11–15].

In the past decade, a large set of selenoprotein families have been experimentally or computationally identified in prokaryotes. Several selenoprotein families, such as SelD, deiodinase-like, glutathione peroxidase (GPx)-like and selenoprotein W (SelW)-like proteins, are also present in eukaryotes (details on eukaryotic selenoproteins can be found in other chapters in this book). However, the majority of them occur almost exclusively in bacteria. In this chapter, identification of prokaryotic selenoproteins using bioinformatics tools and comparative genomics analysis of full sets of selenoproteins (selenoproteomes) in different prokaryotic organisms will be briefly described.

12.2 Computational Identification of Selenoproteins in Prokaryotes

Selenoproteins were initially searched for (in the 1980s and 1990s) and identified by demonstrating the presence of Se in these proteins. For example, selenoproteins were metabolically labeled with ^{75}Se, wherein the resulting labeled selenoproteins were visualized on gels using a PhosphorImager [16, 17]. More recently, much progress in sequencing technologies provided an opportunity and valuable resources for new selenoprotein discovery. To this end, several computational algorithms have been developed to identify selenoprotein genes in prokaryotic genomes and metagenomic datasets.

In selenoprotein genes, the SECIS element is essential and highly specific for Sec insertion, which contains a relatively common secondary structure core and conserved segments. These features can be utilized for the prediction of selenoprotein genes in genomic sequences. In bacteria, the SECIS element is a stem-loop structure and is located immediately downstream of Sec-encoding UGA codon within selenoprotein mRNAs [18]. Both the stem-loop structure and the distance between the in-frame UGA codon and the apical loop of SECIS are important for Sec incorporation [19]. Thus, a SECIS-based approach for prediction of bacterial selenoprotein genes is a reasonable strategy.

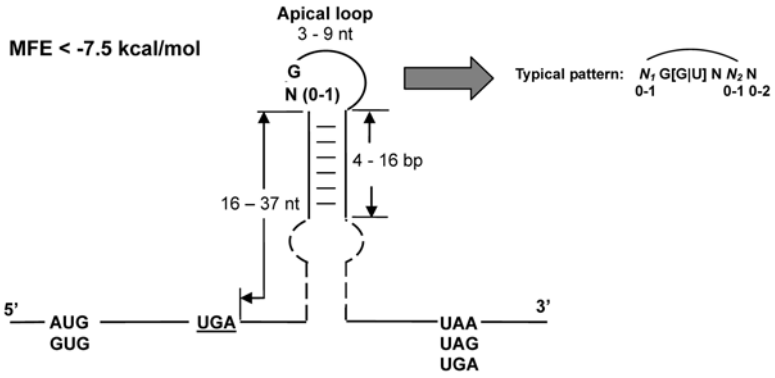

Fig. 12.1 A SECIS structural model in bacteria. The core of this model includes a 3–9 nucleotide (nt) apical loop and a 4–16 bp upper-stem. At least one guanosine (G) among the first two nucleotides (N means any nucleotide) in the apical loop, the majority of which contain the N_1(0-1)G[GU] NN_2(0-1)N(0-2) pattern, wherein N_1 and N_2 form a base pair. The distance between the Sec-encoding UGA codon and the apical loop is 16–37 nt. The minimum free energy (MFE) is less than −7.5 kcal/mol

A previous study suggested a common structural model of bacterial SECIS elements [20]. In this model, the primary sequence is not strictly conserved except for a single guanosine (G), often followed by a uridine (U), which is present among the first two nucleotides in the apical loop of SECIS element (Fig. 12.1). In addition, the distance between the UGA codon and the apical loop is also limited (16–37 nucleotides). Based on this model, a bioinformatics program, named bSECISearch, was developed to predict selenoprotein genes in bacterial genomes [20]. This program initially examined the occurrence of candidate SECIS elements downstream of each UGA codon in the genome on the basis of this bacterial SECIS structural model. Additional criteria, such as open reading frame constraint, segment-based SECIS profiling and analysis of conservation of UGA-flanking regions, were used to filter out most false positives. All remaining candidates were further analyzed in regard to the location of the UGA codon, occurrence of Sec-containing and cysteine (Cys)-containing homologs and presence of possible SECIS elements in other Sec-containing orthologs. Finally, a set of selenoprotein genes was identified for further verification.

Considering that most selenoproteins have homologs in which Sec is replaced with Cys, additional SECIS-independent bioinformatics approaches were developed to search for selenoprotein genes in bacterial genomes and environmental samples. These tools employ Cys-containing proteins in large protein databases (e.g., non-redundant protein database in NCBI) to search against nucleotide sequence datasets for selenoprotein genes [21, 22]. The key step is to identify UGA-containing nucleotide sequences which, when translated, are homologous to Cys-containing protein sequences such that the conserved Cys residues could align with translated UGA codons and these Cys/UGA-containing pairs should be flanked by

conserved regions. One deficiency in this approach is that selenoproteins with no Cys-containing homologs (although very rare) cannot be identified.

With the use of SECIS-dependent and SECIS-independent algorithms, many new selenoprotein genes have been identified in various bacterial genomes. Table 12.1 shows a list of reported selenoprotein families in this kingdom.

In archaea, SECIS elements differ from those in bacteria and are most often located in the 3′-UTR of selenoprotein genes. These SECIS elements appear to have a GAA_A unpaired region and additional conserved structural features [23, 24]. A program called archaeal SECISearch was then developed to recognize the archaeal consensus SECIS element in sequence databases [24]. In addition, a SECIS-independent method was also applied to identify archaeal selenoprotein genes [24]. Compared to bacteria, only a small number of selenoprotein families are known in archaea (Table 12.1).

12.3 Prokaryotic Selenoproteins

As described above, different computational approaches have identified a large set of selenoprotein families in both sequenced prokaryotic genomes and environmental genome projects, which revealed a wide distribution of selenoprotein families in prokaryotes [20–22, 24–26]. Homologs of some prokaryotic selenoproteins are also present in eukaryotes, such as SelD (named SPS2 in eukaryotes), GPx, deiodinase, SelW and methionine-S-sulfoxide reductase (MsrA). Below, we describe the major prokaryotic selenoprotein families.

Formate dehydrogenase alpha subunit (FdhA) is responsible for the oxidation of formate to carbon dioxide [27, 28], and is the most abundant and widespread prokaryotic selenoprotein [29, 30]. In this enzyme, Sec is coordinated to molybdenum (Mo) and is involved in the catalytic activity. FdhA is the only selenoprotein in many bacterial species, implying that it may play an important role in maintaining the Sec utilization trait in these organisms [29, 30].

SelD is another important selenoprotein family in prokaryotes. The function of SelD is to synthesize selenophosphate, a highly active Se donor compound, from selenide and ATP [31]. This enzyme is essential for Sec biosynthesis in prokaryotes and is homologous to eukaryotic SPS2 protein.

Formylmethanofuran dehydrogenase subunit B (FmdhB) is a distant homolog of FdhA and has a similar function, but uses formylmethanofuran as the substrate. Similar to FdhA, Sec is coordinated to Mo in the active site [32].

Some hydrogenases were found to contain Sec, such as coenzyme F420-reducing hydrogenase (alpha and delta subunits, FrhA and FrhD) and methylviologen-reducing hydrogenase alpha subunit (VhuA). In these selenoproteins, Sec is coordinated to nickel [33]. In some Sec-containing forms of FrhD (such as *Methanococcus jannaschii*), two Sec residues could be observed [23].

Table 12.1 Prokaryotic selenoprotein families

Bacteria	Archaea
Formate dehydrogenase alpha subunit (FdhA)	FdhA
Formylmethanofuran dehydrogenase subunit B (FmdhB)	FmdhB
Selenophosphate synthetase (SelD)	SelD
Coenzyme F420-reducing hydrogenase alpha subunit (FrhA)	FrhA
Coenzyme F420-reducing hydrogenase delta subunit (FrhD)	FrhD
Methylviologen-reducing hydrogenase alpha subunit (VhuA)	VhuA
HesB-like	HesB-like
Heterodisulfide reductase alpha subunit (HdrA)	HdrA
Glycine reductase selenoprotein A (GrdA)	
Glycine reductase selenoprotein B (GrdB)	
Peroxiredoxin (Prx)	
Thioredoxin (Trx)	
Glutaredoxin (Grx)	
Proline reductase PrdB	
Deiodinase-like	
Prx-like thiol:disulfide oxidoreductase	
Thiol:disulfide interchange protein	
GPx-like	
SelW-like	
MsrA	
DsbG-like	
Fe-S oxidoreductase (GlpC)	
DsbA-like	
DsrE-like	
AhpD-like	
Arsenate reductase	
Molybdopterin biosynthesis protein MoeB	
Glutathione S-transferase-like (GST-like)	
OsmC-like	
NADH:ubiquinone oxidoreductase subunit E	
Sulfurtransferase COG2897	
Sulfurtransferase COG0607	
Rhodanase-related proteins	
Methylated-DNA-protein-cysteine methyltransferase	
organic mercuric lyase MerB2	
UGSC-containing protein	
Arsenite S-adenosylmethyltransferase	
CMD-containing protein	
Ferredoxin-thioredoxin reductase	
Trypsin-like serine protease	
Putative regulatory protein, FmdB family	
Prx-like proteins	
Trx-like proteins	
Grx-like proteins	
Different Trx-fold-containing families	
Other predicted selenoproteins	

Glycine reductase selenoprotein A (GrdA) and B (GrdB) are two selenoprotein components of a multiprotein glycine reductase complex involved in the reduction of glycine, sarcosine, betaine and other substrates [34, 35]. GrdA is the only known prokaryotic selenoprotein for which no Cys-containing homologs have been detected [4]. GrdB protein is substrate-specific and binds a single GrdA [36].

Proline reductase (PR) catalyzes the reductive cleavage of the D-proline ring to yield δ-aminovaleric acid [37]. This enzyme contains two components, PrdA and PrdB. PrdB was found to be a selenoprotein in some bacteria [38]. PrdB exhibits similarities to GrdB, especially the region around the Sec residue.

Peroxiredoxin (Prx) is a ubiquitous family of antioxidant enzymes that control peroxide levels and thereby mediate signal transduction in cells. These proteins are present in essentially all organisms. The Sec-containing forms of Prx have been identified in some bacteria such as *Eubacterium acidaminophilum* [39].

Thioredoxin (Trx) is a major intracellular thiol-disulfide reductase that plays a role in many biological processes in all organisms. The Sec-containing Trxs have been found in certain bacteria and marine environmental samples [21, 24, 25]. It has been shown that selenoprotein Trx confers higher catalytic activity than its Cys homologs [40].

Glutaredoxin (Grx) is another well studied thiol-disulfide oxidoreductase which uses glutathione as a cofactor. Glutaredoxins are oxidized by substrates and reduced non-enzymatically by glutathione. The Sec-containing forms of Grx were identified in some bacterial species [21, 24, 25].

HesB-like protein is a distant homolog of HesB (or IscA) proteins and is a selenoprotein in certain archaea and bacteria [24, 41]. HesB proteins are possibly involved in iron-sulfur cluster assembly [42], but the function of their selenoprotein homolog has not been characterized thus far.

SelW-like protein is a homolog of eukaryotic SelW protein [43]. In mammals, SelW is the smallest selenoprotein whose function is not clear. It has been reported that the Sec-containing form of SelW-like protein is the most widespread selenoprotein family in the marine microbial world [25].

MsrA reduces free and protein-based methionine-S-sulfoxide to methionine, and is widely present in many organisms, from bacteria to humans [44]. The selenoprotein MsrA was only detected in very few organisms including green algae (*Chlamydomonas* and *Ostreococcus* species) and certain bacteria such as *Clostridium oremlandii* (previously named as *Alkaliphilus oremlandii*) [45–47]. Structural analysis of the overall structure of MsrA from *C. oremlandii* revealed that this protein consists of two domains, the N-terminal catalytic domain and the C-terminal distinct helical domain [48]. Grx can act as a reductant of MsrA, which directly forms a complex with this enzyme via a disulfide bond [49].

Other prokaryotic selenoproteins that have been identified or predicted in previous studies are listed in Table 12.1. These include heterodisulfide reductase, deiodinase-like, DsbA-like, DsbG-like, DsrE-like, GPx-like, AhpD-like, arsenate reductase, glutathione S-transferase-like, OsmC-like protein, rhodanese-related proteins, methylated-DNA-protein-cysteine methyltransferase, UGSC-containing protein, arsenic methyltransferase, and a variety of Prx/Trx/Grx-like proteins which contain a Trx-like fold [20–22, 24, 25]. Many of these selenoproteins are predicted

by bioinformatics tools and their functions are not clear. However, considering that almost all selenoproteins whose functions are known are redox proteins and that most of the computationally identified selenoproteins are homologous to proteins with known functions, it is very likely that the majority of these uncharacterized selenoproteins are also redox proteins.

12.4 Comparative Genomics of Selenoproteomes in Prokaryotes

In past decade, bioinformatics approaches have been successfully used to identify all or almost all selenoproteins in sequenced prokaryotic genomes and marine environmental samples [20–22, 24–26]. These studies allowed a detailed view on the composition and evolution of selenoproteomes in bacteria and archaea, providing insights into the requirement of Se for these organisms. In addition, Sec is a superior catalyst in selenoenzymes, compared with Cys [5, 40]; however, it is used selectively by proteins and organisms. Thus, comparative analysis of selenoproteomes may also help understand the contradiction between the wide distribution of Sec and its restricted use.

An early comparative genomics study examined all Sec-utilizing organisms and their selenoproteomes based on the analysis of nearly 350 completely sequenced bacterial genomes [30]. It was observed that less than one-fourth of sequenced bacteria utilized Sec, whose selenoproteomes had 1–31 selenoproteins. Selenoprotein-rich organisms (containing six or more selenoproteins) were mostly Deltaproteobacteria or Firmicutes/Clostridia. FdhA and SelD were the most widespread selenoprotein families in bacteria. In most selenoprotein families, selenoproteins appeared to have evolved from their Cys-containing ancestors. However, specific selenoprotein gene loss events were observed in a number of sister species of selenoprotein-rich organisms. These observations suggested a highly dynamic and delicate balance between Sec acquisition and selenoprotein loss, which may partially explain the discrepancy between the catalytic advantages offered by Sec and its limited use in nature. Finally, several environmental factors might influence Sec utilization and/or selenoproteomes, e.g., decreases in oxygen concentration and/or increases in growth temperature appeared to preserve, and even expand the use of Sec, and the former also promoted the evolution of new selenoprotein genes [30].

Another comparative analysis was carried out on Sec utilization in prokaryotes and eukaryotes [47]. In this study, approximately 600 bacterial and archaeal genomes were examined. In bacteria, the size of selenoproteomes varied from 1 to 39 selenoproteins, with the largest selenoproteome identified in *Syntrophobacter fumaroxidans*. On the other hand, very few archaea (only in Methanococcales and Methanopyrales) utilized Sec and the archaeal selenoproteomes varied only slightly, most often consisting of 7–10 selenoproteins).

A recent study analyzed the evolution of the complete machinery for Sec biosynthesis and the selenoproteome of several *Helicobacter pylori* strains and related

Epsilonproteobacteria [50]. It was found that the Sec incorporation system was recently lost prior to the split of *H. acinonychis* and *H. pylori*. In addition, the authors predicted a new selenoprotein family, the DUF466 family, in Epsilonproteobacteria, in which the predicted Sec is a carboxyl-terminal residue [50].

Another important comparative analysis of the evolutionary history of SelD/SPS genes in prokaryotes and eukaryotes provided a map of selenoprotein function spanning the whole tree of life [51]. In bacteria, the occurrence of SelD was in good agreement with the other gene markers for Se utilization and also with selenoprotein presence. In Gammaproteobacteria/Pasteurellales, a Cys-to-Sec conversion of SelD proteins was identified, which is the first clearly documented case, although cases of conversion of Cys to Sec have been proposed [30]. Moreover, different fusions between SelD and other genes were observed. In all cases, the fusions were on the N-terminal side of the SelD genes, and these were always Cys-containing SelD with the exception of NifS-SelD in *Geobacter sp. FRC-32*, which contained Sec. These extended SelD genes were thought to retain the original selenophosphate biosynthetic function. In metazoans, the SPS phylogeny constitutes a prototypical case of functional evolution, in which dual function is segregated to different loci through independent gene duplications and subsequent convergent subfunctionalization [51].

Very recently, we analyzed more than 5200 genomes in attempting to identify new evolutionary patterns of Se utilization and selenoproteomes in bacteria [52]. This study generated a global species map of all known Se utilization traits (including Sec biosynthesis and other Se utilization pathways), which demonstrated the most detailed understanding of Se utilization in bacteria thus far. Consistent with previous results, the majority of sequenced organisms (~80 %) lacked the ability to use Sec. By characterizing the selenoproteomes of all Sec-utilizing organisms, a new selenoprotein-rich phylum, Synergistetes, and additional selenoprotein-rich organisms were observed (Fig. 12.2). Re-investigation of the relationship between environmental factors and Sec utilization suggested new macro-evolutionary trends

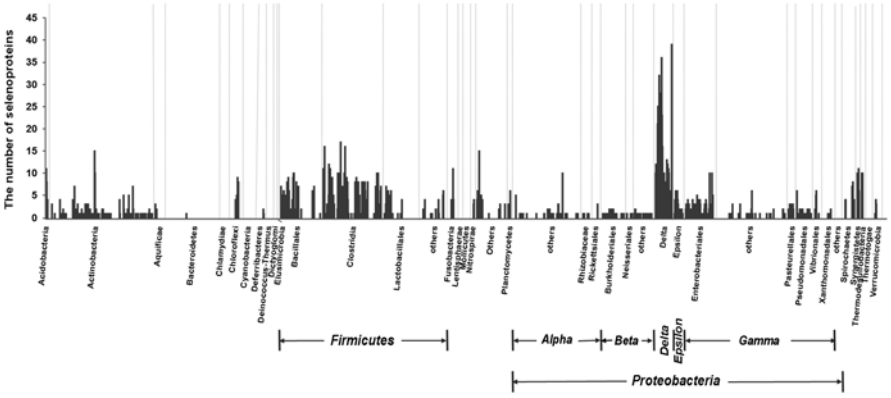

Fig. 12.2 Distribution of selenoproteomes in bacteria. More than 5000 bacterial genomes were analyzed. The majority of selenoprotein-rich organisms belong to three phyla: Deltaproteobacteria, Clostridia and Synergistetes

of Sec utilization in bacteria. The Sec utilization trait appeared to favor a host-associated condition. More importantly, we could not identify the strong relationship previously reported between oxygen level and Sec utilization [30, 47], as a significant number of both aerobic and anaerobic organisms possess this trait [52]. Considering that the majority of selenoprotein-rich organisms are anaerobic organisms, low oxygen levels might contribute to the evolution of new selenoprotein genes. In the future, it would be important to identify additional factors that may influence the evolution of individual selenoprotein families.

12.5 Concluding Remarks

In this chapter, we discussed the use of computational approaches to identify new selenoprotein genes and examine the evolution of Sec utilization in prokaryotes. More than 70 selenoprotein families or subfamilies have been reported in recent decades. The majority of these selenoproteins appear to be thiol-based oxidoreductases that contain a Trx-like fold. Several comparative genomics studies have analyzed the utilization and evolution of both the Sec utilization trait and selenoproteomes in a variety of sequenced prokaryotes. These efforts may not only help unravel the general principles of Sec utilization across the three domains of life, but they may also help explain how Sec utilization changed during evolution and which environmental factors played a role in such a process. Further studies on identification of new selenoprotein genes and their functions should assist us in understanding new biological processes and pathways dependent on this micronutrient.

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Chapter 13
Functional Genomics of Selenoproteins and Se-responsive Pathways

Catherine Méplan and John Hesketh

Abstract Nutrigenomics approaches have contributed to our understanding of how selenium impacts metabolic pathways, homeostatic control and disease risk. In this chapter, we discuss the known genetic polymorphisms in genes encoding selenoproteins and components of the selenoprotein synthesis machinery. Furthermore, the consequences of these genetic variants on the synthesis and activity of individual selenoproteins within the context of the metabolic pathway in which they are involved, including the impact of these variants on the overall selenoproteome, as a result of the shared synthesis machinery between selenoproteins are discussed. The evidence for the association of these genetic variants with several chronic diseases is presented, with a specific emphasis on functional variants.

Keywords Association study • Cancer • Nutrigenomics • Selenoprotein • Selenoprotein hierarchy • Single nucleotide polymorphisms

13.1 Introduction

Selenoproteins play pivotal roles in many biochemical pathways and in particular in the response to oxidative stress and endoplasmic reticulum (ER) stress. In a nutritional context, it is important to consider the effect of selenium (Se) intake on the overall balance of selenoproteins and related pathways; similarly, from a genetic perspective, the effects of inter-individual variations need to be assessed in the overall Se pathway. Here, we discuss how genetic studies are providing novel perspectives about the mechanisms by which Se intake and metabolism are related to disease risk.

Single Nucleotide Polymorphisms (SNPs) represent 90 % of the genetic variations among individuals, and some genetic variants alter gene or protein regulation or protein activity and are thus functionally relevant. During evolution, the ability of our ancestors to survive relied on their capacity to combat metabolic stresses and

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infections and ensure their reproduction; these functions are strongly supported by various selenoproteins and therefore highly dependent on Se intake. It is possible that genetic variants which modulate selenoprotein synthesis or activity in different conditions of Se supply could have been selected as a result of the disparate geological distribution of Se and exposure to various pathogens and stresses. Thus, the pressure of selection imposed by environmental forces may partially explain the variations in allelic frequencies currently observed among different populations. Supporting this hypothesis, several studies have identified SNPs in selenoprotein genes corresponding to signatures of recent genetic adaptation, suggesting a local adaptation to low Se content in the soil. In Asian populations living in Se-deficient regions, it was reported that a selective sweep occurred recently at the *GPX1* locus [1]. Similarly, allele frequency shifts for SNPs in selenoprotein genes were observed in populations living in the severe Se-deficient regions of China [2]. Moreover, a recent study identified a strong positive natural selection in individuals of European decent, for known functional variants in *GPX1*, *SELENBP1*, *GPX3, GPX2* and *SELO* genes suggesting local adaptation to low soil Se [3].

With the increase in human lifespan, genetic variations in Se metabolism have been proposed to be associated with risks for several age-related diseases, including cancer, diabetes, immunological or neurological disorders and cardiovascular diseases. Many of these disorders share a common basis for their development such as impaired cellular maintenance mechanisms and responses to stress. These metabolic pathways involve many selenoproteins. Thus, genetic factors affecting selenoprotein activity or synthesis, have the potential to modify individual risk to chronic disease (Fig. 13.1).

Three types of studies have shown links between genetic variants in selenoprotein genes and disease susceptibility (Tables 13.1, 13.2, 13.3, and 13.4): (1) the study of a small number of candidate SNPs of known functional relevance in which it is assumed that the functional consequences of the SNP on the gene/protein regulations could contribute to the disease risk; (2) the broader study of SNPs in several selenoprotein genes or screening of genes across the Se metabolic pathways, usually using a combination of tagSNPs and functional variants; and (3) large scale genome-wide association studies (GWAS) in which selenoprotein genes were associated with a disease trait. Focussed studies of well-characterized SNPs provide mechanistic backing to epidemiological studies and also often allow measurements of biomarkers of Se status and stress, particularly important in the case of Se since intake varies across different populations [4]. Screening studies involving a wider range of SNPs regardless of known functionality and large-scale GWAS have the advantage of wide genetic coverage but are less likely to have environmental and dietary data available.

13.2 Selenium Metabolism and Transport

Selenoprotein synthesis depends upon Se intake, Se transport from the liver to other organs, Se conversion to selenocysteine (Sec) and its incorporation into selenoproteins (Chaps. 2 and 4). Genetic variants in two genes involved in Sec synthesis (Table 13.4),

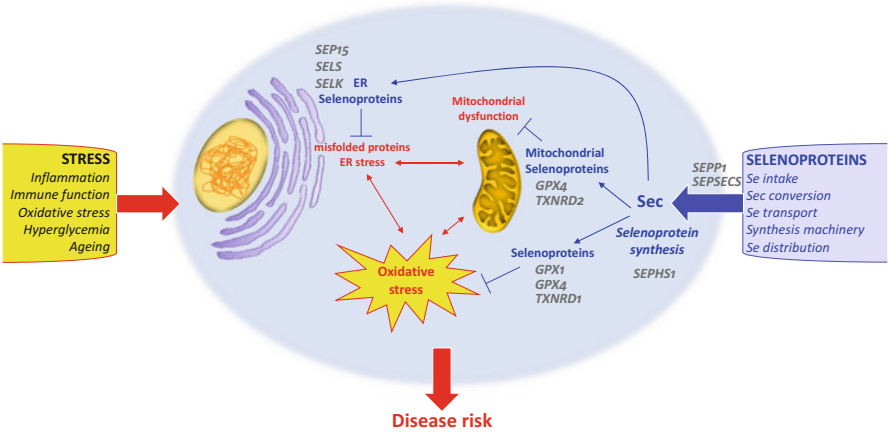

Fig. 13.1 Schematic diagram illustrating interactions of selenoprotein SNPs and Se supply on selenoprotein function and disease risk. The diagram illustrates how SNP-Se interactions could affect selenoprotein synthesis and activity and consequently downstream biochemical pathways known to be crucial in the response to oxidative and ER (endoplasmic reticulum) stress and in the maintenance of mitochondrial redox status. As disruption of these biochemical pathways is commonly observed in many chronic disorders, the existence of genetic associations linking these SNP with diseases indicate the importance of selenoproteins' function in these pathways. Genes (*dark grey*, *italic*) in which functional SNPs have been associated with disease risk are indicated at subcellular location in which the corresponding selenoproteins function is required

Sec tRNA synthase *(SEPSECS)* and selenophosphate synthetase 1 *(SEPHS1)*, have been linked to Crohn's Disease in a Caucasian population from New Zealand with suboptimal Se intake [5, 6]. Moreover, SNPs in *SEPP1*, the gene coding for selenoprotein P (SePP, SEPP1), which transports hepatic Se to other tissues, have been shown to affect Se delivery, Sec incorporation throughout the body and disease risk [7]. In particular, two genetic variants, rs3877899 and rs7579 (*SEPP1*), affect the plasma SePP isoform pattern, response to Se supplementation in healthy individuals with sub-optimal Se intake and selenoprotein synthesis [8, 9]. In support of this, expression of SePP and GPx1 was affected by both variants in patients with mild cognitive impairment supplemented with Se [10]. Moreover, rs3877899 and rs7579 were associated with risk for several cancers (Table 13.3) in different European populations [7, 11–13] and various *SEPP1* variants were linked to colorectal (CRC) [14] or breast (BC) cancer in Native Americans [15]. In Europeans, risk of prostate cancer (PCA) or advanced PCA was linked to rs7579 (*SEPP1*) [12, 13] and modulated by an interaction between rs3877899 (*SEPP1)* and rs4880, a known functional SNP in *SOD2* (manganese superoxide dismutase) [16]. In a US population, rs13168440 (*SEPP1)* was also shown to affect PCA risk [17], but other studies have failed to link SNPs in *SEPP1* to PCA [13, 18, 19] or disease recurrence [20]. In addition, both rs3877899 and rs7579 (*SEPP1*) were also shown to influence body mass index, blood pressure, peripheral arterial

Table 13.1 Genetic association of SNPs in the *GPX1* gene with disease risk[a]

SNP	Base change	Cases/controls	Target/location	Functionality	Population	Association	Reference
Breast cancer							
rs1050450	C>T	1038/1088	Pro198Leu	Enzyme activity Pro>Leu	USA	None	[62]
		1229/1629			USA	None	[61]
		79/517			USA	T allele: ↑ BC risk	[46]
		399/372			Canada	None	[63]
		2293/2278			UK	None	[86]
		4371/0			UK	No association with BC survival risk	[41]
		377/377			Denmark	T allele: ↑ BC risk	[47]
		933/959			Denmark	T allele: ↑ non-ductal BC; interaction with rs3877899(SEPP1); ↓ eGPx activity	[11]
Prostate cancer							
rs1050450	C>T	745/0	Pro198Leu	Enzyme activity pro>Leu	USA	None	[87]
		500/1391			USA	None	[88]
		247/487			Germany	T allele: ↓ PCA risk with ↑ serum Se levels	[12]
		82/123			Macedonia	T allele: ↓ PCA risk	[50]
		262/435			New Zealand	T allele: ↑PCA risk	[51]
rs1800668	C>T	951/25408	TagSNP/promoter	TagSNP/high LD with rs1050450	Netherlands	TT: ↓advanced (stage III/IV) PCA risk	[13]
rs17650792	A>G	952/25426	TagSNP/promoter	unknown	Netherlands	GG: ↑advanced (stage III/IV) PCA risk	[13]

rs1050450	C>T	656/743	Pro198Leu	Enzyme activity pro>Leu	USA	No association with advanced distal colorectal adenoma	[14]
		981/397			Norway	None	[89]
		375/779			Denmark	None	[90]
		832/705			Czech Republic	No association SNP alone, but genetic interaction with rs37413471 (*SELS*)	[7]
		827/733			Korea	None	[76]
Lung cancer							
rs1050450	C>T	237/234	Pro198Leu	Enzyme activity pro>Leu	USA	CC :↑ lung cancer in smokers>80 years of age	[52]
		315/313			Finland/men	T allele: ↑ risk	[53]
		95/176			Poland	T allele: ↓ risk	[40]
		432/798			Denmark	T allele: ↓ risk	[54]
		186/207			Germany	T allele: ↓ risk	[91]
Laryngeal cancer							
rs1050450	C>T	111/213	Pro198Leu	Enzyme activity pro>Leu	Poland	T allele: ↓ risk	[40]
Bladder cancer							
rs1050450	C>T	224/0	Pro198Leu	Enzyme activity Pro>Leu	USA	T allele: ↑ bladder cancer recurrence risk	[56]
		213/209			Japan	T allele: ↑ risk	[55]

(continued)

Table 13.1 (continued)

SNP	Base change	Cases/controls	Target/location	Functionality	Population	Association	Reference
Cardiovascular disease							
rs1050450	C>T	184/0	Pro198Leu	Enzyme activity pro>Leu	Japan (diabetic)	T allele: ↑ CVD risk in diabetic patients and ↑ intima-media thickness	[92]
Kashin-Beck disease							
rs1050450	C>T	638/324	Pro198Leu	Enzyme activity pro>Leu	China	None	[93]

[a]The table presents results from association studies between functional and tagSNPs in the *GPX1* gene and disease risk. The disease, SNP, allele or genotype associated with disease risk or progression is indicated together with the studied population and the known functional consequences of the SNP on the protein function or expression

Table 13.2 Genetic association of SNPs in the *GPX4* gene with disease risk[a]

SNP	Base change	Cases/controls	Target/location	Functionality	Population	Association	Reference
Breast cancer							
rs713041	C>T	2182/2264	3′UTR, near SECIS	Sec-insertion efficiency C>T	UK	None	[86]
		4356			UK	T allele: ↑ risk of mortality by BC	[41]
		939/960			Denmark	T allele: ↓ eGPx activity	[11]
Prostate cancer							
rs713041	C>T	739/0	3′UTR, near SECIS	Sec-insertion efficiency C>T	USA	None	[87]
		245/490			Germany	None	[12]
		260/439			New Zealand	None	[51]
Colorectal cancer							
rs713041	C>T	745/758	3′UTR, near SECIS	Sec-insertion efficiency C>T	USA	No association with advanced distal colorectal adenoma	[14]
		252/187			UK	TT:↓ CRC risk	[36]
		832/705			Czech Republic	CT: ↑ CRC risk; interaction with rs4880 (*SOD2*), rs9605031 (*TXNRD2*) and rs3877899 (*SEPP1*)	[7]
		827/733			Korea	None	[76]

(continued)

Table 13.2 (continued)

SNP	Base change	Cases/controls	Target/location	Functionality	Population	Association	Reference
Lung cancer							
rs713041	C>T	95/176	3′UTR, near SECIS	Sec-insertion efficiency C>T	Poland	T allele: ↓ risk	[40]
Laryngeal cancer							
rs713041	C>T	325/287	3′UTR, near SECIS	Sec-insertion efficiency C>T	Poland	T allele: ↓ risk	[40]
Kashin-Beck disease							
rs713041	C>T	219/194	3′UTR, near SECIS	Sec-insertion efficiency C>T	China	None; ↓ GPX4 mRNA level in KBD patients	[39]
haplotype rs713041 -rs4807542		219/194			China Han	Haplotype A-T: ↓ KBD risk	[39]

[a]The table presents results from association studies between functional and tagSNPs in the *GPX4* gene and disease risk. The disease, SNP, allele or genotype associated with disease risk or progression is indicated together with the studied population and the known functional consequences of the SNP on the protein function or expression

Table 13.3 Genetic association of SNPs in the *SEPP1* gene with disease risk[a]

SNP	Base change	Cases/controls	Target/location	Functionality	Population	Association	Reference
Breast cancer							
rs3877899	G>A	937/957	Ala234Thr	Plasma SePP isoforms, Se bioavailability	Denmark	AA:↓ BC and ductal BC risk	[11]
rs7579	G>A	937/957	3′UTR	Plasma SePP isoforms, Se bioavailability	Denmark	None	[11]
rs230812	A>C		TagSNP		Native American	CC: ↑ BC risk in women of 28-70 % Native American ancestry Strong LD with rs230813 (associated with oxidative stress)	[15]
rs6865453	A>C		TagSNP			In LD with rs7579 AC/CC: ↓BC risk in women of 28-70 % Native American ancestry	[15]
Prostate cancer							
rs3877899	G>A	2643/1570	Ala234Thr	Plasma SePP isoforms, Se bioavailability	Sweden	None	[16]
		248/492			Germany	None	[12]

(continued)

Table 13.3 (continued)

SNP	Base change	Cases/controls	Target/location	Functionality	Population	Association	Reference
		951/25409			Netherlands	Genotype interacts with Se status: ↓advanced PCA risk	[13]
		259/436			New Zealand	None	[51]
rs7579	G>A	248/492	3′UTR	Plasma SePP isoforms, Se bioavailability	Germany	AA :↑ PCA risk; interaction with plasma SePP	[12]
		951/25408			Netherlands	A allele: ↓advanced (stage IV) PCA risk; genotype interacts with plasma Se status to ↓advanced PCA risks	[13]
rs13168440	T>C		TagSNP	Unknown	USA	C allele: interacts with plasma Se to ↓PCA risk	[17]
Colorectal cancer							
rs3877899	G>A	193/127	Ala234Thr	Plasma SePP isoforms, Se bioavailability	Germany	None	[94]
		832/705			Czech Republic	No association SNP alone, but interaction with rs5859(*SEP15)* and rs713041 (*GPX4)*	[7]
		827/733			Korea	None	[76]

rs7579	G>A	832/705	3′UTR	Plasma SePP isoforms, Se bioavailability	Czech Republic	AA:↑ CRC risk, interaction with rs5859 (*SEP15*)	[7]
		827/733			Korea	None	[76]
Promoter (−4166), Exon 5 (rs3877899, rs6413428), 3′UTR (rs12055266, rs2972994, rs3797310)		772/777			USA	Association global *SEPP1* variants with advanced distal colorectal adenoma	[14]
Type 2 diabetes							
rs28919926, rs146125471, rs16872779, rs7579		2446			Hispanics, European American, African American	Associated with fasting insulin and first phase insulin response	[25]

[a]The table presents results from association studies between functional and tagSNPs in the *SEPP1* gene and disease risk. The disease, SNP, allele or genotype associated with disease risk or progression is indicated together with the studied population and the known functional consequences of the SNP on the protein function or expression

Table 13.4 Genetic association of SNPs in other selenoprotein genes with disease risk[a]

Gene symbol	SNP	Base change	Cases/ controls	Target/ location	Functionality	Population	Association	References
Prostate cancer								
SEP15	rs5859	G>A	1195/1186	3′UTR	Sec-insertion efficiency	USA	None	[84]
			248/492			Germany	AA :↓ GPX3 activity	[12]
SEP15	rs5845	G>A or C>T	259/436	3′UTR	Sec-insertion efficiency	New Zealand	AA ↑ PCA risk	[51]
SEP15	rs561104	G>A	1195/1186	TagSNP	Unknown	USA	AA: ↑risk of mortality by PCA	[84]
SELK	rs9880056	T>C	248/492	TagSNP	Unknown	Germany	C allele: interacts with serum SePP & serum Se to ↓ risk advanced and high grade PCA	[19]
TXNRD1	rs7310505	C>A	248/492	TagSNP	Unknown	Germany	CC: interacts with serum SePP & serum GPX activity to ↑ risk of advanced PCA	[19]
TXNRD2	rs9605030	C>T	248/492	TagSNP	Unknown	Germany	T allele: interacts with serum Se status to ↑ high grade PCA risk	[19]
TXNRD2	rs9605031	C>T	248/492	TagSNP	Unknown	Germany	T allele: interacts with serum Se status to ↓ high grade PCA risk	[19]
Colorectal cancer								
GPX2	rs4902347	G>A	570/762	TagSNP	Unknown	USA	GA/AA: ↓risk of rectal cancer (but not colon or adenoma)	[70]

GPX3	rs3828599	C>T	582/773	TagSNP	Unknown	USA	T allele: ↓risk of rectal cancer (but not colon or adenoma)	[70]
	rs736775	C>T	582/773	TagSNP	Unknown	USA	T allele: ↓risk of rectal cancer (but not colon or adenoma)	[70]
	rs8177447	C>T	582/773	TagSNP	Unknown	USA	T allele: ↓risk of rectal cancer (but not colon or adenoma)	[70]
SEP15	rs5859		832/705	3′UTR, SECIS	Sec-insertion efficiency	Czech Republic	no association SNP alone, but interaction with rs3877899, rs7579, rs3797310, rs12055266 in *SEPP1*	[7]
			827/733			Korea	A allele:↑ CRC risk	[76]
SEP15	rs5845		827/733	3′UTR	Sec-insertion efficiency	Korea	None	[76]
	rs35009941		772/777	C>G		USA	G allele:↓ CRC, alone and in association with rs34195484, rs4077561, rs1128446, rs5018287, rs6539137,rs10778322 and rs35776976 in *TXNRD1*	[14]

(continued)

Table 13.4 (continued)

Gene symbol	SNP	Base change	Cases/ controls	Target/ location	Functionality	Population	Association	References
TXNRD1	rs35009941	C>G	772/777			USA	G allele:↓ CRC, alone and in association with rs34195484, rs4077561, rs1128446, rs5018287, rs6539137, rs10778322 and rs35776976 in *TXNRD1*	[14]
Lung cancer								
SEP15	rs5859		325/287	3′UTR, SECIS	Sec-insertion efficiency	Poland	A allele: ↑risk in individuals with low Se status	[85]
SEP15	rs5845		325/287	3′UTR	Sec-insertion efficiency	Poland	None	[40]
Laryngeal cancer								
SEP15	rs5845		325/287	3′UTR	Sec-insertion efficiency	Poland	None	[40]
Cardiovascular disease								
SELS	rs28665122 rs4965814,rs2862845, rs7178239			TagSNPs		European Americans/ diabetic	Associated with measures of vascular calcification in European American families enriched for type 2 diabetes	[95]

Crohn's disease								
SEPHS1	rs7901303	G>T	351/853	TagSNP	Unknown	New Zealand-Caucasians	SNP-Serum Se interaction affecting Crohn's disease risk	[5]
	rs17529609	A>G	351/853	TagSNP	Unknown	New Zealand-Caucasians	SNP-Serum Se interaction affecting Crohn's disease risk	[5]
SEPSECS	rs1553153	G>A	351/853	TagSNP	Unknown	New Zealand-Caucasians	SNP-Serum Se interaction affecting Crohn's disease risk	[5]
Type 2 diabetes								
IDI2	rs225014		721	Thr92Ala		Brazil	Ala variant: less active, associated with type 2 diabetes, interaction with PPARγ2 Pro12Ala	[80–82]

[a]The table presents results from association studies between functional and tagSNPs in selenoprotein genes not presented in Tables 13.1, 13.2, and 13.3 and disease risk. The disease, SNP, allele or genotype associated with disease risk or progression is indicated together with the studied population and the known functional consequences of the SNP on the protein function or expression

disease occurrence and abdominal aortic aneurysm development in overweight and obese subjects [21].

Moreover, considerable mechanistic evidence suggest a role for SePP in glucose and insulin metabolism [22–24]. In support of this, four SNPs in *SEPP1* (rs28919926, rs146125471, rs168727790 and rs7579) were shown to be associated with an altered fasting or acute insulin responses in two different Hispanic cohorts [25].

It is interesting to note that the effects of genetic variants in factors involved in Sec conversion and transport on disease risk are often influenced by Se status and/or ethnicity (hence genetic background), consistent with evolutionary adaptation of populations to geographical differences in Se distribution.

13.3 The Effects of SNPs on the Sec Incorporation Machinery

Sec incorporation requires the recoding of a UGA codon for Sec and the binding of factors from the selenoprotein synthesis machinery to the Sec insertion sequence (SECIS) stem-loop structure within the 3′untranslated region (3′UTR) of selenoprotein mRNAs (reviewed in Chap. 2). In addition, SECIS-binding protein 2 (SBP2) plays a major role in this process.

The 3′UTR regions of selenoprotein mRNAs play a key regulatory role in the so-called selenoprotein hierarchy [26], a mechanism by which the synthesis of the various selenoproteins is affected differently by limiting conditions of Se supply [27, 28]. The hierarchy reflects the fact that all selenoproteins share the same incorporation machinery and the same tRNA carrying Sec for their synthesis and therefore there is in essence a competition between the 25 selenoprotein mRNAs for the available synthesis machinery and Sec. As a result, a genetic variant in the 3′UTR of one selenoprotein has the potential to affect synthesis, not only of the selenoprotein coded by that mRNA, but also synthesis of other selenoproteins. Similarly, a SNP in a gene coding for factors involved in the selenoprotein biosynthesis machinery have the potential to affect the synthesis of all selenoproteins.

In particular, mutations in the selenoprotein N gene (*SELN*) within the gene region corresponding to the SECIS region of the 3′UTR were associated with reduced binding affinity of SBP2 for SECIS, lower expression of SelN and congenital muscular dystrophy [29]. Moreover, missense mutations in *SBP2* led to poor Sec incorporation, poor thyroid function or muscular dystrophy, low expression of all selenoproteins and increased sensitivity to oxidative stress [30, 31]. However, mutations in selenoprotein genes that directly cause genetic disease are rare. On the contrary, common SNPs may have more subtle effects on selenoprotein metabolism, but in conjunction with other factors, such as dietary Se intake, can lead to altered risk for many diseases. Three functional genetic variants, one in *GPX4* (rs713041) [32] and two in *SEP15* (rs5859 and rs5845) [33], which induce base changes in a region nearby or within the SECIS element in corresponding transcripts, have been shown

to reduce the efficiency of Sec incorporation in the corresponding protein and to affect the selenoprotein hierarchy and disease risk [27, 33–35].

Originally identified as a C/T variant in the gene region corresponding to the 3′UTR region of *GPX4* mRNA [32], rs713041 illustrates how SNPs in selenoprotein genes can be studied from a functional perspective and how a SNP affecting the 3′UTR region impacts selenoprotein hierarchy. The C variant was shown to induce reporter gene expression to a greater extent than the T counterpart [36], to have a stronger binding affinity for the selenoprotein synthesis machinery in RNA-protein binding assays [35] and to alter the pattern of selenoprotein synthesis, especially during Se-depletion [36, 37]. Moreover, in healthy individuals, rs713041 was shown to affect expression of blood selenoproteins in response to Se supplementation, consistent with an effect on the selenoprotein hierarchy in vivo [35]. Finally, human umbilical vein endothelial cells from individual donors and monocytes [38] expressing the T-variant showed an increased expression of vascular cell adhesion protein 1 and adhesion to monocytes compared with cells from the CC individuals.

13.4 Genetic Variants Affecting Redox-Active Selenoproteins

Many selenoproteins are involved in the control of cellular redox balance and anti-oxidant defense and can be divided into two main classes of redox-active selenoenzymes, the *GPX* and thioredoxin reductases (*TXNRD)*. Functional SNPs in *GPX1-4* and *TXNRD1-2* have been shown to affect anti-oxidant defense and disease risk (Tables 13.1, 13.2, 13.3, and 13.4).

13.4.1 Genetic Variants in GPX4

As mentioned above, rs713041 (*GPX4*) was shown to affect the selenoprotein hierarchy [35], as well as the sensitivity to oxidative challenge [37, 38]. Interestingly, in a Se-deficient Chinese population (Table 13.2), rs713041 together with rs4807542, another *GPX4* SNP in high linkage disequilibrium with rs713041, were found to affect Kashin-Beck disease (KBD) risk, with *GPX4* mRNA levels being reduced in KBD patients compared with controls [39]. Moreover, rs713041 has been linked to risk of CRC in Czech and Scottish populations [7, 36], to lung and laryngeal cancers in a Polish population [40] and risk of BC mortality in a British population [41]. In the Czech population, CRC risk was affected by polymorphisms in both *SEPP1* (rs7579) and *GPX4* (rs713041) [7] and was further modulated by significant genetic interactions between SNPs in *SEPP1 (rs7579* and rs3877899), known to affect Se bioavailability, and variants in *SEP15 (rs*5859) or *GPX4* (rs713041), known to affect Sec incorporation [7]. These interactions suggest that in carriers of the combined genotypes, the altered pattern of synthesis of selenoproteins affects the

individual's ability to respond to stress. Similarly, genetic interactions between rs713041 in *GPX4,* rs960531 in *TXNRD2* and rs4880 in *SOD2* mirror the interactions of these enzymes in mitochondrial redox function and suggest that the genetic interactions could affect an individual's ability to counteract oxidative stress in the mitochondria [7]. Additionally, two independent GWAS linked the *GPX4* locus to Crohn's disease [42, 43], consistent with involvement of GPx4 in inflammatory responses and NF-kB regulation [44, 45].

13.4.2 Genetic Variants in GPX1

Cellular GPx1 is an important antioxidant enzyme in mammals. In humans, the enzyme activity is affected by rs1050450, a coding C/T SNP in the *GPX1* gene, inducing a Pro (CC) to Leu (TT) amino acid change at position 198 of the amino acid sequence. The Leu variant exhibits lower activity compared with the Pro counterpart [46, 47] and, during Se-supplementation, GPx1 activity is stimulated less in TT carriers compared with CC carriers [48, 49]. Moreover, significantly higher levels of DNA oxidation were observed in Leu carriers, probably as a result of reduced GPx1 activity [48, 49]. Surprisingly, however, an increased susceptibility to DNA strand breaks was observed in CC subjects, but not TT, during Se withdrawal [48].

Many studies have linked rs1050450 to risk for several disorders (Table 13.1), including various cancers [12, 13, 40, 46, 50–56], Alzheimer's disease [57], metabolic syndrome and obesity [58, 59], and KBD [60]. In particular, rs1050450 was linked to BC risk in some [46], but not other US populations [61–63], although a meta-analysis found an increased BC risk among women of African descent [64]. However, the association was replicated in several European populations [11, 47, 65]. The consistent differences observed between studies of European and North American populations suggest that the effect of rs1050450 on BC risk may be partially influenced by Se status. In a Danish population, the Leu variant was associated with reduced GPx1 activity, increased risk of BC and a higher grade of ductal tumors [11, 47]. Additionally, pre-diagnostic erythrocyte GPx1 activity was lower in Leu females, following hormone replacement therapy and who develop BC later in life, compared with controls [11]. Furthermore, a GCG repeat polymorphism in *GPX1,* resulting in variant protein sequences containing between 5 and 7 alanines, was linked to BC risk [63], supporting a role for GPx1 activity in protecting breast tissue from carcinogenesis.

Variants in the *GPX1* gene have also been linked to PCA, another cancer influenced by sex-hormones [13]. Moreover, rs1050450 together with rs18006688 (a *GPX1* SNP in high linkage disequilibrium) modified the association between lead exposure and glioblastoma [66], suggesting that the reduced GPx1 activity of the Leu variant could impair the protection against oxidative damage generated by lead [66]. Like *GPX4*, GWAs have linked the *GPX1* locus to inflammatory conditions such as Crohn's disease [42], inflammatory bowel disease [43] and ulcerative colitis [67].

13.4.3 Genetic Variants in TXNRDs

Mammalian cells have three isozymes of thioredoxin reductases, including cytoplasmic and nuclear TXNRD1 and mitochondrial TXNRD2. The relationship between diseases and genetic variants in *TXNRD1* and *TXNRD*2 has been investigated in studies using tagSNPs including a GWAs [68, 69], in a study investigating association of SNPs in several selenoprotein genes on PCA risk, grade and recurrence [20], and in a study investigating the association of polymorphic variants in Se metabolism with PCA risk [19]. In the latter study, carried out in a German population with low Se intake, tagSNPs in *SELK*, *TXNRD1* and *TXNRD*2 were found to interact with plasma Se or SePP status to modulate risk of advanced disease [19] (Table 13.4).

In addition, SNPs in other selenoproteins involved in the protection against oxidative damage were linked to disease. In a US population, three SNPs in *GPX3* and one in *GPX2* were significantly associated with risk of rectal cancer, but not with either colon cancer or adenoma [70] (Tables 13.3, and 13.4). In the *MSRA* gene, coding for methionine sulfoxide reductase A, rs10903323, was associated with coronary artery disease risk in a Chinese population [71].

13.5 Genetics of Endoplasmic Reticulum Selenoproteins

In eukaryotic cells, the ER is not only responsible for the synthesis, post-translational modification and correct folding of membrane and secreted proteins, but also for intracellular Ca^{2+} homeostasis and lipid biosynthesis [72]. Alteration of protein folding caused by changes in intracellular Ca^{2+} levels, redox state, nutrient status, protein synthesis rate or inflammatory stimuli, can result in ER stress and activation of the unfolded protein response to remove misfolded proteins [72]. ER dysfunction and prolonged ER stress have been implicated in diseases such as cancer, diabetes and Alzheimer's disease [72]. Seven human selenoproteins have been shown to be associated with the ER, which are the 15-kDa selenoprotein (Sep15), type 2 iodothyronine deiodinase and selenoproteins K, M, N, S, and T [73]. Selenoprotein S is a component of the ER-associated protein degradation pathway and is involved in the removal of misfolded proteins from the ER lumen [73]. Sep15 has been implicated in the formation of disulfide bonds and the quality control of protein folding [34], while SelK plays a key role in calcium signalling in immune cells [74].

Genetic polymorphisms in *SELS, SELK* and *SEP15* have now been linked to various cancers and inflammatory conditions, consistent with these SNPs affecting correct ER function (Table 13.4). In *SELS* gene, rs34713741 affects levels of pro-inflammatory cytokines, IL-6, IL-1β and TNF-α [75], and risk of CRC in Korean [76] and Czech [7] populations. In the Czech population, rs34713741 was associated with greater CRC risk, and in the Korean population a second variant in close proximity led to increased risk (in females only). The replication of the association

in these two diverse populations strongly indicates that, independently of lifestyle and dietary factors, SNPs in *SELS* influence CRC risk. Moreover, supporting a role of SelS in gastrointestinal function, rs34713741, was also linked to gastric cancer risk [77, 78]. No association was identified between six genetic variants in *SELS* and type 1 diabetes [79]. In contrast, rs225014 in (type 2 deiodinase gene), results in a Thr to Ala amino-acid change at position 92 in the protein sequence, with the Ala variant being less active and associated with type 2 diabetes [80–82].

In the *SEP15* gene, rs5859 and rs5845, known to reduce Sec incorporation efficiency and Sep15 synthesis, affect BC risk in a Se-dependent manner in African American women, but not in Caucasians [33]. In breast tumors, a loss of heterozygosity at the *SEP15* locus was observed [33, 83], indicating a potential tumor suppressor role of Sep15 in breast tissue. In addition, rs5859 and rs5845 were associated with increased risk of PCA in German [12] and New Zealand [51] males with low Se intake, and other SNPs in *SEP15* were also linked to PCA mortality (Table 13.4) [20, 84]. CRC risk was also affected by rs5859 in a South Korean population [76] and by genetic interactions between SNPs in *SEPP1* and rs5859 in *SEP15* [7]. Moreover, rs5859 was linked to lung cancer in Polish individuals with low Se status [85]. The influence of these SNPs on disease risk and progression provides insight into the potential role of ER stress and protein folding control in disease etiology as well as on the importance of key selenoproteins in maintaining a healthy tissue.

13.6 Perspectives

Two main lessons can be learned from the studies described above. First, there is now considerable evidence for a number of genetic variants affecting selenoprotein synthesis and, in a limited number of cases, this has been linked to effects on response to dietary Se intake. Genetic epidemiology studies suggest that a number of these variants, notably those in *SEPP1, SELS*, *GPX1*, *GPX4* and *SEP15*, modulate risk of various chronic diseases. In particular, these results suggest that variants in *SEPP1, SEP15* and *GPX1* affect PCA risk and progression, SNPs in *SELS* influence CRC risk and variants in *GPX1* BC risk. However, the evidence linking these variants with disease risk comes largely from relatively small studies that often lack accompanying measures of Se status and often require replication. In addition, observed effects are often inconsistent between study populations, a phenomenon that likely reflects differences in the characteristics of the study populations, notably Se status. Further work is necessary to be confident of the clinical relevance of selenoprotein SNPs in different populations, both in terms of Se intake and ethnicity. Larger studies, combining genetics and biomarkers of Se status should provide a clearer picture of the links between Se, selenoprotein genetics and disease risk.

Second, identifying that SNPs in selenoprotein genes affect risk for several chronic diseases is compatible with the observation that most of these multifactorial diseases share a common basis, with the disruption of biochemical pathways involved

in the response to oxidative and ER stress. As a result, these genetic associations help elucidate potential pathways affected in the etiology of disease for an individual. Selenoprotein synthesis depends on the distribution of Se between selenoproteins and the use of common synthesis machinery. Thus, the observation of genetic associations of a SNP with a disease may reflect an effect of this particular SNP on the whole selenoproteome, inviting us to combine genetic association studies with expression of selenoproteins in a tissue.

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Chapter 14
Selenium Regulation of the Selenoprotein and Non-selenoprotein Transcriptomes in a Variety of Species

Roger A. Sunde

Abstract Selenium (Se) status regulates the expression of selenoproteins, not only by availability of Se for incorporation into these selenoproteins, but also by regulation of the selenoprotein transcriptome. Selenoprotein transcripts can be used as "molecular biomarkers" for assessment of Se deficiency in rodents, and we have expanded these studies to avian species and the nematode to better investigate the hierarchy of Se incorporation into selenoproteins. In addition, we are investigating the impact of high-Se levels on selenoprotein transcripts, and the impact of high-Se intake on the expression of non-selenoprotein transcripts. These studies indicate that increased reactive oxygen species are associated with Se deficiency and high-Se intake, and that panels of non-selenoprotein transcripts have potential as biomarkers of supranutritional and toxic-Se status.

Keywords Biomarker • *Caenorhabditis elegans* • Chicken • Collagen • Hierarchy • Mouse • Rat • Reactive oxygen species • Requirement • Turkey

14.1 Introduction

Selenium (Se) status regulates the expression of selenoproteins, not only by availability of Se for formation of the key cofactor, selenocysteine (Sec), in these selenoproteins, but also by regulation of the selenoprotein transcriptome. This regulation translates into use of selenoproteins and selenoenzymes as biomarkers of Se status and requirements, and also offers the "selenotranscriptome" as a "molecular biomarker" of Se status. The hierarchy of selenoenzyme biomarkers in rodents was reviewed in the third edition of this book [1].

Right after the discovery that glutathione peroxidase (GPX) was a selenoenzyme [2], dietary Se was shown to regulate the level of GPX activity [3]. Dietrich Behne was the first to describe the differential incorporation of ^{75}Se into selenoproteins in

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various tissues as a hierarchy of Se supply to tissues and selenoproteins [4]. Subsequent researchers also reported differential Se incorporation or expression of selenoproteins as new selenoproteins were discovered. Exploring the underlying mechanism for Se regulation of GPX activity, we found that GPX protein could also be used as a biomarker for Se status [5, 6]. That led to investigating *Gpx* mRNA levels, which were surprisingly found also to be regulated by Se status [7]. Relative to *Gpx* mRNA, differential regulation by Se status was reported for transcripts for selenoprotein P (*Sepp1*), 5′-deiodinase (*Dio*), *Gpx4*, *Gpx2*, and other known selenoproteins [8–12]. These additional selenoproteins expanded the array of both biochemical and transcript-based biomarkers of Se status in our studies [13–17]. The bioinformatics identification of the complete selenoproteome by Gladyshev and colleagues [18] allowed us to use selenoprotein transcripts as "molecular biomarkers" for assessment of Se deficiency in rodents [19–22]. These studies have now been expanded to avian species, such as turkeys and chickens [23–25], and nematodes [26, 27], to better investigate the hierarchy of Se incorporation into selenoproteins. In addition, this approach of investigating the effect of high-Se intake (supranutritional and toxic) on selenoprotein transcripts in these species was examined to investigate the impact of high-Se status on the expression of non-selenoprotein transcripts.

14.2 Animal Models for Se Regulation of Selenoprotein Transcripts

To study the effect of Se deficiency on selenoprotein expression, weanling rats and mice from Se-adequate dams were typically fed a basal 30 % torula yeast-based diet supplemented with 0.4 % additional methionine (Met) to meet the sulfur amino acid requirements, and supplemented with 100 mg/kg of vitamin E, which provides twice the vitamin E requirement [28]. This diet allowed adequate growth and prevented liver necrosis, so that the impact of Se status was studied without impaired growth or subsequent effects of disease. Since selenomethionine is an excellent analog of Met, it is readily aminoacylated to Met tRNA and incorporated in place of Met into the nascent polypeptide. Furthermore, dietary Met alters the incorporation of Se from selenomethionine and, therefore, these studies were usually conducted using inorganic Se [29, 30].

These basal torula yeast diets consistently contained 0.005–0.007 μg Se/g, which were then supplemented with Se as sodium selenite. For most studies, multiple, graded levels of supplemental Se were used so that the impact of Se status on the target Se biomarkers could be titrated. Importantly, the plateau levels for the biomarker could be better established, indicating that Se is no longer the rate-limiting factor controlling expression of the biomarker. The multiple levels of dietary Se within the plateau also helped establish the variability and effectiveness for detecting biologically-important differences. Specifically, a "Se-response curve" was constructed for each biomarker using sigmoidal or hyperbolic regression analysis including all individual values at each dietary Se treatment as described previously [13, 17, 19]. The "plateau break-

point" for each Se-response curve, defined as the intersection of the line tangent to the point of steepest slope and the plateau, was calculated to estimate the "minimum dietary Se requirement" necessary to obtain the plateau response.

14.3 Se Regulation of Conventional Se Biomarks

In rodents, Se-deficient diets as compared to Se-adequate diets, had no effect on animal growth. However, liver Se concentration fell to three and 10 % of Se-adequate levels in rats and mice fed these basal diets, respectively, demonstrating that the diets were in fact Se-deficient. Similarly, in rats and mice fed these diets for 4 weeks, liver GPX1 activity fell to two and three percent of Se-adequate levels, plasma GPX3 activity fell to two and 37 % of Se-adequate levels, but liver GPX4 activity fell to 47 and 55 % of Se-adequate levels, respectively [19]. Liver thioredoxin reductase (TXNRD) activity fell to 15 % of Se-adequate levels in Se-deficient rats [16]. Clearly, there is a differential effect of Se deficiency on the activity levels of these selenoenzymes.

In this context the minimal dietary Se required to achieve hepatic GPX1 plateau activity was ~0.1 μg Se/g diet, and was ~0.05 μg Se/g diet for liver GPX4, liver TXNRD activity, and plasma GPX3 activity in rats [19]. Similar minimal Se requirements were found in mice [21, 31]. These studies thus showed that there was a Se requirement hierarchy for these selenoenzyme activity biomarkers. This raised the question about underlying Se regulation of the selenoprotein transcriptome.

14.4 Se Regulation of Selenoprotein Transcripts in Rats

The discovery that Se deficiency dramatically down-regulated the level of *Gpx1* mRNA was the start of our focus on Se regulation of transcript levels [7]. Early studies reported differential down-regulation of selenoprotein transcripts in Se-deficient relative to Se-adequate rat liver to 36 % for *Dio* [8], to 67 % for *Sepp1* [8], but only to 90–110 % for *Gpx4* [32], clearly indicating that a hierarchy exists for Se regulation of transcript abundance. Subsequent studies using multiple graded levels of Se supplementation showed that liver *Gpx1* mRNA levels fell to 10–11 % of Se-adequate levels in both Se-deficient male and female rats [13, 14], even though Se-adequate female rat liver had twice the level of GPX1 activity and mRNA as male rat liver [33]. *Gpx4* mRNA levels, in contrast, only fell non-significantly to 61 % and 82 % of levels in 0.1 and 0.2 μg Se/g supplemented rats, respectively [9], illustrating the importance of fully assessing multiple plateau levels for transcript abundance. *Txnrd1* transcripts in Se-deficient rat liver were 70 % of Se-adequate levels [16].

In all of the above studies, the Se-response curves demonstrated that selenoprotein transcript levels reach well-defined plateaus with increasing Se supplementation. Furthermore, as expected, plateau levels of selenoprotein transcripts reached plateau levels at dietary Se concentrations roughly half or less than dietary Se concentrations

necessary for plateau levels of corresponding selenoprotein enzyme activity. Thus, some selenoprotein transcripts, such as *Gpx1,* were suggested to be used as "molecular biomarkers" to detect Se deficiency [34, 47], although resulting dietary Se levels would not provide sufficient Se to maximize selenoenzyme activity.

The report by Gladyshev and colleagues in 2003 [18] of a bioinformatics approach to identify the complete selenoproteome in species was a huge boost for Se research. Quantitative polymerase chain reaction (qPCR) was used to assess transcript abundance for all 24 selenoproteins in rats supplemented with deficient to supranutritional Se (0.007–0.8 μg selenite/g diet) and demonstrated that the majority of liver selenoprotein mRNAs were not significantly regulated by Se status [19]. In liver, *Gpx1* mRNA remained the most highly down-regulated transcript in Se deficiency, falling to 10 % of Se-adequate levels. *Sepw1*, *Selh*, and *Selk* mRNAs were also highly down-regulated to <25 % of Se-adequate levels, whereas *Sepp1* mRNA was only down-regulated to 50 %, while the *Gpx4* mRNA level was not significantly altered by Se deficiency [19]. This hierarchy of Se regulation is shown in Fig. 14.1.

The resulting Se-response curves showed that all regulated selenoprotein transcripts were down-regulated by Se deficiency and reached well-defined plateaus with increasing supplemental Se, and all had plateau breakpoints in a tight range of 0.04–0.07 μg Se/g diet [19]. This tight range suggested that one underlying mechanism

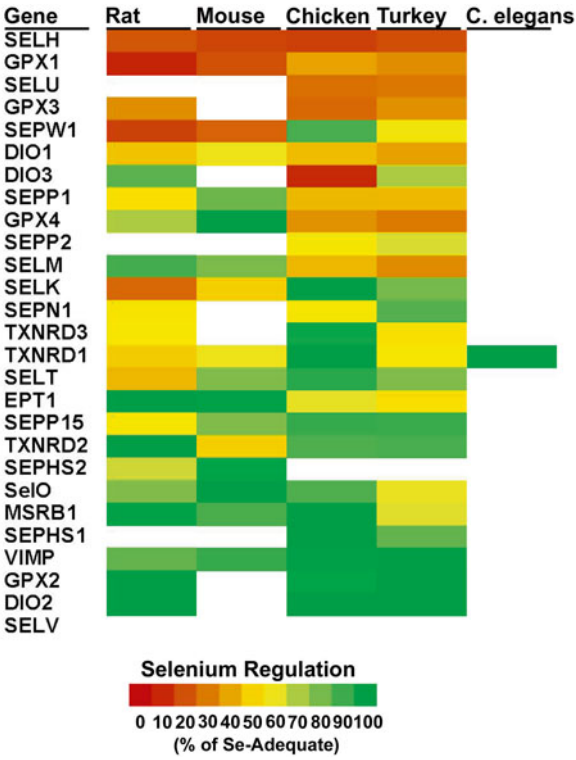

Fig. 14.1 Se regulation hierarchy. Heat map of the hierarchy of down-regulation of selenoprotein transcript abundance in Se-deficient liver, shown as percent of Se-adequate levels for rats [19], mice [21], chickens [25], turkeys [24], and *C. elegans* (total organism) [26], determined by qPCR or microarray analysis as reported in these references. *White bars* indicate missing data or genes. Chicken *DIO3* abundance is not significantly different from Se-adequate

was in play for regulation of selenoprotein mRNA levels [21]. In addition, supplementation of up to 0.8 μg Se/g diet showed that, above the plateau breakpoint, none of the liver selenoprotein mRNA levels were altered by such Se supplementation. This indicated that none of these selenoprotein mRNAs could be used as biomarkers for supranutritional or anti-carcinogenic levels of Se.

14.5 Se Regulation of Selenoprotein Transcripts in Mice

Similar studies were conducted in mice, assessing transcript levels by northern [31] and microarray analyses followed by qPCR confirmation [21]. The impact of Se deficiency on mouse liver selenoprotein mRNA levels is shown in Fig. 14.1. Microarray analyses showed that nine of the 17 selenoprotein mRNAs present on the array were significantly down-regulated by Se deficiency. In mouse liver, *Gpx1*, *Selh* and *Sepw1* mRNAs were highly down-regulated to ~20 % by Se deficiency, whereas *Sepp1* and *Gpx4* mRNA levels were not regulated, as confirmed by qPCR [21]. Se-response curves showed that the plateau breakpoint for mouse liver *Gpx1* mRNA was at 0.04 μg Se/g diet, with no further increase up to 0.5 μg Se/g [31]. However, *Gpx4* and *Sepp1* mRNA levels were not altered by Se supplementation level from deficient to supranutritional.

14.6 Se Regulation of Selenoprotein Transcripts in Turkeys

The Se requirement for turkeys is 2–3 times higher than that of rodents [35]. An early study indicated that some of the apparent GPX1 activity in turkeys might be due to GPX4 [36]. Thus, GPX1 was separated from GPX4, which demonstrated that 47 % of the H_2O_2 activity in Se-adequate turkey liver was due to GPX4, and also determined a factor for calculation of GPX4- and GPX1-specific enzyme activities [37]. Day-old male turkey poults fed torula yeast-based diets supplemented with 0–0.5 μg Se/g for 28 days were found to require 0.05 μg Se/g for maximum growth. Liver Se concentration, liver GPX1 activity, and liver GPX4 activity fell to 15 %, 3 % and 7 %, respectively, of Se-adequate levels, thus showing that Se biology in the turkey is distinctly different from that of rodents. Se-response curves using GPX1 and GPX4 activity in liver showed that the minimum dietary Se requirement in the growing male turkey was 0.3 μg Se/g diet, or three-times that for rodents. The level of Se-adequate liver GPX1 activity in turkeys was 10 % of the level in rat liver, and the level of GPX4 activity is six-times the level in rat liver [37]. In these studies, *GPX1* and *GPX4* transcripts were decreased to ~30 % of Se-adequate levels [37].

The sequence of the turkey selenoprotein transcriptome was completed recently, and expressed transcripts for 24 turkey selenoproteins were found [23]. Notably, *SELV* and *SEPHS2* appeared to be missing, but two additional selenoprotein transcripts, *SELU* and *SEPP2*, which are paralogs of *SELV* and *SEPP1*, with in-frame UGA codons and SECIS elements for Sec incorporation were also observed. *SEPHS1*, a homolog of

SEPHS1 in mammals and also a non-selenoprotein, was present. Importantly, these sequences averaged 96 % sequence identity (range 92–99 %) with the corresponding chicken sequences [23].

These sequences were used to assess Se regulation of selenoprotein transcripts in turkey poults fed 10 graded levels of Se from deficient to 1.0 μg Se/g diet [24]. qPCR analysis found that five of the 24 turkey selenoprotein transcripts were down-regulated, four significantly by Se deficiency to ≤35 % of Se adequate levels, *SELH, GPX4, GPX1, SELU,* and *GPX3*, whereas 6 of 24 were only decreased to 35–50 % and 11 more to >70 % (Fig. 14.1). Resulting Se-response curves showed that all down-regulated selenoprotein transcripts reached well-defined plateaus with increasing supplemental Se, and all had plateau breakpoints in a tight range of 0.05–0.09 μg Se/g diet. Furthermore, Se supplementation up to 1.0 μg Se/g showed that none of these selenoprotein mRNAs can be used as biomarkers for supranutritional Se status [24].

14.7 Se Regulation of Selenoprotein Transcripts in Chickens

In a parallel study in chicks [25], day-old male broilers were fed the torula yeast diet supplemented with 0–1.0 μg Se/g for 29 days. Only 0.025 μg Se/g were required for maximum growth, and liver GPX1 activity and liver GPX4 activity fell to 2 % and 10 %, respectively, of Se adequate levels, showing that Se biology in the chicken, like the turkey, is distinctly different from that of rodents. Se-response curves using GPX1 and GPX4 activity in liver showed that the minimum dietary Se requirement in the growing male turkey was 0.13 and 0.10 μg Se/g diet, respectively, or ~0.05 μg Se/g higher than that for rodents. The level of Se-adequate liver GPX1 and GPX4 activities in the chick were very similar to the levels in turkey poults [25].

qPCR analysis of chick liver selenoprotein transcript levels revealed a pattern of Se regulation similar to that of the turkey. The same five selenoprotein transcripts as in the turkey, *SELH, GPX3, SELU, GPX4,* and *GPX1*, were significantly down-regulated by Se deficiency to <40 % of Se adequate levels, whereas 6 of 24 were only decreased to 40–50 % and 13 more to >70 % (Fig. 14.1). Resulting Se-response curves further showed that all significantly-regulated selenoprotein transcripts reached well-defined plateaus with increasing supplemental Se, and all had plateau breakpoints in a tight range of 0.07–0.11 μg Se/g. Furthermore, Se supplementation up to 1.0 μg Se/g diet showed that none of these selenoprotein mRNAs can be used as biomarkers for supranutritional Se status [25].

14.8 Se Regulation of Selenoprotein Transcripts in *Caenorhabditis elegans*

The selenoproteome in *C. elegans* consists of a single selenoprotein, thioredoxin reductase (designated as *trxr-1*), as well as a Cys-containing paralog, *trxr-2. C. elegans* can be grown in axenic (bacteria-free) media, allowing careful control of Se supplementation.

Low media Se, however, had no effect on growth, and only decreased apparent TRXR activity to ~80 % of Se-supplemented levels [26]. Deletion of *trxr-1*, *trxr-2*, or both, in *C. elegans* was without phenotype under standard conditions [26, 38], and only blocked the increase in apparent TRXR activity associated with Se supplementation [26]. Low media Se had no effect on *trxr-1* mRNA levels relative to Se supplemented media (Fig. 14.1) [26].

14.9 Overall Selenoprotein Transcript Regulation

Figure 14.1 shows a similar hierarchy of a "Se-deficiency regulon" among the four animal species with respect to down-regulation by Se deficiency. The upper half of the heat maps, representing *SELH* to *SELK* transcript levels, shows *SELH*, *GPX1*, and *GPX3* as the transcripts most commonly down-regulated to <40 % of Se adequate levels. In avian species, this group includes *SELU*. *Sepw1* and *Selk* are also highly down-regulated by Se deficiency in rodents, but not in avian species, whereas *GPX4* and *SELM* are more highly regulated in avian species. Although not presented here, there are usually fewer selenoprotein transcripts down-regulated by Se deficiency in non-hepatic tissues of these animals.

The complete mechanism responsible for Se regulation of selenoprotein transcript abundance remains unclear. Nonsense-mediated decay clearly is involved, but the various proposed models to explain this regulation continue to fall short when the profile of Se regulation of the complete selenotranscriptome is examined in a whole animal. As previously discussed in detail, the position of the UGA codon adjacent to the splice junction does not match with the hierarchy of the Se-deficiency regulon [1, 21]. It is important to recognize that the studies reported here were all conducted in whole animals, not cultured cells. As elegantly shown by Maquat and colleagues [39], rodent *Gpx4* mRNA is highly regulated in cultured cells but not in rat liver, clearly demonstrating that other factors are in play in whole animals.

One notable feature of the Se-deficiency regulon from these studies is that, for highly regulated Se transcripts, the plateau breakpoints all resided in a tight group on the Se-response curves. Furthermore, these tight groups were all similar, 0.03–0.07 μg/g diet for the rat, 0.04 μg/g for the mouse, 0.05–0.09 μg Se/g for the turkey, and 0.07–0.11 μg Se/g for the chick, in spite of much larger differences in the level of dietary Se required for maximal selenoenzyme activity. This suggested that a common mechanism is present when Se status regulates selenoprotein mRNA levels in all species [21], and that other factors are also important which subsequently modulate Se availability for incorporation into proteins.

Relative transcript abundance may play a role. It was clear that *Gpx1* mRNA was highly abundant in Se-adequate rat liver, perhaps 20-fold higher than *Gpx4* mRNA and twice as high as *Sepp1* mRNA [40], providing more range for changes in abundance as well as saturation of Se incorporation rates. In contrast, *GPX4* mRNA is three-fold higher and *SEPP1* mRNA was 12-fold higher than *GPX1* mRNA in turkey and chick liver than in rodents. This variable Se-responsive transcript abundance would suggest that Se incorporation into SEPP1 protein and Se

export from liver would dominate over Se incorporation into resident hepatic selenoproteins in avian species.

Collectively, the above studies suggested that whole animal Se metabolism is likely to play a role in the differences between the minimum Se requirement for plateau transcript level and the requirement for plateau level of the selenoprotein. High levels of liver SEPP1 transcripts may out-compete levels with transcripts for GPX1 and other selenoproteins, facilitating export of Se to other tissues, and thus, extending the amount of dietary Se necessary to maximize Se incorporation into hepatic selenoproteins.

14.10 Se Regulation of Non-selenoprotein Transcripts in Rodents

Changes in nutrient levels as well as other factors can have a dramatic effect on the full transcriptome. For instance in yeast, changes in media amino acid concentration or other environmental factors can alter a third of the yeast transcriptome [41]. It was initially anticipated that modest changes in dietary Se supplementation, from deficient to twice the minimum Se requirement, might elicit Se-specific changes as an animal switches from Se retention to Se excretion. Surprisingly, feeding mice 0 vs. 0.2 μg Se/g had a negligible effect on the total transcriptome as determined by microarray analysis [22]. Only three and five transcripts (all selenoproteins) in mouse kidney and liver, respectively, were decreased significantly by Se deficiency, and only three non-selenoprotein transcripts in mouse liver and three in kidney were increased significantly by Se-deficiency; five of the six up-regulated transcripts were nuclear factor (erythroid-derived 2)-like 2 (NFE2L2 or NRF2) targets, indicating that increased reactive oxygen species is associated with Se deficiency [22].

Se toxicity is another aspect to consider in the above studies. When rats were fed a Se-deficient diet supplemented with 0, 0.08, 0.24, 0.8, 2.0 and 5.0 μg Se/g (0 to 50-times the dietary Se requirement), 5.0 μg Se/g, but not 2.0 μg Se/g diet, significantly altered growth (20 % decrease), indicating a mild onset of Se toxicity at 5.0, but not 2.0 μg Se/g. Liver GPX1, liver GPX4 and plasma GPX3 activities all reached plateau levels by 0.24 μg Se/g, and were not further increased by additional dietary Se [22]. When total liver RNA from these rats was subjected to microarray analysis, fewer than 10 out of >30,000 transcripts were significantly altered by 0, 0.08, 0.8 and 2.0 μg Se/g treatments relative to 0.24 μg Se/g-fed rats. As in mice, only selenoprotein transcripts were down-regulated by Se deficiency in rat liver, and Se deficiency increased only two non-selenoprotein transcripts significantly in rat liver [22].

Supplementation with 2.0 μg Se/g altered just six transcripts significantly in rat liver; none were selenoprotein transcripts, and none changed up or down more than fourfold. In contrast, supplementation with 5.0 μg Se/g significantly altered 1193 transcripts or four percent of the transcriptome. As shown in Fig. 14.2, filtering this 1193 high-Se transcript dataset against a calorie-restriction dataset plus a drug toxicity dataset resulted in a 715 “toxic-Se specific” transcript dataset [22]. Additional filtering against a dataset of 1076 NFE2L2-targeted genes [42, 43] showed that

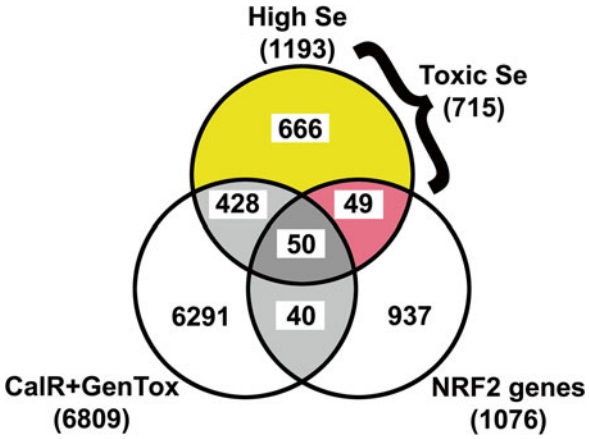

Fig. 14.2 Toxic-Se rat liver transcripts, including NFE2L2 (NRF2) targets. The 1193 high-Se transcripts changed significantly by 5.0 μg Se/g as compared to 0.24 μg Se/g [22] were filtered against a combined "CalR + GenTox" probe set (6809 transcripts changed significantly by caloric restriction (CalR) [51] combined with a general drug toxicity (RatTox FX 1.0, GenTox) probe set), and filtered against a "NRF2 genes" probe set (1076 transcripts for known NRF2-regulated genes [42, 43]). The result was the set of 715 "toxic-Se specific" transcripts, including 49 transcripts of NRF2 target genes (in *red*) plus 666 additional toxic-Se transcripts (in *yellow*)

8.3 % of the 1193 transcripts (99 of 1193) were known NFE2L2 targets, including 49 in the 715 toxic-Se specific dataset. The prevalence of NFE2L2-regulated genes in the dataset of genes significantly altered by Se toxicity as well as in the small set of genes up-regulated by Se deficiency indicated that Se excess as well as Se deficiency increases oxidative stress.

The 715 toxic-Se specific transcript dataset contained 667 unique transcripts, with 542 being up- vs. 125 down-regulated. GOMiner analysis identified 33 biological processes significantly enriched in a Se-specific filtered dataset. These were nearly all related to cell movement/morphogenesis, extracellular matrix, and development/angiogenesis, including a number of collagen-related genes [22].

Interestingly, four selenoprotein transcripts in the 1193 high-Se transcripts were significantly up-regulated by 5 μg Se/g diet. *Selm* and *Sepw1* mRNAs were up-regulated 2.5-fold relative to Se-adequate levels and *Txnrd1* and *Gpx3* were up-regulated 1.5-fold relative to Se-adequate levels [44]. Note that GPX3 activity, presumably primarily from kidney, was not elevated at 5.0 μg Se/g [22]. These four selenoprotein transcripts can thus be considered candidate molecular biomarkers for toxic-Se status, but it is unclear whether these increases were due to primary Se toxicity, or were changes secondary to mild toxicity.

Unsupervised hierarchical clustering of the Se-specific transcript in rats fed 5.0 and 2.0 μg Se/g vs. lower Se intakes was used to identify clusters of potential biomarkers of high-Se intake [22]. Three clusters containing 117 transcripts (72 genes) were up-regulated to some extent by 2.0 as well as 5.0 μg Se/g, and one distinct cluster containing 44 transcripts (25 genes) was down-regulated to some extent by 2.0 as well as 5.0 μg Se/g diet. Functional analysis revealed a set of genes within the

clusters that were up-regulated by 2.0 and 5.0 μg Se/g that are involved in glucose transport, insulin signaling, or glycoprotein biosynthesis.

14.11 Se Regulation of Non-selenoprotein Transcripts in *C. elegans*

Initial studies with *C. elegans* showed that culturing in 0.2 and 0.4 mM Se resulted in a significant delay in growth as compared to 0, 0.05, or 0.1 mM Se, indicating Se-induced toxicity [26]. A microarray study in *C. elegans* cultured in axenic media supplemented with five levels of Se from 0 to 0.4 mM selenite was used to characterize Se regulation of the full transcriptome. Worms were staged to mid-L4 larval stage to minimize developmental differences [27]. Relative to 0.1 mM Se treatment, culturing *C. elegans* at 0, 0.05, 0.2, and 0.4 mM Se resulted in 1.9, 9.7, 5.5, and 2.3 %, respectively, of the transcriptome being altered at least twofold. Filtering these datasets found 295 overlapping transcripts that were altered by both 0.2 and 0.4 mM Se, but not by other treatments. A "toxic-Se specific" dataset of 182 genes was then identified by filtering against gene sets for sulfur [45] and cadmium toxicity [46]. Genes in this toxic-Se dataset were significantly enriched in functions related to oxidoreductase activity, and significantly depleted in genes related to structural components of collagen and the cuticle [27]. These results suggest that Se toxicity in *C. elegans* also caused an increase in reactive oxygen species and stress responses, marked by increased expression of oxidoreductases and reduced expression of cuticle-associated genes, which together underlie impaired growth.

14.12 Se Regulation of Non-selenoprotein Transcripts in Avian Species

In supplemented chicks and turkeys with graded levels of Se up to 1.0 μg Se/g, 1.0 μg Se/g had no effect on growth [24, 25]. In a preliminary study, total RNA pooled from four chicks supplemented with Se-adequate and high Se (0.3 and 1.0 μg Se/g) diets was subjected to microarray analysis using the Affymetrix Chicken Genome Array (32,773 transcripts). Because an average of 96 % sequence identity was found between the turkey and chicken selenoprotein transcripts [23], total liver RNA pools from turkeys fed Se-adequate and high Se (0.4 and 1.0 μg Se/g) diets were also analyzed. Filtering expression to 20–100 % showed significant expression of 30,524 probe sets for chicken RNA, and 30,458 probe sets for turkey RNA, indicating that the chicken array could be used for turkey RNA analysis. As in the studies with rats fed ≤2.0 μg Se/g, there were very few changes associated with high-Se feeding. Only 78 liver transcripts (31 up, 47 down) were changed ≥2-fold in chicks fed 1.0 vs. 0.3 μg Se/g; only 55 liver transcripts (14 up, 41 down) were changed ≥2-fold in turkeys fed 1.0 vs. 0.4 μg Se/g. Along with lack of growth depression due to feeding 1.0 μg Se/g to these

young birds, the near complete lack of changes in transcript abundance clearly showed that this level of dietary Se was not toxic, and that homeostatic mechanisms not linked to changes in gene expression were sufficient to readily accommodate this level of Se ingestion.

14.13 Molecular Biomarker Panels

The finding that Se status regulates GPX1 mRNA levels [7] and that this transcript can be used as a "molecular biomarker" to assess Se status and requirements suggested that individual transcripts or panels of transcripts have potential as biomarkers of nutritional status [34, 47]. In rats, total RNA isolated from whole blood or erythrocytes has been used successfully for assessing Se status [48]. Attempts to extend this to humans, however, have not been successful; plasma Se concentration and plasma GPX3 activity as well as mRNA biomarkers from a European population, ingesting an average of 46 μg Se/day, all resided on the plateau region of the corresponding Se-response curves [49].

Better biomarkers for determining upper limits for Se intake are needed [50]. The lack of major changes in non-selenoprotein transcript abundance, as well as selenoprotein transcript abundance, in rats fed ≤2.0 μg Se/g, and in chickens and turkeys fed ≤1.0 μg Se/g, however, showed that homeostasis is readily able to manage the elevated Se intake. The small set of transcripts with altered abundance in rats fed 2.0 μg Se/g provides an example of how transcript panels might be used as biomarkers for establishing upper nutrient limits. In this rat study, when individual transcript levels for the six transcripts regulated by 2 μg Se/g plus the five transcripts regulated by Se deficiency were analyzed by step-wise multiple regression [34, 47], the resulting equation predicted liver Se concentration with an overall correlation coefficient of 0.9988 ($P < 10^{-6}$), accounting for 99 % of the variation in liver Se concentration over the full range from 0 to 5 μg Se/g. This example illustrated that panels of selenoprotein and non-selenoprotein transcripts clearly have potential as molecular biomarkers of nutrient status [34, 47].

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Chapter 15
^{77}Se NMR Spectroscopy of Selenoproteins

Jun Liu and Sharon Rozovsky

Abstract One of the most essential contributions of selenium to biology is the specialized chemistry performed by selenoproteins. Elucidating the mechanisms by which selenoproteins govern the reactivity of their selenocysteine (Sec) requires exploring how the protein environment primes Sec interactions with substrates, prevents inactivation, and otherwise optimizes the use of this unique amino acid. ^{77}Se nuclear magnetic resonance (NMR) spectroscopy is a particularly powerful technique to study the chemical properties of selenocysteine, its conformational preferences and mobility, and the molecular interactions by which it is stabilized. Recent advances have simplified sample preparation and data analysis, extending the utilization of ^{77}Se in NMR studies of biological samples. These improvements include the development of efficient procedures for enriching proteins with the ^{77}Se isotope, the reports on NMR parameters of different selenoproteins that greatly expand the available basis for data analysis, and the progress in utilizing theoretical calculations for data interpretation. We discuss these areas of progress in ^{77}Se NMR of biological systems, and we consider the range of questions for which ^{77}Se NMR is most useful.

Keywords ^{77}Se NMR • Selenium NMR • Selenocysteine • Selenocystine • Selenopeptides • Selenoproteins • Selenoredox motifs

15.1 NMR Spectroscopy of Biological Macromolecules

NMR spectroscopy is a superb probe of the molecular environment and an unparalleled tool for understanding its electronic and chemical structure. It is routinely used to study the atomic structures, conformational mobility, and supramolecular organization of biological macromolecules. In addition, it can characterize their interactions with ligands, drugs and protein partners. Routine biological NMR relies on the nuclei ^{1}H, ^{13}C, ^{15}N, and ^{31}P, whose behavior in different environments is extensively documented. Theoretical calculations allow the NMR observables of these nuclei in

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different chemical environments to be predicted, and their relation to the structural and functional properties of macromolecules to be elucidated [1].

Biological NMR utilizes a variety of other, less ubiquitous nuclei (for example, metals) that yield rich information about function. Likewise, ^{77}Se NMR is employed to study selenoproteins, since the chemistry centers on selenium's high reactivity and thus it is advantageous to directly probe it. Furthermore, ^{77}Se NMR is used as a spectroscopic surrogate of sulfur because the only NMR-sensitive isotope of sulfur, ^{33}S, is a low-sensitivity quadrupolar nucleus that cannot be utilized to study biological systems. This chapter focuses mostly on recent progress in the studies of selenoproteins because the fundamentals and historic perspectives of ^{77}Se NMR of biological systems have been summarized in a recent review [2].

15.2 Selenium's NMR Properties

Nuclear spin is an intrinsic property of the nucleus that must be larger than zero for the nucleus to interact with a magnetic field and be detected by NMR spectroscopy. Among selenium's six naturally occurring isotopes, only ^{77}Se, whose nuclear spin quantum number is ½, is NMR-active [3]. In general, the detection sensitivity is higher for spin ½ nuclei than for nuclei with larger spin numbers and their spectra are simpler to record and understand [4]. It is therefore fortuitous that ^{77}Se NMR is spin ½ and, like proton, carbon and nitrogen, can be readily detected with routine hardware and pulse sequences.

NMR is sensitive to the electronic structure because the local magnetic field near the observed nucleus reflects the interactions between the electron cloud and the external magnetic field [5, 6]. This results in the chemical shifts that report not only on covalent bonds, but also on non-covalent interactions, solvent structures, nearby charges, and other forces that influence the electron cloud [7]. Selenium has more electrons than carbon and nitrogen and those are held further away from the nucleus and occupy more orbitals. Thus, the external (spectrometer) magnetic field exerts a more extensive effect on its electron cloud. Therefore, while the chemical shift range for ^{13}C spectra in diamagnetic samples is ca. 300 ppm, the range for ^{77}Se spectra is over 3000 ppm (Fig. 15.1). This large range reflects ^{77}Se NMR's high sensitivity to the local environment, making it an excellent spectroscopic reporter on bonding, geometry, and electronic structure [2, 8–10]. However, this sensitivity comes at a price, as the large response of the electron cloud to the external magnetic field leads to broad resonances. These line widths, ranging from 50 to 500 Hz, depending on the intrinsic mobility of the specific site and the strength of the external magnetic field, decrease detection sensitivity and can potentially cause overlap of different resonances. Nevertheless, since the number of ^{77}Se sites in biological samples is typically low, this overlap is usually not a problem. Thus, the ^{77}Se spectrum is straightforward to detect with satisfactory sensitivity and is rich in information due to its pronounced chemical shift response, as exemplified in the following sections.

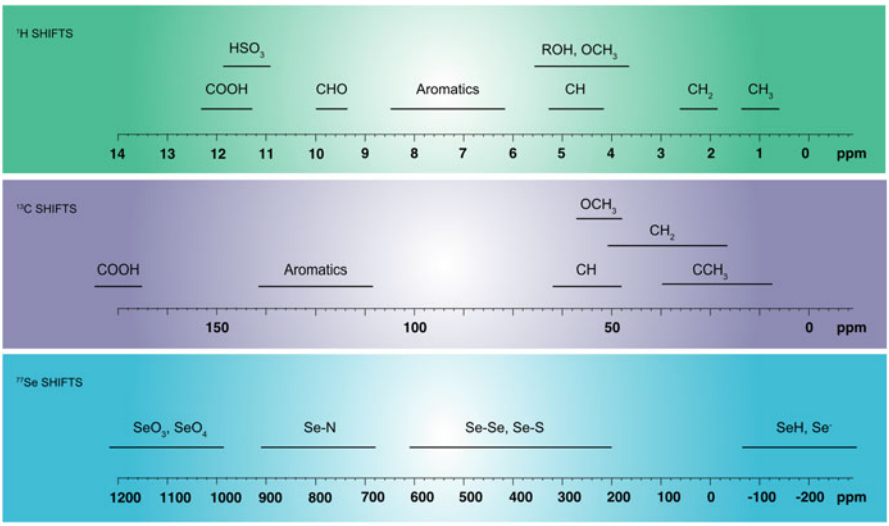

Fig. 15.1 Characteristic chemical shifts for biologically relevant ^{1}H, ^{13}C and ^{77}Se chemical groups. ^{77}Se chemical shifts cover a range of over 3000 ppm. The figure was adapted from references [4, 8, 9]

^{77}Se NMR is abundantly used in studies of materials, small molecules and selenopeptides. Extensive surveys describing ^{77}Se NMR of small compounds and materials can be found in comprehensive reviews [8–13]. However, only a handful of publications have utilized ^{77}Se NMR for studies of biological systems [14–17] and ^{77}Se is far from being routinely exploited. This can be attributed to the following major bottlenecks: a lack of protocols to enrich proteins with the NMR-active ^{77}Se isotope, and the challenge of interpreting NMR data in the absence of a significant body of research. Recent advances in sample preparation and data analysis [11–13, 18, 19] discussed in the following sections, however, render ^{77}Se NMR broadly applicable for biological systems.

15.3 ^{77}Se Isotopic Enrichment of Proteins for NMR Studies

Since not all nuclei are NMR active or their sensitivity is low, biological samples typically need isotopic enrichment with NMR-active nuclei. Similar to ^{13}C and ^{15}N, whose NMR-active isotopes make-up only a small percentage of the total isotopes in the natural abundance sample, ^{77}Se natural abundance is only 7.63 %, and, hence, isotopic enrichment is required. For ^{13}C and ^{15}N enrichment, recombinant proteins are usually prepared using ^{13}C-glucose and ^{15}N-ammonium chloride as the sole carbon and nitrogen sources in *Escherichia coli* growth media. *E. coli* then synthesizes the amino acids from these basic building blocks. A similar approach can be undertaken for ^{77}Se enrichment, but high concentrations of selenocompounds are toxic to

E. coli necessitating the development of labeling protocols with suitable yield and affordable building blocks.

We have developed a ^{77}Se isotopic labeling method for replacing sulfur with ^{77}Se by heterologous expression in *E. coli* [20]. This technique relies on commercial elemental ^{77}Se instead of the expensive and hazardous synthesis of selenocompounds, thus providing a facile and cost-effective route for the insertion of the NMR-active isotope ^{77}Se into proteins. In essence, the method replaces all sulfur sources in the media with selenium using ^{77}Se-selenite. The incorporation is random, leading to a substitution of all sulfur sites and generating ^{77}Se-substituted proteins that contain multiple Sec and selenomethionine (Sem) residues. By fine-tuning the ratio of sulfur to selenium in the growth media, it is possible to control how much sulfur in proteins is replaced with selenium. In other words, for proteins that originally had disulfide or selenylsulfide (Se-S) bonds, it is possible to poise the sample for dominance of either diselenide (Se-Se) or selenylsulfide bonds, as desired.

A second method for ^{77}Se isotopic labeling by heterologous expression in bacteria relies on harnessing *E. coli* genetic incorporation machinery to overexpress selenoproteins. In this procedure, the ancillary proteins that assist Sec synthesis are overexpressed to meet the high level demand for Sec incorporation [21]. A sulfur-rich growth medium is supplemented with low concentrations of ^{77}Se-selenite as the sole source of selenium. *E. coli* then synthesizes and incorporates ^{77}Sec into the target protein in a site specific fashion [13, 22]. While this method allows for the presence of multiple, unaltered cysteines (Cys), it can only label one Sec residue in the protein. In addition, it is realistically restricted to the subset of proteins with a C-terminal located Sec (for additional discussion see reference [2]). This procedure has been employed to generate a series of non-native selenoproteins with representative selenoredox motifs for ^{77}Se NMR investigation [13].

Heterologous expression is not the only path for ^{77}Se enrichment of proteins. Initially, investigations of biological systems by ^{77}Se NMR relied on reacting proteins with ^{77}Se-labeled compounds [15–17]. Another early incorporation method was purification of erythrocyte glutathione peroxidase from lambs fed with ^{77}Se-selenite in their diets [14], but this route was limited as only a few selenoproteins can be readily purified from natural sources. Hence, it became increasingly popular to employ peptide synthesis using ^{77}Sec [12]. ^{77}Sec and chemically protected forms that are compatible with peptide synthesis are not commercially available, but procedures for their synthesis have been published [23]. However, this approach is constrained by the size and properties of the peptide.

An alternative method to prepare selenoproteins for biophysical studies is native chemical ligation (NCL), which relies on an amide-forming reaction to generate peptides and proteins from their respective fragments [24]. This approach has long been employed by selenium biochemists [25], but because it relies on solid-phase peptide synthesis, its scope is limited by the properties of the selenopeptide. Indeed, NCL has only been reported for one native selenoprotein so far, thioredoxin reductase 1 [26], and has never been employed for enrichment with ^{77}Se. Recently we have devised a novel expressed protein ligation procedure that is compatible with the production of

^{77}Se-labeled proteins [J Liu, Q Chen, S Rozovsky, manuscript submitted]. Instead of utilizing peptide synthesis, we produce selenoprotein fragments for ligation in *E. coli*, alleviating the size restriction. This flexible approach allows for the preparation of selenoproteins with Sec at any location in the protein. It is also compatible with labeling of more than one Sec residue, tolerating the presence of Cys residues, and isotopic labeling with additional nuclei such as ^{13}C and ^{15}N.

15.4 Identification of Chemical Species by ^{77}Se NMR

With the realization that selenium is covalently incorporated in proteins came an interest in recording the identity and diversity of chemical species that partake in biochemical reactions. Hilvert and coworkers reported the chemical shifts of a selenosubtilisin that was prepared by reacting the catalytic serine of the bacterial protease subtilisin with ^{77}Se enriched hydrogen selenide [17]. They charted the chemical shifts of selenosubtilisin selenolate (R-Se$^-$), seleninic acid (R-O_2H), and selenylsulfide forms (R-Se-S-R′) under native conditions. This work was groundbreaking in demonstrating that it is possible to collect NMR spectra with sufficient detection sensitivity for this 27.4 kDa protein at what is now considered low magnetic fields of 200 and 300 MHz. Another seminal study was performed by Gettins and coworkers on erythrocyte glutathione peroxidase and other non-native selenoproteins [14, 15]. Even though these were conducted under denaturing conditions, they demonstrated the potential of biological ^{77}Se NMR.

However, these early studies were, for the most part, restricted to proteins that were prepared by chemical synthesis. Thus, it remained unclear if protein samples containing multiple Sec and Sem residues would yield significant signals at moderate to high magnetic field strengths, and to what extent the broad signals would lead to signal overlap. Our group reported the first NMR detection of a ^{77}Se-substituted protein that contained numerous Sec and Sem residues. These multiple selenium resonances were detected at moderate and high magnetic fields of 14.1 and 19.97 T (^{1}H frequency of 600 MHz and 850 MHz, respectively). Most importantly they were highly sensitive to changes in the chemical environment [11]. We studied augmenter of liver regeneration (ALR), a sulfhydryl oxidase in the mitochondrial intermembrane space. ALR employs a CXXC motif, in conjunction with a flavin cofactor, to catalyze the formation of disulfide bonds (Fig. 15.2). In its secreted form, it is a 32 kDa homodimer that employs six Cys residues in each monomer to form interfacial and internal disulfide bonds. Thus ALR contains both structural and catalytic Cys. The sulfur sites of all six Cys and two Met residues were substituted to selenium, using a defined medium with a prefixed ratio of selenium/sulfur. By controlling this ratio, it was possible to poise the proportion of diselenide and selenylsulfide bonds in the selenium-substituted ALR. To better understand the ramifications of substituting sulfur to selenium, we determined the thermal stability and activity of selenium-substituted ALR and the X-ray crystal structure of ALR enriched with 90 % selenium [20, 27] to a 1.5 Å resolution (PDB entry 3U5S). This high

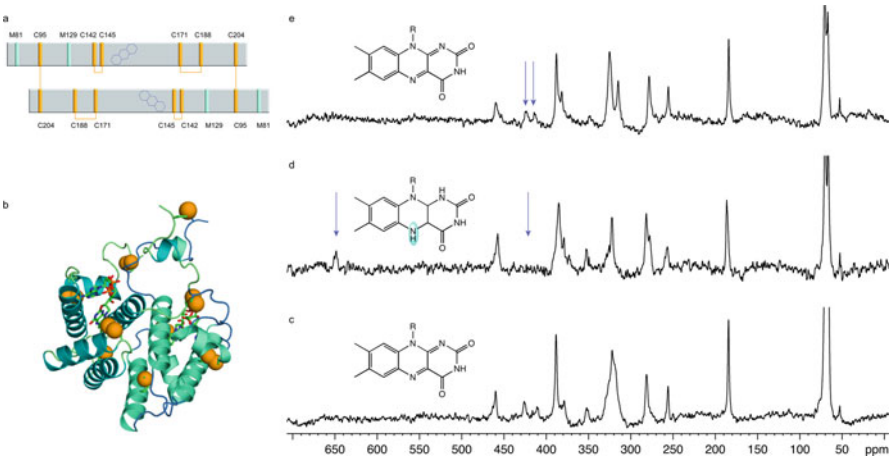

Fig. 15.2 ^{77}Se NMR investigations of the sulfhydryl oxidase ALR. (**a**) ALR is a 32 kDa homodimer enzyme with 12 Cys (*yellow*) and four Met (*green*) residues. Each active site contains a flavin cofactor (*purple*), and two catalytic Cys residues (C142–C145). Selenium-substituted ALR maintains its activity. (**b**) The selenium in different Sec (*orange spheres*) are shown in the crystal structure of selenium-rich ALR (PDB entry 3U5S). (**c**–**e**) ^{77}Se NMR characterization of the flavin reduction in ALR. For ^{77}Se NMR, the sulfur was replaced with selenium to create a protein rich in selenylsulfide bonds. All NMR spectra were acquired at 20 °C and 14.1 T (^{1}H frequency of 600 MHz). Sem resonances between 0 and 150 ppm are truncated for clarity. (**c**) Proton-decoupled ^{77}Se spectrum of ALR with 50 % selenium enrichment. The *inset* shows oxidized FAD. (**d**) Spectrum of ALR with reduced FAD under anaerobic conditions. *Arrows* indicate missing resonances at 412 and 426 ppm and a new resonance at 651 ppm. The *inset* shows reduced FAD. (**e**) Spectrum of ALR when FAD was reoxidized following removal of the reducing agent. The two resonances missing in (**d**) reappeared, suggesting that they arise from the catalytic residues in the immediate vicinity of the FAD. The *inset* shows oxidized FAD. Adapted from reference [20]

resolution allowed us to contrast the bond length and dihedral angles between the sulfur-based and selenium-rich proteins.

For 90 % ^{77}Se-substituted ALR, it was possible to collect overnight data on a high magnetic field of 19.97 T (^{1}H frequency of 850 MHz) with adequate signal to noise ratio [2]. For a 50 % ^{77}Se-substituted ALR at the moderate magnetic field of 14.1 T (^{1}H frequency of 600 MHz), the acquisition time was close to 2 days. Therefore, it is possible to acquire informative ^{77}Se NMR spectra using conventional acquisition times. In addition to acquiring ^{77}Se spectra with resolved resonances and a wide range of chemical shifts, we further demonstrated that these resonances are responsive to the reduction of ALR's flavin adenine dinucleotide (FAD) cofactor. The FAD was specifically reduced with sodium dithionite in an anaerobic NMR tube, changing the environment next to the CXXC active-site motif. Figure 15.2 displays the spectra of ALR before, during, and following reduction of the FAD. Several resonances shifted upon reduction of FAD, and two of these (between 400 and 450 ppm) disappeared, but are restored when the reductant is removed. These two resonances were tentatively assigned to be the catalytic residues next to the FAD. A new resonance appeared downfield at 651 ppm in the presence of sodium dithionite, and can be

attributed to a reversible modification on a cysteine. Accordingly, this resonance disappeared when the reducing agent was removed. Hence, the Sec is acting as a sensitive probe of the chemical transformations in the active site and the FAD state.

15.5 The Connectivity of Diselenide Bonds

The connectivity of a diselenide bond (i.e., the identity of the residues that form the diselenide bond) can be traced using NMR experiments that examine the properties of the selenium bonds. Such approaches rely on measuring the scalar coupling, a property of the chemical bond. Scalar coupling (also called J coupling and indirect spin-spin coupling) arises from the influence of electrons in the chemical bond on the tendency of the atoms' spins to align [4]. This coupling can only be measured if atoms are bonded together or through a small number of chemical bonds. The sign and magnitude of scalar coupling depends on the molecular geometry and thus can be used to extract information on which atoms participate in a given bond, and what are the bond and torsion angles form. This approach was first demonstrated for the 37-mer insecticidal neurotoxin k-ACTX-Hv1c [12]. The vicinal disulfide bond of this spider toxin was converted to a diselenide bond using solid-phase peptide synthesis. Diselenide connectivity was measured in the k-ACTX-Hv1c by both ^{1}H–^{77}Se Heteronuclear Multiple Bond Correlation (HMBC) and ^{77}Se-^{77}Se Correlation Spectroscopy (COSY) [12, 28]. Since then, additional seleno-toxins have been characterized in a similar fashion [29].

In selenoproteins, the selenylsulfide bonds are significantly more important for the catalytic cycle than the diselenide bonds [30, 31]. For these selenylsulfide bonds, it is only possible to correlate the selenium and hydrogen atoms so HMBC is the experiment of choice. We have shown that it is possible to record HMBC for the selenylsulfide bond in selenoproteins, specifically in a 12 kDa protein containing a Gly-Cys-Sec-Gly redox motif, see Figure 4 of reference [2].

15.6 Measurements of Sec pK_a in Selenoproteins and Selenopeptides

NMR spectroscopy is useful for measuring the pK_a of residues including that of Sec. The chemical shift difference between protonated selenols and the deprotonated selenolate is in excess of 100 ppm, allowing easy differentiation between the two. This chemical shift difference can be plotted against pH, and analysis of the titration curve yields pK_a values. Hilvert and coworkers established that in selenosubtilisin, the pK_a of Sec was lower than 4.0, but they could not extend these studies to lower pH due to the instability of the native fold of the protein [17]. However, selenopeptides lend themselves to investigations at acidic pH and thus can provide information on the contributions of nearby residues. The pK_as of two Sec residues in the 9-mer

peptide selenovasopressin (UYFQNUPRG) were reported as 3.3 and 4.3 at positions 1 and 6, respectively, and these pK_a values were used for selective alkylation [18].

An intriguing possibility offered by these experiments is that a similar approach can be employed to record the redox potentials of selenopeptide and selenoproteins. However, this application is still awaiting demonstration.

15.7 Conformational Preferences and Dynamics of Sec

In addition to its sensitivity to the chemical environment, the strength of NMR lies in its ability to probe conformational dynamics on all time scales relevant for function of proteins (10^{-9}–10^{2} s). The ability of selenium to report on the conformational preferences and mobility of Sec at different sites was examined with a series of proteins whose local Sec environment was systematically changed. Specifically, we have recorded the NMR spectra of proteins into which we had placed Sec in different redox motifs (i.e., the patterns of amino acids next to Sec) [13]. In these selenoproteins, the reactive Sec formed a selenylsulfide bond with a neighboring Cys. The reactivity of Sec is influenced by the size of the ring formed in the oxidized state and the identity of the residues forming this redox motif. Using solution-state ^{77}Se NMR spectroscopy, we probed the conformations of these redox motifs in proteins, and the flexibility of Sec in both the reduced and oxidized states. As the size of the ring formed in the oxidized state increased, the NMR spectra display evidence for increased mobility at the selenium site, and also the presence of additional ring conformations. At the reduced state, the width of resonances suggests that serine in the redox motif can form a hydrogen bond to Sec.

A unique aspect of this study [13] is that theoretical calculations of the magnetic shielding were used to understand the conformational preferences of the oxidized selenylsulfide-containing rings. Not only do they illustrate the range of chemical shifts expected in Sec in similar protein environments, but they also demonstrate how conformation and thus weak non-bonding molecular interactions can be inferred from ^{77}Se NMR. This study is the first to combine experimental measurements and Density Functional Theory (DFT) calculations to investigate the origin of selenium NMR parameters in biological systems. These calculations provide a comprehensive interpretation of chemical shifts of selenium and insight into their origin. Overall this work deepens our understanding of the chemical reactivity of selenoproteins.

15.8 ^{77}Se NMR Sensitivity to the Local Environment

As discussed, the chemical shift contains information on how the electrons in the molecules respond to the external magnetic field, and thus report on the electronic structure, the chemical bond, and the protein environment [32]. This shift is not merely a single number, but has orientation-dependent values (which are usually

summarized in a tensor) that reflect how the molecule is positioned in the external magnetic field [6]. In solution-state NMR, the rapid molecular tumbling averages this orientation dependence of the chemical shifts. In contrast, in solid-state NMR (SSNMR), the arrested motion of the molecules allows the individual elements of the chemical shift tensor to be recorded. SSNMR samples can be large proteins, protein complexes, protein crystals, and other molecular states that are incompatible with solution-state NMR [33]. However, despite SSNMR's high information content and extensive flexibility with sample conditions, SSNMR has not yet been applied to biological systems with ^{77}Se.

In preparation for applying ^{77}Se SSNMR to macromolecules, we recently reported the selenium magnetic shielding tensor of the model compound L-selenocystine [19]. This study is notable, because DFT calculations of the selenium magnetic shielding tensor were utilized to explain the reported L-selenocystine chemical shift tensor (Fig. 15.3). We found that even though the crystal structure of L-selenocystine is unknown, the Se-Se torsional angle can be deduced using calculations of the magnetic shielding (i.e., chemical shift). Thus, this study demonstrates the ability to extract structural information from the chemical shift tensor for selenium-containing molecules with unknown molecular geometry. More importantly, it presents a systematic investigation of the fit of the experimental data to the calculations when various structural effects, such as protonation state, protein environment, substituent, or conformation, are varied. Consequently, this work provides fundamental information for future studies of Sec and Sem in different protein environments.

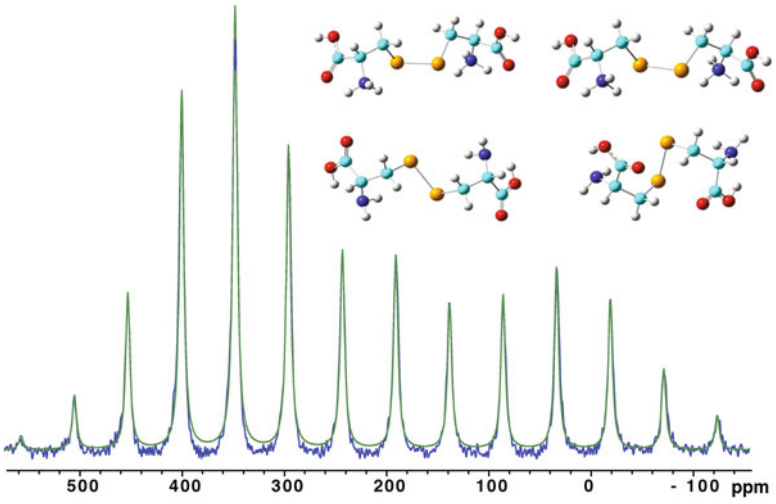

Fig. 15.3 ^{77}Se solid-state NMR spectra of L-selenocystine. *Blue*: ^{77}Se-^{1}H cross polarization magic angle spinning spectra of L-selenocystine recorded at 11.75 T, a temperature of 261 K, and a spinning rate of 5 kHz. *Green*: best fit simulated spinning chemical shift anisotropy sideband patterns for L-selenocystine at a spinning rate of 5 kHz. *Inset* shows models of representative selenocystine structures used for DFT calculations. Adapted from reference [19]

15.9 Data Interpretation

Due to the paucity of biological ^{77}Se studies, there is no comprehensive knowledge base to help determine the influence of the local environment (i.e., neighboring residues, hydrogen bonding, disulfide/diselenide/selenylsulfide interchange and their dynamics, local pH environment, etc.) on selenium chemical shifts in macromolecules. Consequently, the bottleneck for biological ^{77}Se NMR is neither sample preparation nor data acquisition, but rather data interpretation.

Fortunately, quantum calculations of chemical shifts in proteins are transforming NMR data interpretation, and thus can aid data interpretation in ways such as assignments of individual resonances and charting their molecular environment. These calculations have become an invaluable tool for biological NMR and they are now practically indispensable for the assignment of proteins' chemical shifts [34], the determination of their structure, characterization of minor structural conformers, and studies of ligand-protein interactions [1, 35, 36]. However, the magnetic shielding of ^{77}Se is somewhat more difficult to calculate than that of lighter nuclei such as ^{13}C and ^{15}N, since for higher atomic number nuclei, it is necessary to incorporate a more extensive description of electron correlation [37]. Thus, in the case of ^{77}Se, when the crystal structure is taken into account, the theoretical and calculated values differ typically by 5–15 % [38]. For biological systems it is more difficult to predict and understand chemical shifts. However, good agreement was found between calculations of isotropic chemical shifts for selenoproteins and selenol amino acids [39–41] and the experimental data reported by Odom, Gettins, and Hilvert [14–17] (see Sect. 15.4).

We have worked to build the knowledge base and to advance data interpretation by recording ^{77}Se chemical shifts through systematic surveys of ^{77}Se NMR parameters in biological systems [13]. In this work, theoretical calculations of the isotropic chemical shifts have been used to understand the conformational preferences of the oxidized selenylsulfide-containing rings. This was the first study to infer nonbonding interactions of Sec and its conformations (see Sect. 15.7). We also employed DFT calculations to understand ^{77}Se chemical shifts of the model compound L-selenocystine (see Sect. 15.8). This has advanced our understanding of how different variables in DFT calculations influence prediction of ^{77}Se chemical shifts of Sec and Sem residues measured in proteins.

15.10 Concluding Remarks

^{77}Se NMR of biological systems has become broadly accessible due to the development of high-yield and low-cost methods for ^{77}Se-isotopic enrichment, as well as the reports on the detection of Sec and Sem residues in various proteins. In addition, the quantum mechanical calculations of ^{77}Se magnetic shielding continue to improve in accuracy and ease, allowing for their routine implementation in studies of

biological systems. The rise in ^{77}Se NMR studies of different classes of biological macromolecules is leading to improved data interpretation and better integration of biological ^{77}Se NMR into routine use.

New directions include the use of ^{77}Se as a local probe of conformational changes. Selenium is an excellent probe for molecular motion since its magnetic shielding interaction can be used to detect dynamics that occur on the microsecond time scale, a range relevant to many biological processes. Other particularly exciting directions are investigations of native selenoproteins by both solution and solid-state NMR to understand general thematic questions pertaining to selenoproteins.

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Chapter 16
Thioredoxin Reductase 1 as an Anticancer Drug Target

Edward E. Schmidt and Elias S.J. Arnér

Abstract Several genetic, biochemical and pharmacological studies suggest that the cytosolic selenoprotein thioredoxin reductase 1 (TrxR1, TXNRD1) can serve as a promising anticancer drug target. This notion is in part based upon potent and selective targeting of the selenocysteine residue in TrxR1 by electrophilic anticancer agents. Whereas normal somatic cells typically can survive without TrxR1, the metabolic and proliferative realignments associated with cancer increase the oxidative stress and thus an increased reliance upon reducing pathways in cancer cells, thereby increasing their dependence upon TrxR1 activity. Intricate functional links between TrxR1 and transcription factors such as Nrf2, NF-kB and p53, and interaction with other growth-promoting signaling pathways, further underpin anticancer therapies involving TrxR1 targeting. However, caveats exist, as some effects of TrxR1 inhibition may promote cancer rather than counteract its progression. Although encouraging advances are being made, the field is clearly not yet ready for clinical trials evaluating novel specific TrxR1 inhibitors in anticancer treatments. In this chapter we present and discuss the major aspects of this topic.

Keywords Cancer • Therapy • Thioredoxin reductase

16.1 Introduction

The thioredoxin (Trx) system is an important reductive enzyme system that acts in conjunction with enzymes of the glutathione (GSH) system [1–4]. The Trx system involves isoenzymes of Trx and thioredoxin reductase (TrxR), the latter using

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NADPH to reduce the active site disulfide of Trxs, which subsequently act upon a wide range of downstream targets that are required for reductive pathways and regulate cellular function. In mammals, the Trx system is selenium-dependent due to the fact that the three mammalian TrxR isoenzymes are among the approximately 25 selenoproteins that exist in mammals. The reader is referred to other literature for detailed information on the wide physiological roles and biochemical features of the enzymes that constitute the Trx system [1, 2, 4–7]. Here we shall instead focus upon the role of this system in cancer and the potential therapeutic effects of inhibiting the cytosolic TrxR1 enzyme for anticancer therapy.

16.2 The Multiple Roles of the Thioredoxin System in Cancer

Many studies have indicated that the Trx system should be considered an important player in carcinogenesis and cancer progression [1, 5, 8–15]. It is less clear, however, which, if any, of the specific molecular mechanisms of the Trx system that may be required for cancer development and, conversely, may be selectively targeted by inhibitors for use in improved anticancer therapy. In this chapter we aim to discuss consequences of TrxR targeting that may indeed be beneficial for anticancer therapy, while other effects could instead promote cancer. Indeed, "antioxidant enzymes" such as TrxRs are increasingly understood to have either health-promoting or health-detrimental effects, depending upon their diverse roles in specific physiological settings and contexts [16]. The main question thus becomes whether drug-mediated inhibition of TrxR1 may be possible to develop for beneficial, specific, and potent anticancer therapy. That question is yet to be answered by clinical trials, but we here aim to provide a discussion of promising observations for development of TrxR-based therapy as well as the potential pitfalls for such efforts.

16.3 The Interplay Between Thioredoxin Reductase and Nrf2

Nuclear factor erythroid-2-related factor 2 (Nrf2, NFE2L2) is a transcription factor that can activate a wide range of cellular antioxidant systems [17–20] and that is increasingly being recognized to have major importance in cancer. Indeed, Nrf2 is typically highly activated in cancer cells and as such thought to be required for the survival of cancer cells, wherein it acts to counteract their endogenously increased levels of oxidative stress [17, 21–24]. It has, on the other hand, also been shown that anti-tumoral immune system functions may require Nrf2 activation [19, 25–27]. Interestingly, a wide range of inhibitors of TrxR1 also activate Nrf2, which is likely indicative of a direct functional link between TrxR1 and Nrf2-driven enzyme systems [28]. We propose that this functional link between TrxR1 and Nrf2 is one important factor determining the outcome of pharmacological treatment of cancer based upon drug targeting of TrxR1.

16.4 Impact of Thioredoxin Reductase Targeting in Cancer and Its Potential for New Anticancer Therapy

TrxR1 is targeted by a wide range of compounds that inhibit the enzyme. The relative ease of targeting TrxR1 mainly results from its accessible and exceptionally reactive Sec residue in the C-terminal active site [29]. Indeed, in most cases characterized to date, inhibition of TrxR1 activity is associated with covalent derivatization of the Sec residue [3, 28, 30–34]. Nevertheless, TrxR1 is a complex enzyme with other functional domains and many amino acids whose activities are required in the catalytic cycle. Thus, despite the relatively specific and strong target provided by the Sec residue, it should not be overlooked that TrxR1 inhibition could also be achieved by reversible or irreversible targeting of other motifs in TrxR1.

It is notable that a very large number of compounds that inhibit TrxR1 have anticancer effects and, also, that several clinically used anticancer agents inhibit TrxR1 [1, 5, 13, 14, 30, 31, 34–51]. The questions still not answered are whether efficient anticancer therapy can solely be based upon TrxR1 drug targeting, whether counteracting cancer-promoting effects may be triggered by TrxR1 inhibition, and/or what are the specific consequences of TrxR1 targeting that could form the basis for a successful anticancer therapy. In Fig. 16.1, we have schematically outlined

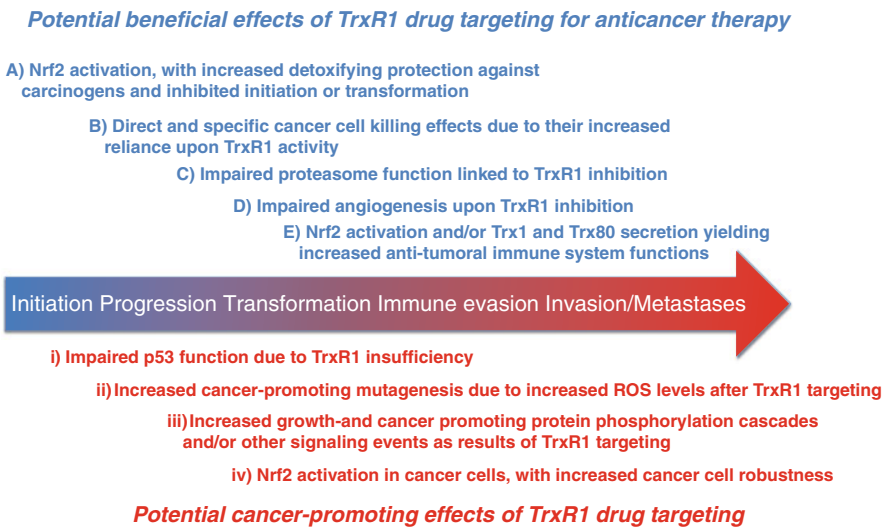

Fig. 16.1 Potential anticancer effects as well as cancer promoting effects of TrxR1 drug targeting during different phases of cancer progression. This figure schematically summarizes potential beneficial consequences of TrxR1 drug targeting for anticancer therapy (*blue text*) as well as caveats for such therapy (*red text*) during different phases of carcinogenesis (*arrow*). Each listed effect is further discussed in the text

possible consequences of TrxR1 targeting for initiation, promotion, transformation, progression or immune system evasion in cancer, with either beneficial (*blue*) or detrimental (*red*) effects in view of therapeutic efficacy. These effects shall now be discussed in further detail, also referring in each sub-section the reader to relevant literature for further in-depth information.

16.5 Potential Beneficial Effects of TrxR1 Drug Targeting for Anticancer Therapy

It should be emphasized that several of the potential therapeutically beneficial effects of TrxR1 targeting that are discussed in this section remain to be confirmed in a clinical setting, and are thus yet mainly hypothetical, although they are implied from basic studies as reported in the literature.

16.5.1 Nrf2 Activation, with Increased Detoxifying Protection Against Carcinogens and Inhibited Initiation or Transformation

As mentioned above, it is clear that a large number of drugs that inhibit TrxR1 also activate Nrf2, suggesting a functional link between these two events [28]. A question is whether Nrf2 activation in normal cells may have anticancer consequences? It seems improbable that Nrf2 activation lessens the progression of cancer, since most data in the literature suggests that cancer cells depend upon Nrf2 activation as a means of cancer cell survival in view of the increased oxidative stress typically related to the cancer cell phenotype [21, 23, 24]. This suggests that high Nrf2 activity may, in fact, worsen cancer progression rather than being a therapeutic advantage (see discussion on this aspect below). However, it is possible that Nrf2 activation can protect normal cells from cancer *initiation*, as a result of increased antioxidant capacity upon Nrf2 activation, thus leading to lower oxidative stress and less likelihood of carcinogenic mutations due to oxidative DNA damage. This effect relates to the potentially chemopreventive properties of Nrf2 activation, which is a widely studied topic. Interestingly, it has been shown that drug-mediated TrxR1 targeting in normal cells is, perhaps at first seemingly paradoxically so [16], not detrimental but instead *protective* against oxidative challenges as a result of an "overshoot" in Nrf2 activation [52–54]. This effect may possibly explain how TrxR1-inhibiting compounds such as curcumin [31, 50] or isothiocyanates [55–58] can be chemopreventive agents against cancer, which would be an effect linked to the cancer preventive properties of Nrf2 claimed for normal cells [19, 48, 56, 59–65].

16.5.2 Direct and Specific Cancer Cell Killing Effects Due to Their Increased Reliance upon TrxR1 Activity

The notion of cancer cells having an inherently increased level of oxidative stress due to their distorted energy metabolism, proliferative drive and abnormal cellular phenotype, is rapidly gaining wider recognition [8, 35, 66–70]. This notion, in turn, should explain why cancer cells typically exhibit high endogenous Nrf2 activities with resulting increased levels of enzymes in the GSH and Trx systems, as a means of surviving in spite of their abnormally high levels of cellular oxidative stress [19, 21–23, 61]. In view of such a cellular phenotype, it should thus be a natural consequence that TrxR1 inhibition in cancer cells can lead to an impairment of the required antioxidant capacity, particularly in these cells, thereby triggering cell death in cancer cells while normal cells typically survive loss of TrxR1 activity [5, 8, 13, 35, 62, 69]. This may be one major mechanism by which TrxR1 inhibition using small compounds can lead to anticancer efficacy and reduction of tumor mass, which is further corroborated by the fact that genetic deletion or knockdown of TrxR1 in cancer cells was shown to impair their capacity for tumor development [11, 12, 71, 72]. An additional effect of drug targeting of TrxR1 in cancer cells, which may contribute to tumor cell death, is conversion of the enzyme to toxic pro-oxidant forms, named SecTRAPs, that can further aggravate the oxidative stress in cancer cells, which has been shown for several different inhibitors of the enzyme [28, 37, 49, 73, 74].

16.5.3 Impaired Proteasome Function Linked to TrxR1 Inhibition

Proteasome inhibitors are a promising class of antitumor agents, mainly active against myeloma, including other forms of cancer, which seem to be linked also to oxidative stress [75–77], and interestingly, targeting of TrxR [78–80]. Targeting of the Trx system has indeed been implicated to have major impact on proteasome function [81, 82] and it should thus be of interest to further study whether effects on the proteasome could contribute to anticancer efficacy of TrxR targeting drugs. This possibility awaits conclusive scrutiny.

16.5.4 Impaired Angiogenesis upon TrxR1 Inhibition

Drug-mediated inhibition of angiogenesis is being intensively discussed as a potential principle for anti-tumoral treatment [83–86]. It is in this context interesting to note that mitochondrial TrxR2 expression in tumor cells seems to be required for

tumor growth and tumor-promoted angiogenesis [87]. Also, the potent TrxR inhibitor auranofin prevents angiogenesis in zebrafish [88], which was also seen with another gold compound [89], and a third class of gold compound inhibitors of TrxR could inhibit tumor growth and angiogenesis in a mouse melanoma model [90]. It is also interesting that thioredoxin-interacting protein (TXNIP) was shown to be essential for VEGF-triggered angiogenesis in mice, while inhibition of TrxR1 in endothelial cells increased expression of VEGF and promoted angiogenesis [91, 92]. These observations suggest that modulation of TrxR1 or TrxR2 activities can impact angiogenesis in a cell- and possibly cancer type-specific manner. Whether this inhibited angiogenesis might be part of therapeutic anticancer effects upon use of TrxR inhibitors must, however, be studied further.

16.5.5 Nrf2 Activation and/or Trx1 and Trx80 Secretion Yielding Increased Anti-Tumoral Immune System Functions

Anti-tumoral efficacy of the immune system is important for final eradication of cancer in cancer therapies [93–95]. Importantly, the Trx system might regulate the effectiveness of the immune system against cancer cells by two different mechanisms. First, activation of Nrf2 and the resultant increase in reducing power seems to be important for optimal immune system anti-tumoral activities [25, 26, 66, 93, 94], which may thus be another potential consequence of TrxR1 inhibition particularly in immune cells, as discussed above. Second, if TrxR1 is inhibited in cancer cells and yields increased tumoral oxidative stress, this might increase secretion of Trx1 as well as its C-terminally truncated form Trx80 to serum; both proteins acting as co-cytokines and chemokines that may thereby help to attract anti-tumoral immune cells to the tumor [4, 13, 96–99]. We propose that these possible mechanisms for anticancer effects of TrxR1 targeting should be further addressed in forthcoming studies, as they may provide a basis for final tumor eradication upon TrxR1 inhibition.

16.6 Potential Cancer-Promoting Effects of TrxR1 Drug Targeting

With redox systems affecting many different cellular pathways, it might also be that drug-mediated inhibition of TrxR1 could *aggravate* cancer growth, rather than yielding therapeutic benefits (as summarized with red text in Fig. 16.1). Here we shall discuss some possible mechanisms by which TrxR1 inhibition may be

detrimental in cancer therapy. It should be noted that, as above in Sect. 16.5, several of these effects of TrxR1 targeting remain to be confirmed in a clinical setting, and are thus yet mainly hypothetical.

16.6.1 Impaired p53 Function Due to TrxR1 Insufficiency

Native p53 functions as an important cancer-preventive checkpoint mechanism, explaining why p53 is mutated or by other means impaired in most cancer cells. Interestingly, p53 is also sensitive to redox alterations [100] and, specifically, is impaired by lowered TrxR1 activity, likely due to accumulation of incorrectly folded and nonfunctional p53 in these conditions [101–103]. This could suggest that if TrxR1 becomes inhibited in normal cells, the p53 maturation in those cells might be impaired and thus leave room for increased propensity of carcinogenesis due to loss of p53 checkpoint activity. On the other hand, TrxR1 and p53 also seem to be intimately linked in promoting cancer cell death related to increased oxidative stress, at least upon some types of drug therapy [35, 39, 103–106]. The complex interplay between p53 and TrxR1 activities following TrxR1 targeting thereby remains uncertain in relation to anticancer efficacy, and should thus be studied further.

16.6.2 Increased Cancer-Promoting Mutagenesis Due to Increased ROS Levels after TrxR1 Targeting

As discussed above, a therapeutic principle for TrxR1 targeting in anticancer therapy is to increase oxidative stress in cancer cells beyond the point of no return, thereby leading to cancer cell-specific induction of cell death. Normal cells, by contrast, should survive TrxR1 targeting, as they will have an excess of antioxidant capacity, in part from Nrf2 activation, and may even "overshoot" and become more resistant to oxidative stress than cells having normal activity of TrxR1 (see discussion above). This hypothesis for cancer cell-specific oxidative stress-induced cell death upon TrxR1 inhibition has, however, not yet been adequately supported in vivo or from the outcomes of relevant clinical trials, and is in need of further evaluation of its potential. A caveat to approaches that increase oxidative stress in cancer cells would be the possibility that some cancer cells might both survive the TrxR1 inhibition-induced oxidative stress and escape clearance by the anti-tumoral effects of the immune system (see above). Could the increased oxidative stress in such cells trigger additional mutations, which may promote cancer evolution and aggravate cancer progression? This would be a detrimental effect that needs to be taken into account. Indeed, since most clinical cancer therapies, including both

chemotherapy regiments as well as radiotherapy, are known to induce cellular oxidative stress [66, 69], this same caveat should be considered for nearly all conventional cancer therapies.

16.6.3 Increased Growth- and Cancer Promoting Protein Phosphorylation Cascades and/or other Signaling Events as Results of TrxR1 Targeting

Abnormally high cancer cell proliferation and growth promotion is typically maintained by growth factor-related protein phosphorylation cascades and/or other signaling events. This may, among other pathways, include increased activities in PDGF-, EGF- or NF-kB signaling pathways [107, 108]. Importantly, such signaling pathways are modulated by the Trx system and can be stimulated upon TrxR1 inhibition, as a result of downstream consequences such as diminished activities of protein tyrosine phosphatases [109–111] or other intricate and yet insufficiently understood regulation of transcription factor activities [22, 112]. Thus, there might be a risk that TrxR1 inhibition could promote cancer cell growth through such mechanisms, which naturally needs to be considered in relation to cancer therapies based on targeting TrxR1.

16.6.4 Nrf2 Activation in Cancer Cells, with Increased Cancer Cell Robustness

The typically high activity of Nrf2 in cancer cells and their dependence upon this effect for survival in relation to an increased endogenous oxidative stress provides a mechanistic rationale for anticancer therapy based upon TrxR1 targeting, as discussed above in Sect. 16.6.1. It may, however, be possible that some types of cancer cells present lower levels of endogenous oxidative stress and thus lower Nrf2 activity, as for example, in the case of slower-growing cancers. Indeed, it was found for melanoma cells that these could be classified into two patient groups, where the "low oxidative-stress" group displayed better prognosis [25]. It was also shown that different cancer cell types indeed display different basal Nrf2 levels but, interestingly, drug-mediated Nrf2 activation in cancer cells did not seem to provide the protective effects seen upon genetically determined Nrf2 activation [113]. Such differences might possibly be explained by the fact that Nrf2 interacts functionally with other factors that are often genetically altered in cancer, such as BRCA1 [114] or p38 and p53 [115]. Thus, it is not certain that TrxR1 drug targeting, which typically induces Nrf2 activation [28], will activate Nrf2 in cancer cells having lower Nrf2 activity. However, this risk must nonetheless be considered and ruled out, as it would potentially aggravate cancer growth rather than providing the basis for anticancer therapy.

16.7 Concluding Remarks

We have herein discussed the promising potential of using drug-mediated inhibition of TrxR1 as a basis for anticancer therapy, which is a notion that has already been widely discussed in the literature, but not yet proven in clinical trials. We have also pointed out a number of potential detrimental consequences of TrxR1 targeting that could, in some cases, exacerbate cancer progression and must therefore be very carefully considered and evaluated. It is possible, or even likely, that the distinction between anticancer effects *vs*. cancer growth promoting effects may be cancer cell- or tissue type-specific, which is a question that needs to be addressed in future studies. It is furthermore plausible that therapies using TrxR1 inhibitors in combination with other cancer treatment regimes could minimize unwanted cancer promoting-consequences and pave the way for more efficient and improved anticancer therapies. The potential benefits, as well as the potential pitfalls of targeting TrxR1, as discussed in this chapter, provide strong arguments not only for further investigation, but also for improved diagnostics and streamlined development of personalized therapies, such that each cancer and each cancer patient can be provided with optimal care for their particular situation. Studies of optimal combination therapies involving TrxR1 inhibition have yet to be reported, but will hopefully become a major focus in the near future.

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Chapter 17
Basics and News on Glutathione Peroxidases

Leopold Flohé and Regina Brigelius-Flohé

Abstract The catalytic mechanism of glutathione peroxidases and its variations in the subfamilies are reviewed and biological roles of the individual enzymes are compiled. The oxidative part of the catalytic cycle involves a water-mediated charge separation in the reaction center leading to dissociation of the selenocysteine (or cysteine) residue and binding of the delocalized proton in a highly energized position. In this environment, a suitably bound H_2O_2 is cleaved without any energy barrier in a concerted reaction yielding water and a selenenic (sulfenic) acid. Depending on family subtype and physiological conditions, the unstable oxidized enzymes form intramolecular disulfide or selenenylamide bonds. The reductive part of the cycle involves the reaction of selenenic (sulfenic) acid with a thiol and (selena) disulfide exchange. Trivial in principle, the reduction steps are most variable within the family, which explains its diversified specificities ranging from GSH to thioredoxin, disulfide isomerases, and particular SH groups of other proteins. The versatility in substrate and co-substrate use predestines these proteins for redox regulation, either as competitors for hydroperoxide utilization by other regulatory proteins or as sensor(s)/transducer(s) in hydroperoxide-initiated signaling cascades.

Keywords Apoptosis • Carcinogenesis • Diversity • Glutathione peroxidases • Mechanism • Oxidative protein folding • Redox regulation • Spermiogenesis • Specificity

17.1 Introduction

Cellular glutathione peroxidase, now known as GPx1, was the first and for a decade the only mammalian protein that had been identified as a selenoprotein. Its functional, chemical, and structural characterization was instrumental to our present understanding of selenoprotein biosynthesis and enzymatic selenium catalysis and also helped to clarify some aspects of selenium deficiency syndromes. Early studies

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on this enzyme and their implications for the entire field of selenium biochemistry have recently been compiled in an amply-referenced retrospective [1] and will not be reiterated herein. GPx1 is only one member of a large peroxidase family present in all kingdoms of life. Only a minority of these proteins carry out selenium catalysis (SecGPx). In most of them, the active site selenocysteine (Sec) is replaced by cysteine (CysGPx). CysGPxs prevail in plants, archaea, bacteria, protists, and non-vertebrate species in general, while in vertebrates, four or five of the eight GPxs are selenoproteins [2]. The number of exceptions to this rule, however, appears to be growing. SecGPxs have been discovered in remote species such as the platyhelminths *Schistosoma mansoni* [3], *S. japonica* [4], *Echinococcus granulosus* [5], the lung fluke *Paragonimus westermani* [6], and probably other worms [7], the green alga *Chlamydomonas rheinhardtii* [8], the fresh water polyp *Hydra vulgaris* [7], the myriapod *Strigamia maritima* [9], different crab and shrimp species [10–12], the arachnidal tick *Rhipicephalus* (*Boophilus*) *microplus* [7], but rarely in bacteria [13] or in insects with the blood-sucking *Rhodnius prolixus* being the only exception thus far [14]. Members of the GPx family, as defined by sequence homology and the highly conserved catalytic tetrad comprising Sec (or Cys), Gln, Asn and Trp residues [15], have adopted diverse functions during evolution. The 2-Cys-GPx subfamily common in bacteria, plants, protists, and insects primarily uses thioredoxin (Trx) instead of glutathione (GSH) as a reductant [16, 17], and the glutathione peroxidase activity of GPx1 appears not to be the predominant role of all vertebrate GPxs either. The scenario is further complicated by the glutathione peroxidase activities of structurally unrelated enzymes of the GSH-*S*-transferase and peroxiredoxin families [18].

The scope of this chapter is to outline general mechanistic features of the canonical GPxs, to describe the diversifications and to discuss their established and potential biological functions. For more detailed information, we refer to the chapters of this book focusing on individual GPxs (see Chaps. 18, 38, 43 and 49) and recent reviews [19–21].

17.2 The Glutathione Peroxidase Reaction

In general, GPxs catalyze the reduction of hydroperoxides by thiols. In the mammalian SecGPxs1-4, the reductant primarily used is GSH as shown in Equation 17.1 (Eq. 17.1).

$$\mathrm{ROOH} + 2\mathrm{GSH} \rightarrow \mathrm{ROH} + \mathrm{H_2O} + \mathrm{GSSG} \qquad (17.1)$$

The specificity for GSH appears to gradually decline from GPx1 to GPx4 in line with the loss of Arg residues implicated in GSH binding [22, 23], and in the CysGPxs7 and 8 and many non-mammalian CysGPxs, the substrates most widely used are dithiol proteins such as protein disulfide isomerase (PDI) or Trx, respectively (Eq. 17.2).

$$ROOH + Prot(SH)_2 \rightarrow ROH + H_2O + ProtS_2 \quad (17.2)$$

With all SecGPxs so far investigated, and with many of the CysGPxs, a kinetic pattern is observed that does not comply with the Michaelis-Menten theory: a ping-pong pattern with infinite V_{max} and K_M values [24]. Such kinetics, however, are not unusual for oxidoreductases and were, in fact, initially anticipated in the systematics of multi-substrate enzymatic reactions in 1957 [25], when also the first GPx was discovered [26]. They simply reveal that: i) we have to consider the GPx reaction as a sequence of independent bimolecular reactions; and ii) typical enzyme-substrate complexes are either not formed or the monomolecular downstream reactions within the complexes are faster than the formation of these complexes. The latter option is more realistic in view of the weak, but obvious substrate specificities of the GPxs.

A recent re-investigation of the GPx reaction using the Density Functional Theory (DFT) corroborates this interpretation of the kinetic pattern [27] and simultaneously explains the extreme reaction rates of GPxs [28]. By employing DFT, an active site model can be calculated from seven amino acids comprising the reacting Sec (Cys) in the conserved NVAxxU/C motif, the Trp and Asn of the conserved WNF motif, and the essential Gln [15, 29]. The tetrad residues therein are connected by multiple hydrogen bonds, which facilitate delocalization or shuttling of protons [24, 27]. If this active site model is complemented by a single water molecule, the proton of the Sec selenol migrates via water to the imino nitrogen of the Trp. By this charge separation, two prerequisites for a fast oxidation of Sec are met: i) the selenol function is fully dissociated; and ii), more importantly, the delocalized proton is bound in a highly unstable and reactive position. The hydroperoxo group of the substrate is accommodated between the selenium and Trp. Within this complex, the delocalized proton is prevented from shuttling back to selenium. When it proceeds along the electrochemical gradient, it hits the peroxo bond, and, in a concerted reaction, H_2O (or ROH, respectively) is formed as an ideal leaving group, while Sec becomes oxidized to a selenenic acid, as shown in Eq. 17.3.

$$[E-Se^- \rightarrow {}^{H}O-O^{H} \leftarrow H-OH \leftarrow H^+-Trp] \rightarrow [E-Se-OH \bullet 2H_2O \bullet Trp] \quad (17.3)$$

As a corollary, the calculations revealed that this reaction within the unstable GPx-H_2O_2 complex proceeds without any energy barrier. The rate-limiting step in the oxidative part of the GPx cycle is not the decay of the enzyme-substrate complex, as presumed in the Michaelis-Menten theory, but the binding of the hydroperoxide to the active site, which is reflected in the measurable rate constant, k_{+1}. Interestingly, DFT-calculations qualitatively yielded the same solution for CysGPx, which complies with surprisingly high k_{+1} values observed with natural CysGPxs [28]. Similar conditions prevail in the reductive part of the SecGPx reaction. To bind a flexible and multiply charged molecule, such as GSH, in a way that its SH function is oriented towards the Se-O^- function of the oxidized enzyme [23] requires time. Yet once this has been achieved, the reaction leading to the Se-glutathionylated intermediate (E-Se-SG in Eq. 17.4) will proceed fast enough to prevent accumulation of

the E_{ox}-GSH complex. Similarly, generation of the symmetric disulfide GSSG is likely faster than a productive binding of GSH to the intermediate (Eq. 17.5).

$$[E-Se-O^{-}\bullet H^{+}\bullet GSH] \rightarrow E-Se-SG+H_2O \tag{17.4}$$

$$[E-Se-SG\bullet GSH] \rightarrow [E-Se^{-}\bullet H^{+}]+GSSG \tag{17.5}$$

Thus, in all these individual steps of the catalytic cycle, the complexes do not accumulate, which leads to the unusual lack of saturation kinetics. This peculiarity of SecGPxs is, however, not shared by all CysGPxs. In particular, 2-Cys-GPxs often display typical Michaelis-Menten-type saturation kinetics (for examples, see [28]). This GPx subfamily, like the atypical 2-Cys-peroxiredoxins, have a second conserved Cys in a flexible loop. After the peroxidatic Cys (C_P; the one homologous to Sec in SecGPx) has been oxidized by H_2O_2 to a sulfenic acid, it has to build an intramolecular disulfide bridge with a second Cys, before the reductive portion of the catalytic cycle is initiated. The second conserved Cys is therefore called the 'resolving cysteine', C_R. Like in peroxiredoxins, the disulfide form of 2-Cys-GPxs is typically reduced by a redoxin such as Trx or tryparedoxin. Several factors may contribute to the change in kinetic behavior of 2-Cys-GPxs: i) the catalytic efficiency of the C_P is lower than that of the peroxidatic Sec in SecGPxs (by analogy called U_P), which might critically slow down the reaction within the complexes; ii) a rigid molecule such as a redoxin may be bound faster than the flexible GSH; and iii) bridging of C_P to C_R, which, as with peroxiredoxins, requires a substantial conformational change [17, 24, 30, 31] might become the rate limiting step.

In short, the oxidative part of GPx catalysis is similar for all subtypes of the family and appears to be only quantitatively affected by an exchange of Sec by Cys. In contrast, the reductive steps of the catalytic cycle are surprising with perplexing variations [24, 28]. However, apart from these diversifications, a fundamental difference between Sec- and CysGPxs exists in the manner, in which the enzymes evade destruction by their aggressive substrates, the hydroperoxides. The C_P of CysGPxs has a stronger tendency to become over-oxidized than the homologous Sec. In 2-Cys-GPxs, this over-oxidation is likely prevented by the reaction of oxidized C_P with C_R, which simultaneously creates the interaction site for redoxins [17]. In this respect, these CysGPxs mimic 2-Cys-peroxiredoxins, for which the protective role of C_R has been convincingly demonstrated [24]. C_P may also form an intramolecular disulfide with a Cys that is not a C_R in the sense that it is required for the reduction by specific substrates, as is postulated for mammalian GPx7 [32]. Alternatively, intermolecular disulfide bridges may be formed, as is evident from massive polymerization of Cys mutants in mammalian SecGPx4 [27, 33]. Simultaneously, oxidation of C_P to sulfinic and sulfonic acid is also observed [27]. While homologous Se-thiylation appears to be common also in SecGPxs [22, 27, 34], over-oxidation of Sec to a seleninic or selenonic acid ($R\text{-}SeO_2^-$ or $R\text{-}SeO_3^-$, respectively) has never been detected. Instead, SecGPxs, when exposed to hydroperoxide in the absence of any thiol substrate, form a selenenylamide bond with the nitrogen of a peptide bond downstream of Sec (Eq. 17.6). This surprising reaction

has been verified for mammalian SecGPx1 and SecGPx4 [27] and may be assumed to be common to all SecGPxs. Intriguingly, the homologous reaction with CysGPxs was neither suggested by DFT-calculations nor detected by MS analysis [27].

$$E-Se-O^{-}+H^{+}+R_2NH \rightarrow R_2N-Se-E+H_2O \qquad (17.6)$$

The ground state enzyme can be regenerated from this alternate oxidized form, which stores the oxidation equivalents of the labile selenenic acid as a selenenylamide that is stable within the architecture of the active site, but is readily transformed into a dehydro-alanine residue, if the enzyme is denatured [27].

17.3 Diversification Within the GPx Family

17.3.1 Structure and Substrate Specificity

The catalytic tetrad comprising Sec46 (Cys46), Gln81, Trp136 and Asn137 in rats is conserved in all but two of the hundreds known GPx sequences. In mammalian GPx8, the Gln residue is replaced by Ser and in a poplar GPx (GPx5 of *Populus trichocarpa*; *Pt*GPx5) by Glu. In agreement with site-directed mutagenesis results [29, 35], these modifications at the active site indicate that individual tetrad residues, with exception of Sec (Cys), are not absolutely essential for peroxidase activity and may in part complement each other. However, the tetrad Gln has been implicated in productive hydroperoxide binding and, interestingly, a back-mutation of Glu to Gln in *Pt*GPx5 dramatically improved affinity and reactivity with *t*-butyl hydroperoxide [36], which reveals that the canonical tetrad structure is indeed the optimal one for the oxidative portion of GPx catalysis. Apart from the tetrad structure, the quaternary structure may also affect hydroperoxide specificity of a GPx. In the tetrameric mammalian SecGPxs (GPx1, 2 and 3), selenium is localized in a flat valley near the subunit interface, but is freely exposed to the surface in the monomeric GPx4. This is likely one of the reasons, why GPx4-type enzymes are peculiar in efficiently reducing hydroperoxides of complex lipids. Furthermore, GPx4 displays a large, positively charged surface surrounding the reaction center, which predestines it for interaction with the negative polar heads of phospholipids in bio-membranes [28, 37].

While the typical tetrad structure determines the reaction of GPxs with ROOH, the reductive part of the catalytic cycle appears not to depend on the retention of this architectural peculiarity [24, 31, 36]. Extensive Molecular Dynamics calculations [23] indicated that the specificity of the prototype SecGPx1 for GSH is primarily achieved by binding of the carboxyl groups of GSH to the guanidine groups of four Arg residues surrounding the reaction center (for graphical illustrations see [19, 23, 24, 38]). In GPx2, three of the Arg residues are still conserved, and the donor substrate specificity is presumed to be similar to that of GPx1 [39]. In the extracellular

GPx3, only two of the Arg residues are left, and the enzyme accepts Trx and glutaredoxin apart from GSH [40]. Finally, GPx4 has none of the conserved Arg residues, but still is preferentially reduced by GSH, which in this case is achieved by binding GSH to Lys residues [24]. GPx4, however, appears to be the least specific GPx with respect to donor substrates. Apart from a variety of low molecular mass thiols, it accepts protein thiols, including those of GPx4 itself [34], and peptides containing Cys-Cys doublets, as are present in keratin-associated proteins [41]. The mammalian CysGPxs7 has been discussed to bind GSH via hydrophobic interaction [42], but lacks any of the basic residues implicated in specific binding of GSH to GPx1-4 [42–44]. Accordingly, the substrate binding site of GPx7 proved to be versatile: GPx7 is reduced by protein disulfide isomerase (PDI) with a rate constant that is two orders of magnitude higher than that of GSH [32]. GPx7 also interacts with glucose-regulated protein 78 (GRP78) [45]. Furthermore, PDI-specificity is inferred for GPx8 [43]. The donor substrate specificities of the remaining vertebrate GPxs have not yet been systematically investigated. Redoxin-specificity is the domain of the non-vertebrate 2-Cys-GPxs. In these proteins, the reaction with the CxxC motif of the redoxins depends on disulfide formation between the C_P and a C_R, the latter being located in a remote domain. The conformational change required for disulfide formation creates a completely new substrate interaction site, which predisposes the oxidized enzymes for the reaction with alternate substrates.

17.3.2 Diversified Biological Roles

Originally, all GPxs were considered antioxidant enzymes able to ameliorate hydroperoxide challenges, and the individual members were believed to back up or complement each other with their particular specificities. A common denominator indeed appears to be an anti-apoptotic and anti-inflammatory action of these enzymes. Otherwise, this view has to be rated as an over-simplification.

- GPx1 is still considered to be responsible for detoxification of H_2O_2 and other soluble hydroperoxides. Knockout studies reveal that GPx1 is not of vital importance. However, *Gpx1*$^{-/-}$ mice are highly susceptible to oxidative challenges such as exposure to bacterial lipopolysaccharide, macrophage activation, or viral infections [38]. Accordingly, GPx1-deficiency is being widely discussed in the context of diseases linked to oxidative stress [46]. However, over-expression of GPx1 also yielded mice with symptoms of type-II-diabetes or metabolic syndrome [47]. An unexplained finding remains why *Gpx1*$^{-/-}$ hepatocytes are more resistant to peroxynitrite exposure than wild type cells, although GPx1 is able to reduce peroxynitrite [48].
- GPx2, the gastrointestinal form, is believed to sustain the delicate balance of proliferation and apoptosis of the gut epithelium [49]. *Gpx2*$^{-/-}$ mice show enhanced apoptosis in gastrointestinal epithelia and are more susceptible to inflammation-mediated carcinogenesis than wild type mice, yet transplanted

tumors grow faster, if GPx2 was normal [50]. In tumor prevention, GPx1 and GPx2 apparently act synergistically. In double knockout mice, colitis and intestinal tumors develop without any inflammatory challenge apart from gut colonization with non-pathogenic bacteria [51]. Collectively, the results reveal that GPx1 cannot fully compensate for the loss of GPx2 (for more details, see Chap. 38, [20] and [52]).

- Knockout of the extracellular GPx3 did not display an obvious phenotype. Recently, however, accelerated platelet aggregation and multiple thrombi have been observed in *Gpx3*$^{-/-}$ mice [53]. Also, these mice, like *Gpx2*$^{-/-}$ mice, proved to be more susceptible to azoxymethane/dextran sodium sulfate used to model inflammation-mediated colon carcinogenesis [54].
- The *Gpx4* gene is expressed in three distinct ways: all three forms are selenoproteins, all can catalyze the same reactions, but each plays a different role. The nuclear form, nGPx4, has a different N-terminus, since its expression uses a start codon within the first intron. Knockout of *nGpx4* yields a mild phenotype, with impaired chromatin compaction and morphological alterations in sperm as the only established disturbances [55]. For expression of the mitochondrial form, mGPx4, transcription initiates from an upstream start codon. mGPx4 is identical with the cytosolic cGPx4 after cleavage of the mitochondrial import sequence. Knockout of *mGpx4* causes complete loss of male fertility of otherwise healthy mice [56]. Thus, mGPx4 is the expression form that 'moonlights for fertility' in the late phase of spermiogenesis, where it forms the mitochondrial sheath by polymerization and co-polymerization with Cys-rich proteins [34, 41]. It should be stressed that this function of mGPx4 is the opposite of an antioxidant one. Rather, it is an anabolic process: the use of ROOH to build a complex structure that is indispensable for sperm function [34, 57]. Deletion of the complete *Gpx4* gene as well as selective knockout of *cGpx4* leads to embryonic death around day 7 p.c. (reviewed by Conrad [58]). This essentiality of cGPx4 probably reflects a very special 'antioxidant' function, which is its ability to efficiently reduce hydroperoxides within membranes. The still hypothetical explanations for the essentiality of cGPx4 are interrelated: i) cGPx4 can remove all traces of peroxidized lipids, thereby preventing activation of lipoxygenases [21], including 12,15-lipoxygenase [59], which has been implicated in membrane destruction during tissue remodeling [21]; ii) it reduces the products of 12,15-lipoxygenase, which can cause an alternate way of programmed cell death, designated AIF-mediated apoptosis [60]; and iii) it interferes with another type of programmed cell death, ferroptosis, which like AIF-mediated apoptosis involves 12,15-lipoxygenase products, yet leads to LOOH/iron-mediated cell death via initiation of free radical chain reactions [61, 62]. The common denominator of these hypotheses is cell destruction by unbalanced 12,15-lipoxygenase activity, when cGPx4 is missing. Accordingly, one would expect that inactivation of 12,15-lipoxygenase should rescue *Gpx4* knockout mice, which unfortunately remains controversial: pharmacological inhibition of lipoxygenases rescued the mice [60], while disruption of the 12,15-lipoxygenase gene failed to do so [63].

GPx4 also antagonizes RIP3-mediated necroptosis of erythroid precursor cells, which again is driven by oxidation [64] (See also Chaps. 18 and 43).

- GPx5 is an extracellular CysGPx. Its location in the epididymis suggests a role in sperm physiology [65]. Its precise function, however, remains elusive.
- GPx6 is a SecGPx in man, but a CysGPx in rodents and other animals [66]. It is found in the olfactory bulb, and since its discovery, has been suspected to complement the olfactory sensory system [67]. Its precise role remains to be elucidated.
- GPx7 (also known as NPGPx) is a CysGPx with clear preference for PDI as a reducing substrate [32]. It is located at the luminal site of the endoplasmic reticulum (ER) [43, 68]. It is presumed to use the excess H_2O_2 produced by Ero1α (ER oxidoreductin 1α) for oxidative protein folding via oxidation of PDI family members [20]. Moreover, GPx7 has been reported to oxidize GRP78, thereby enhancing the chaperone activity of the latter [45]. Deficiency of GPx7 causes obesity in mice and apparently also in humans [69]. Knockout of *Gpx7* is associated with multiple organ dysfunctions, increased risk of carcinogenesis, and shortened life span [68].
- GPx8 is also a CysGPx and, like GPx7, is located in the ER and implicated in oxidative protein folding [43, 68]. Although its reaction center is similar to that of GPx7, discrete differences in specificities might serve distinct roles of the thiol peroxidases of the ER (GPx7, GPx8 and peroxiredoxin IV) in the complex scenario of protein folding [21, 68, 70, 71]. Moreover, GPx8 appears to be a negative regulator of fibroblast growth factor and insulin signaling [72].

The involvement of GPxs in metabolic regulation is a topical focus of interest. Like the peroxiredoxins, GPxs also can interfere with signaling cascades in multiple ways and, intriguingly, in opposing directions [21]. They can simply remove hydroperoxides that are positive regulators of signaling cascades, as is, for example, discussed for impaired NF-kB activation due to over-expression of GPx1 [73] and GPx4 [74], and for blunted insulin response in GPx1-over-expressing mice [47]. Inversely, peroxidases, because of their extreme reactivity with hydroperoxides, are predestined to act as sensors for H_2O_2 or other ROOH. 'Peroxide sensing', in this case, refers to reacting with hydroperoxide. The oxidized peroxidase then can transduce the oxidant signal by oxidizing a downstream component of the signaling cascade [75]. This regulatory principle has been verified for different types of thiol peroxidases [21], the first example being the direct interaction of a GPx with a transcription factor in yeast: H_2O_2 sensing by a Trx-specific 2-Cys-GPx Orp1, and activation of the transcription factor Yap1 via thiol oxidation and subsequent expression of protective genes [76]. Analogous reaction schemes were found with peroxiredoxins in yeast [77], and more recently, in a mammalian system, wherein Prx2 senses peroxide and transduces the signal by oxidizing the transcription factor STAT3 [78]. The sequence of reaction steps is essentially the same for both types of thiol peroxidases, GPxs and peroxiredoxins: oxidation of C_P and, instead of immediate reduction by Trx or GSH, disulfide formation by reaction of sulfenic acid with an SH function of a target protein as alternate substrate followed by disulfide

exchange reactions. A first example of a GPx as a H_2O_2 sensor in higher animals is the interaction of GPx7 with GRP78 as noted above. Oxidized C_P (Cys57) forms an internal disulfide with Cys86, resulting in an intermolecular disulfide of Cys86 with Cys41or Cys420 of GRP78, and disulfide reshuffling creates the activated chaperone with a Cys41-Cys420 disulfide bond [45]. The reaction scheme is believed to sense excess H_2O_2 by GPx7 and, via GRP78 activation, prevent accumulation of misfolded proteins due to unspecific thiol oxidation by H_2O_2 in the ER [45].

A homologous sensing mechanism has not been described thus far for SecGPxs. However, the metamorphosis of GPx4 in the late phase of sperm maturation from an active peroxidase to an enzymatically inactive structural protein makes use of homologous chemistry: in shortage of the preferred substrate GSH, the oxidized enzyme uses protein thiols as alternate substrates. Instead of activating a regulatory protein, the oxidized GPx4 in spermiogenesis ends up in dead-end intermediates with alternate substrates that, although enzymatically inactive, fulfill a vital function [34, 57]. Therefore, in theory, there is no reason why SecGPxs should not be involved in alternate substrate-mediated redox regulation. In fact, the recent discovery of selenenylamide formation in oxidized SecGPxs [27] adds a new perspective, i.e., these alternate intermediates of the catalytic cycle are only formed under pronounced deficiency of reducing substrate and they display an active site architecture distinct from the regular one and, thus, could be suspected to be specifically designed for alternate substrate reactions.

17.4 Concluding Remarks

Research on glutathione peroxidases has experienced many unexpected turns. The discovery of an erythrocyte enzyme, "which protects hemoglobin from oxidative breakdown" [26], did not predict that it marked the beginning of a dramatic development. Whereas early studies were limited to oxidative damage, GPx research appears to have influenced the entire field of selenium biochemistry [1], resulting in a more nuanced comprehension of redox biochemistry comprising the obvious hazards of aerobic life, but equally the pivotal role of peroxides in host defense, redox regulation and cell differentiation [79, 80]. Over the last few decades, GPx family members kept surprising us with new biological roles such as the diabetogenic potential of GPx1, the involvement of GPx4 in spermiogenesis and special types of programmed cell death, cross-talk of GPxs with the Trx system, the contribution of GPx7 and 8 to oxidative protein folding and the sensor function of GPx7. Despite the 16,096 PubMed hits on "glutathione peroxidase" (by January 1, 2016), the still steadily increasing knowledge involving this fascinating class of proteins suggests that the summit of related discoveries in the seemingly old GPx family has yet to be reached.

Our current understanding of the catalytic cycle of an archetypal SecGPx in mammals is summarized in Fig. 17.1.

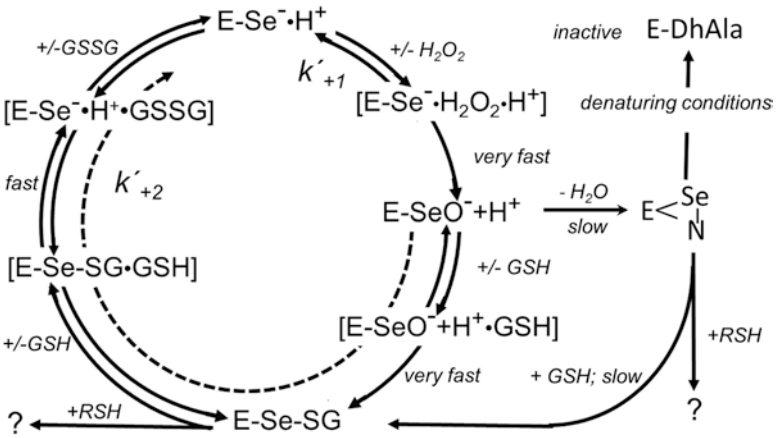

Fig. 17.1 Update of the catalytic cycle of a typical mammalian SecGPx. Reduction of H_2O_2 by GSH is chosen as example (*left* site of the scheme). The apparent rate constants k'_{+1} and k'_{+2} are those that are easily assessed by steady state kinetics. k'_{+1} is the net forward rate constant for the formation of the complex between the ground state enzyme and H_2O_2 ($>10^7$ $M^{-1}s^{-1}$), k'_{+2} is the net forward rate constant for the entire reductive part of the cycle (*dashed line*; $>10^5$ $M^{-1}s^{-1}$). "very fast" means that the partial reaction within a complex (shown in *square brackets*) is faster than the formation of the latter. Alternate substrate reactions with suitable thiols (RSH) are possible starting from each of the catalytic intermediates (*no brackets*). DhAla designates dehydroalanine in irreversibly inactivated enzyme that results from β-cleavage of Sec under denaturing conditions

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Chapter 18
Glutathione Peroxidase 4

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Abstract Glutathione peroxidase 4 (GPx4) is a selenocysteine (Sec)-containing glutathione peroxidase. GPx4 catalyzes the reduction of hydroperoxides and the oxidation of thiols through a ping-pong mechanism in which the redox transitions are faster than the formation of enzyme-substrate complexes; thus, K_m and V_{max} are infinite. The formation of a charge separation in the redox center accounts for this extremely fast reaction. In the absence of reducing substrate, the oxidized selenium is stabilized, forming a bond with a nitrogen atom in the backbone. This reaction, which protects the enzyme from inactivation, is particular of Sec and does not take place when Cys substitutes for Sec. The glutathione (GSH)-dependent reduction of phospholipid hydroperoxides accounts for the vital function of GPx4 and links the peroxidase to a new subroutine of cell death, named ferroptosis. This reaction is also related to protection from cardio-metabolic disorders and promotion of viral spread and infectivity. Finally, GPx4 is also competent for the oxidation of specific protein thiols when GSH is permissively low. This reaction accounts for midpiece stability and chromatin compaction in spermatozoa.

Keywords Ferroptosis • Glutathione peroxidase 4 • Glutathione • Lipid hydroperoxides • Lipid peroxidation • Quantum chemistry • Spermatogenesis • Vitamin E

18.1 Introduction

Glutathione peroxidase 4 (GPx4, E.C. 1.11.1.12) is a selenocysteine (Sec)-containing GPx (Se-GPx). GPx4 was first purified through chromatographic steps following an activity present in cell sap that leads to inhibition of membrane lipid peroxidation [1]. This "peroxidation-inhibiting protein" was named "phospholipid

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hydroperoxide glutathione peroxidase" (PHGPx) to account for its peroxidase activity on hydroperoxy derivatives of phosphatidyl choline [2]. The systematic name GPx4 was introduced later, following the discovery (in addition to the previously described tetrameric GPx1) of the other tetrameric homologs GPx3 [3, 4] and GPx2 [5]. The most remarkable functional difference between GPx4 and GPx1, and seemingly also GPx2 and GPx3, is the failure of the tetrameric enzymes to reduce hydroperoxide (ROOH) groups in complex lipid substrates in membranes [6].

18.2 Gene Structure

The *GPx4* gene produces three distinct mRNAs that differ at the 5′ ends, encoding mitochondrial (mGPx4), cytosolic (cGPx4), and nuclear (nGPx4) proteins [7]. Although the first two mRNAs result from a longer or shorter transcript of the first 1a exon including an upstream or a downstream translational start, respectively, the mRNA encoding nGPx4 results from an alternate promoter allowing transcription of an alternate 1b exon located within the first intron of the gene. As a consequence, mGPx4 and nGPx4 contain an N-terminal extension that is completely cleaved from the mitochondrial protein and only partially in the nuclear protein. Although mature c- and mGPx4 are identical, and thus, indistinguishable, mature nGPx4 has variable N-terminal extensions. These forms coexist in the nuclei of epididymal spermatozoa [8]. Although the cGPx transcript is found in both somatic and germ cells, mGPx4 is expressed at high levels only in male germ cells, and nGPx4 is expressed at relatively low levels in male germ cells and at even lower levels in somatic cells (see also Sect. 18.9)

18.3 Protein Structure

GPx4, as all other GPxs, shares a thioredoxin fold with members of several families of oxidoreductases [9, 10]. The Trx fold has a typical secondary structure pattern given by four β-strands flanked by three α helices, yielding two layers of an α/β/α sandwich in the final structural scaffold. In the GPx fold, there is an additional α-helix and a small β-sheet between β2 and α2.

Multiple sequence alignments and structural comparative modeling analyses of a large number of homologous GPxs revealed that the monomeric pattern is much more diffuse in nature than the tetrameric pattern. The latter is apparently restricted to vertebrata and descends from the insertion of the additional α-helix generating the inter-subunit interface [11].

18.4 Enzymatic Activity

GPx4 lacks specificity toward the ROOH substrate and accepts either small ROOHs, such as H_2O_2, or more complex ones, such as phosphatidylcholine hydroperoxide (PCOOH) or cholesterol and cholesterol ester hydroperoxides [12, 13]. In the

standardized routine assay for measuring GPx4 activity, the substrate is PCOOH dispersed in Triton X-100 micelles [14].

The interfacial character of the GPx4 reaction on membranes has never been analyzed in depth, although our unpublished evidence indicates that the enzyme binds to the membrane surface by electrostatic interactions and that the ROOH group of esterified fatty acids is exposed to water. The possibility of accommodating large ROOH substrates results from the absence of the loop containing the tetrameric interface, which is indeed a late achievement in evolution of the family [11]. It is tempting to speculate that the formation of tetrameric peroxidases provided a specific advantage, separating the control of ROOH content in the membrane or water phases.

With respect to reducing substrate, GPx4 is unusual in that it accepts several structurally unrelated low-molecular-weight thiols and specific protein thiols when the GSH concentration is low. Protein thiol oxidation by GPx4 has been observed as a critical redox event during sperm maturation [15], but could also be relevant for other, still unexamined, condition of redox signaling, when the ROOH flux exceeds the reducing capacity limited by GSH concentration. Notably, a similar protein thiol oxidation capacity controlled by the amount of GSH was also described for the Cys homolog GPx7 in the endoplasmic reticulum, where protein disulfide isomerase competes with GSH as reducing substrate [16].

18.5 Kinetics

Despite the distinct substrate specificity described above, the kinetic mechanisms of GPx1 and GPx4 are identical with their strictly conserved identical redox center [17, 18]. Steady-state kinetic analysis of the reaction fits the model of a ping-pong mechanism, in which the enzyme and substrate interaction and the release of products are much slower than the redox transitions. This prevents the accumulation of enzyme-substrate complexes and leads to infinite V_{max} and K_m values. For further discussion of GPx kinetics, see Chap. 17.

18.6 Catalytic Mechanism

The catalytic cycle of Se-GPx, in comparison to the Cys-containing GPx (S-GPx), was recently studied using a quantum chemistry approach based on density functional theory (DFT) methods [19]. This computational mechanistic investigation explored the potential energy surface of the system and identified the intermediates and transition states of the reaction energy profile. The complete calculated catalytic cycle for Se-GPx is shown in Fig. 18.1. A stable cluster of seven amino acids (Sec46, Gln81, Trp136, Asn137, Phe138, and Gly47, plus a second Gly residue to mimic a peptide bond linked to Gly47) (numbering is for rat GPx4) was selected as a prototype of the stable catalytic pocket.

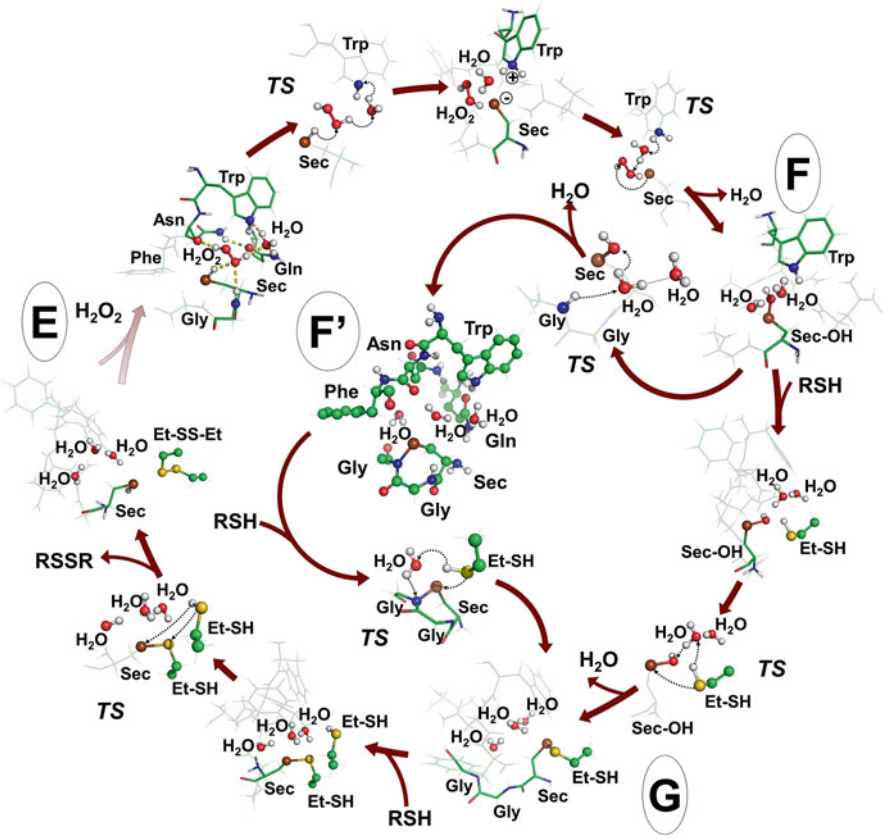

Fig. 18.1 Catalytic cycle of GPx4, focusing on the catalytic center studied using quantum level theory with the transition states (**TS**) of each step. Sec46, Gln81, Trp136, Asn137, Phe138, and Gly47, plus a second Gly to mimic a peptide bond linked to Gly47, are shown (numbering is for rat GPx4). The starting point is the reduced enzyme, **E**, followed by **F**, the enzyme containing Sec oxidized to selenenic acid, and **G**, the selenenylsulfide/disulfide intermediate. In the center of the figure, the formation of the eight-membered ring generated by the interaction of selenenic acid with the amide in the backbone (**F′**) is highlighted. **GSH**, modeled for simplicity as ethanethiol (Et-SH), is the reducing substrate, whereas **GSSG**, modeled for simplicity as Et-S-S-Et, is the corresponding disulfide

The coordination of a hydrogen peroxide molecule (H_2O_2 in Fig. 18.1) is thermodynamically favored and is stabilized by a hydrogen bonding network involving the selenol (or thiol in S-GPx) proton, the peroxide itself, a water molecule, and the indole of the Trp residue. Selenol (or thiol) deprotonation occurs via long-range proton transfer, leading to the formation of Sec^- (or Cys^-) and $TrpH^+$. This charge-separated intermediate is largely destabilized with respect to the reduced enzyme (**E**) and is converted, without appreciable activation energy, to the enzyme form in which Sec is oxidized to selenenic acid (or Cys to sulfenic acid) (**F**). The

reductive part is a two-step process involving two molecules of GSH, modeled for simplicity as ethanethiol (Et-SH), to form the selenenylsulfide (or disulfide) intermediate (**G**), which evolves in the presence of the second molecule of reducing substrate to regenerate **E** and the oxidized form of the reducing substrate (Et-SS-Et). The second reductive step has the highest activation energy and is thus the rate-limiting reaction of the cycle. The complete energy profile of Se-GPx is flatter than that of S-GPx, which influences the turn-over frequency ratio (TOF). TOF is also affected by the endothermicity of the last step, which is much slower when Cys substitutes for Sec (TOF Sec/Cys > 10^2). In Se-GPx, DFT calculations also support the mechanistic path in which the absence of reducing substrate leads to an oxidized intermediate identified by mass spectrometry as 2 amu lighter that **E**. This intermediate, **F′**, is an eight-membered ring generated by the interaction of selenenic acid with the amide in the backbone, one residue downstream of Sec. Thiolysis of the Se-N bond of the selenenylamide in **F′** (when the reducing substrate becomes available again) leads to the formation of **G**, which continues the cycle. The formation of **F′** seemingly protects Se-GPx from inactivation by over-oxidation and/or beta-cleavage leading to dehydroalanine (Dha). Notably, the formation of Dha from **F′** can only take place when the structure of the enzyme is disrupted, supporting the conclusion that the structure of the active site contributes to the stability of the oxidized form.

Evidence demonstrating that dimedone derivatizes selenium only when the reagent is present during the trypsinization of **F′** is fully consistent with and confirms the above mechanism (Zaccarin, M., unpublished). The selenenylamide bond is hydrolyzed only when the structure is disrupted, leading to the release of selenenic acid to react with dimedone. As opposed to Se-GPx, in the absence of reducing substrate, DFT calculations support a further oxidation of the sulfenic acid derivative for S-GPx, first to sulfinic and then to sulfonic acid, indicating that a S-GPx in the absence of a resolving Cys lacks protective mechanisms preventing oxidative inactivation.

18.7 Functions

GPx4 is needed for embryo development, cell survival, inflammation control, and male fertility. Recent evidence suggests roles in metabolism and viral infections, as well.

18.8 Genetic Diseases and Polymorphisms

In mice, ablation of *GPx4* leads to embryonic lethality, with hallmarks of programmed cell death at the gastrula level [20]. In humans, three *GPX4* mutations leading to truncation of GPx4 have been reported in a rare form of spondylometaphyseal dysplasia [21], a lethal neonatal disease characterized by severe

metaphyseal chondrodysplasia with mild limb shortening, platyspondyly, cardiac conduction defects, and severe central nervous system abnormalities. Interestingly, all three mutations, which are predicted to cause premature termination of GPx4, affect the enzyme downstream of the catalytic Sec.

A T/C variation at nucleotide 718 in the *GPX4* gene that corresponds to the 3′-UTR of the mRNA in Asian and Caucasian populations is apparently functional. In a cohort of patients with either adenomatous polyps or colorectal adenocarcinomas, a higher proportion of individuals with the CC genotype was observed in the cancer group [22]. Furthermore, this polymorphism was described as a predictor of stroke in patients with hypertension [23].

18.9 Male Fertility

GPx4 is necessary for survival of the immature male germ cell, stabilization of the spermatozoa midpiece, and chromatin condensation. Each of these functions is mediated by one of the *GPx4* transcripts, which target their products to discrete compartments, namely, the cytosol (cGPx4), mitochondria (mGPx4), or nucleus (nGPx4) (see also Sect. 18.2). Comparison of the phenotypes observed in genetic studies conclusively demonstrated that: i) cGPx4 accounts for the general vital function of GPx4 in cells, including immature male germ cells [24, 25]; ii) mGPx4, although not vital for cells, plays a major role in male fertility, generating the network of oxidized, cross-linked proteins that stabilize the midpiece of spermatozoa [24, 26]; and iii) nGPx4 is neither vital for cells nor critical for male fertility, but contributes to compaction of nuclear chromatin in maturing spermatozoa by oxidizing protamine thiols [27].

The vital function of cGPx4 is accounted for by the inhibition of lipid peroxidation when lipid hydroperoxides (LOOHs) are reduced and GSH is oxidized (see Sect. 18.11). The function of m- and nGPx4 in maturing spermatozoa involves the oxidation of protein substrates rather than GSH. In the final phase of male sperm maturation, mGPx4 indeed does oxidize and polymerize specific proteins of the mitochondrial capsule (i.e., the keratin-like amorphous sheath that surrounds the elongated mitochondria in the midpiece of mature spermatozoa), whereas nGPx oxidizes proteins of nuclear chromatin. This functional shift is seemingly triggered by massive GSH depletion, which occurs during sperm maturation [28]. This implies a physiologic role for ROOHs, albeit of a still unknown nature and source, in GPx4-catalyzed oxidation of protein substrates. As a result of the oxidative polymerization of proteins, mGPx4 eventually becomes entrapped in the oxidized network of the capsule, where it functions as a catalytically inactive structural component [15, 29].

Because the fine structures of both the mature mitochondrial capsule and the chromatin of mature spermatozoa have yet to be resolved, and whether a catalyzed reshuffling of mixed disulfides or selenodisulfides is also required remains unknown. In this respect, it has been suggested that the selenoenzyme, thioredoxin glutathione

reductase, which is the product of the *TXNRD3* gene, plays a role in protein disulfide proofreading [30].

18.10 Inflammation

GPx4 dampens inflammation by inhibiting lipoxygenase (LOX) and cyclooxygenase (COX) activity [31–34]. A comparative study showed that the activities of platelet 12-LOX, 15-LOX, and COX-2 are the most sensitive to GPx4, whereas that of 5-LOX is the least sensitive [33]. Inhibition is seemingly due to removal of LOOH required for the activation of the LOX and COX enzymes [35, 36].

A conditional knockout (KO) model of *GPx4* in neurons, in which mice develop astrocyte inflammation associated with reactive astrogliosis and neurodegeneration [37], confirmed the anti-inflammatory function of GPx4 in vivo. Moreover, transformed cells bearing an increased 12/15-LOX activity resulting from *GPx4* ablation, which surprisingly survive once implanted into mice yield, increased tumor angiogenesis [38]. This suggests that inhibition of eicosanoid production by GPx4 plays a role in biology of cancer progression.

Recent evidence indicates that the control of inflammation linked to the regulation of eicosanoid biosynthesis is a routine function of GPx4 and that regulation of GPx4 expression is part of the complex host-pathogen interaction. It was recently shown that *Salmonella typhimurium* modulates the activity of the host antioxidant machinery by dampening GPx4 expression in intestinal epithelial cells [39]. This occurs via a bacterial Type III secreted effector, SipA, and primes enterocytes to apically produce the pro-inflammatory 12-LOX product hepoxilin A_3, which governs the trans-epithelial migration of polymorphonuclear leucocytes associated with enteric infection. Suppression of GPx4 expression is discussed as a central mechanism governing the ability of *S. typhimurium* to evoke enteritis. Whether this effect is shared with GPx1, the expression of which is also decreased by *Salmonella*, requires further investigation.

18.11 Cell Death and Survival

Consistent with its vital function, GPx4 is also the essential component of a novel subroutine of cell death, designated ferroptosis by Dixon et al. [40, 41]. As it was recently reviewed [42] and is discussed in Chap. 43, ferroptosis will not be addressed in detail here. It is briefly noted that membrane peroxidation is the critical event leading to cell death by ferroptosis and that the series of biochemical events recapitulates the mechanisms of initiation and inhibition of lipid peroxidation, as widely studied in vitro beginning in the 1970s. Along with polyunsaturated lipids and oxygen, lipid peroxidation requires a reduced iron complex and yields, besides LOOH, reactive aldehydes, including malondialdehyde, 4-hydroxynonenal (HNE), and

truncated fatty acid chains. GPx4 and GSH, by reducing LOOH, prevent lipid peroxidation, indicating that initiation of the peroxidative chain reaction requires sparking amounts of preformed LOOH. Notably, the reduction of LOOH also inactivates LOX, because LOOH, seemingly by oxidizing catalytic iron, maintains these enzymes in an active state. Vitamin E (VE) slows down the rate of membrane lipid peroxidation by intercepting the peroxidation-driving lipid hydroperoxyl radicals (LOO•). In this case, the product of the antioxidant reaction of VE is LOOH, and thus, the activity of GPx4 is essential for the antioxidant effect of VE.

Given the pivotal role of traces of LOOH for the initiation of enzymatic and non-enzymatic lipid peroxidation, a relevant question emerges about the mechanism of their formation. A single mechanism has never been unambiguously defined. The most likely candidates involve hydroxyl radical, singlet oxygen, or iron-oxygen complexes. The practical outcome is that aerobic life continuously produces, by different mechanisms, traces of LOOH, from which enzymatic or non-enzymatic lipid peroxidation may be initiated by ferrous iron complexes or LOX. For this reason, we may conclude that the critical event initiating the lipid peroxidation chain reaction is a missed inhibition rather than an increased initiation rate.

Until its roles in specific aspects of cell biology and experimental pathology were brought to light by reverse genetic studies involving *Gpx4* KO, lipid peroxidation was primarily appreciated from a toxicological perspective. Cell death due to membrane lipid peroxidation primed by loss of GPx4 activity (i.e., ferroptosis) is seen today as the common mechanism of several degenerative diseases, including neurodegeneration [37, 43, 44], kidney failure [45], altered hematopoiesis [46], and possibly, ischemia-reperfusion injury [47]. Notably, another example of what today is known as ferroptosis was described in the early 1980s when a study involving single-photon counting in a perfused rat heart model showed that GSH depletion leads to severe contractile failure associated with lipid peroxidation [48].

The critical role of interplay between VE and GPx4 was demonstrated by evidence showing that VE slows down, but does not prevent, iron-induced lipid peroxidation in vitro in the absence of GPx4 and GSH [49]. In contrast, in cellular models, VE was shown to rescue death by ferroptosis [37], at least in the time frame of the experimental conditions. This possible discrepancy requires further consideration about the actual mechanism of VE in vivo. It is indeed worth investigating the hypothesis that VE prevents the formation of the first traces of LOOH by reducing the hydroperoxyl radical (HOO•) produced by protonation of superoxide anion, which may, as the species producing the traces of LOOH, be required for initiation. Notably, inhibition of a specific LOX could also explain the prevention of ferroptosis by VE. In summary, although VE and possibly other free-radical scavengers prevent or delay cell death by ferroptosis under some experimental conditions, the crucial anti-peroxidant mechanism is the reduction of LOOH, which relies exclusively on GPx4 and its substrate, GSH.

Ferroptosis is an appealing target for therapeutic strategies aimed at preventing cancer development and progression or halting degenerative diseases. Indeed, some cancer cells are particularly sensitive to GSH depletion brought about by cystine deprivation [50]. These cells are specifically sensitive to erastin, a compound identi-

fied by a high-throughput screening of small molecules as an inhibitor of the cystine/glutamate antiporter, x_c^-. In addition, an intracellular inhibitor of GPx4, (1S, 3R)-RSL3 (from oncogenic-RAS-synthetic lethality), triggers ferroptosis independent of the intracellular GSH concentration (see also Chap. 43). On the other hand, in a study involving kidney and an ischemia/reperfusion-induced tissue injury model, the spiroquinoxalinamine derivative liproxtatin-1 was shown to rescue cell death by GPx4 depletion by preventing lipid peroxidation; thus, liproxtatin-1 could be useful in preventing GPx4-mediated cellular degeneration [45]. Other compounds interfering with ferroptosis are currently being examined [42].

18.12 Metabolic Diseases

Although the role of GPx4 in ferroptotic cell death was revealed by complete *Gpx4* KO, a haplo-insufficiency transgenic model suggested a protective role in cardio-metabolic disorders related to obesity [51]. Also this effect of GPx4 is related to the inhibition of lipid peroxidation. When fed a high-fat, high-sucrose (HFHS) diet, $Gpx4^{+/-}$ mice exhibit much higher amounts of HNE adducts and protein carbonylation in the liver and heart compared with controls. Moreover, severe glucose intolerance, dyslipidemia, liver steatosis, and cardiac hypertrophy and fibrosis were observed [51].

The formation of reactive aldehydes, seemingly from decomposition of LOOH, was indicated as the primary agent responsible for cardio-metabolic derangements related to obesity. The observation that $Gpx1^{-/-}$ mice are protected from insulin resistance and liver steatosis, despite exhibiting increased systemic oxidative stress associated with diet-induced obesity [52], highlights the relevance of the peroxidase activity of GPx4 in preventing metabolic disorders. Moreover, as a HFHS diet induces transcriptional activation of *Gpx4* in $Gpx4^{+/-}$ mice, the presence and activation of a compensatory response mechanism can be conceived. This mechanism would account for the maintenance of oxidative/anti-oxidative homeostasis until the efficiency of GPx4 declines, leading to a pathologic alteration of the redox steady state [53].

The apparently paradoxical observation that haplo-insufficient $Gpx4^{+/-}$ mice live longer than wild type littermates [54], which is ascribed to an increased 'apoptosis', is consistent with evidence that GPx4 plays a role in controlling intracellular reactive aldehyde production. Seemingly, in $Gpx4^{+/-}$ mice, increased amounts of aldehydes derived from lipid peroxidation could play a protective hormetic function as nuclear factor-E2–related factor 2 activators [55].

18.13 Viral Diseases

A new avenue for GPx4 and VE biology research was uncovered recently in virology. The genomes of positive-stranded RNA viruses such as hepatitis C virus (HCV) are synthesized by a multiprotein replicase complex that assembles in association

with host intracellular membranes known as 'the membranous web' [56, 57]. The integrity of these membranes is vital for viral replication and infectivity, and therefore, endogenous lipid peroxidation is detrimental to viral replication [58]. Most HCV strains replicate poorly in cell culture. However, strain JFH1, a genotype 2a virus recovered from a patient with fulminant hepatitis, was shown to replicate efficiently in cell culture, thus attracting the attention of investigators. In Huh-7 hepatoma cells, JFH1 infectivity and replication are not restricted by oxidative damage or increased by VE [58]. This suggests that the replication and infectivity of strain JFH1 are not affected by lipid peroxidation and supports the hypothesis that the virus manipulates the host antioxidant capacity, inducing resistance. Consistent with these observations, it was recently shown that an adapted JFH1 strain induces both *GPX1* and *GPX4* transcription, although only GPx4 is required for HCV infection. Interestingly, *GPX4* mRNA is indeed elevated in biopsies from chronic hepatitis C patients and returns to baseline upon virus eradication [59]. Nevertheless, it was recently disclosed that in cell culture, replication of pan-genotypic HCV requires the product of the *SEC14L2* gene, tocopherol-associated protein 1, which enhances VE accumulation in cells [60]. In summary, emerging data indicate that VE and GPx4 are essential host components for HCV replication and spread. Whether this is the case for other positive-stranded RNA viruses and whether GPx4 and/or the enzymes of the GSH biosynthetic pathway and/or *SEC14L2* are suitable targets for antiviral therapies are stimulating hypotheses worthy of further investigation.

18.14 Conclusions, Unresolved Questions, and Perspectives

In the more than three decades since the "peroxidation inhibiting protein" was identified, several aspects of the intriguing enzymology of peroxidation have been elucidated, and major insights into the multifaceted physiology of GPx4 have been revealed. GPx4 is an oxidoreductase that reduces LOOHs in membranes at a very fast rate by taking advantage of the unusual chemistry of selenium. The catalyst is regenerated when the oxidized redox center is reduced back by GSH in a reaction in which the unusual reactivity of selenium again maximizes the rate. If GSH is limiting, the oxidizing capability of GPx4 can be directed toward alternative substrates. To date, this remarkable activity has been identified only in the last phase of sperm maturation.

The evolutionary acquisition of Sec in the redox center of GPx provided an unambiguous advantage by lowering the activation energy of each step of the catalytic cycle, leading to a much higher turnover frequency of the catalytic cycle for Sec- versus Cys-supported catalysis. However, although relevant, this is not the unique advantage of selenium versus sulfur. When the reducing substrate is limiting, the sulfenic acid derivative of Cys undergoes further oxidation, first to a sulfinic acid and then to a sulfonic acid derivative. Instead, selenenic acid produced in the catalytic cycle, if not promptly reduced by GSH, rather than undergoing further oxidation, binds to an amide group of the protein backbone, forming a selenenylamide. This species is stable in the active site until the cycle is reactivated by

GSH. An irreversible inactivation by beta-cleavage, which transforms Sec into Dha, may also take place with Sec linked to the amide, but this appears to occur only when the native conformation is lost. Determining whether this inactivation has a pathologic or physiologic significance will be a relevant issue for future studies.

Two aspects of the biochemistry of GPx4 suggests a physiologic scenario: i) inhibition of enzymatic and non-enzymatic lipid peroxidation, which protects cells from death by ferroptosis; and ii) the oxidation of some specific protein thiols, which imparts fertilizing capability to mammalian spermatozoa. Both functions have been independently verified in animal models. Determining whether these functions of GPx4 converge in a coherent physiologic context will be challenging. The most plausible sparking event is the decrease in cellular GSH, at least in the area surrounding GPx4. The overall scenario is that of a redox signal transduction pathway, specifically starting at the surface of membranes—certainly a challenging area for further studies on the "peroxidation inhibiting protein".

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Chapter 19
The 15 kDa Selenoprotein: Insights into Its Regulation and Function

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Abstract The 15 kDa selenoprotein (SEP15) is one of more than 25 selenoproteins found in humans. This protein has been proposed to be involved in redox regulation in the endoplasmic reticulum (ER), although the biological function of SEP15 is still not completely understood. It appears to have strong tissue specificity, and has been shown to have a split personality in terms of cancer initiation and promotion. Polymorphisms in the human *SEP15* gene have been linked to increases in several types of cancer. Thus, among the many selenoproteins, SEP15 continues to generate interest due to its potential implications in human health and disease.

Keywords 15 kDa selenoprotein • Oxidoreductase • Polymorphism • Protein folding • SELM homolog • SEP15 • UDP-glucose:glycoprotein glucosyltransferase

19.1 Introduction

SEP15 belongs to the family of oxidoreductases and has been shown to be involved in quality-control of folding of proteins [1]. It is an endoplasmic reticulum (ER)-resident selenoprotein and a homolog of selenoprotein M (SELM), which is also a selenoprotein with redox activity, thought to be involved in the antioxidant response [2]. *SEP15* was first identified and characterized in human tissues by Gladyshev et al. in 1998 [3]. The *SEP15* gene is located on human chromosome 1p31, a location often deleted or mutated in many cancers. The expression of SEP15, like many other selenoproteins, is regulated by the selenium status of the organism [2]. While the expression of essential, housekeeping selenoproteins,

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such as thioredoxin reductase 1 (TXNRD1), is maintained even at very low levels of organismal selenium, the expression of inducible selenoproteins, such as SEP15, are reduced under conditions of low systemic selenium [4].

19.2 SEP15 Structure and Function

The SEP15 protein consists of 162 amino acids, and the Sec UGA codon (see Fig. 19.1a) is found in exon 3 at position 93 [5]. The SECIS element, a selenocysteine insertion sequence of around 60 nucleotides in length that adopts a stem-loop structure, is present in the 3′-untranslated region (UTR) of its mRNA. There appear to be two alternative transcripts for *SEP15*. The longer transcript variant expresses five exons, whereas the second variant lacks an exon in the 3′ coding region [5]. Human SEP15 was originally isolated and characterized using a human T-cell line and mammalian *Sep15* mRNA was found to be expressed in a wide range of tissues [3] with highest expression levels reported to occur in liver, kidney, testes, thyroid and prostate of human, rat and mouse. *Sep15* appears to be highly conserved in nature [6, 7], and has also been detected in various unicellular eukaryotes [8].

Similar to many other selenoproteins, SEP15 belongs to the class of thiol- oxidoreductases [5, 9]. It contains a thioredoxin-like fold [10], with selenocysteine located in the predicted catalytic position, and also contains an ER-targeting signaling

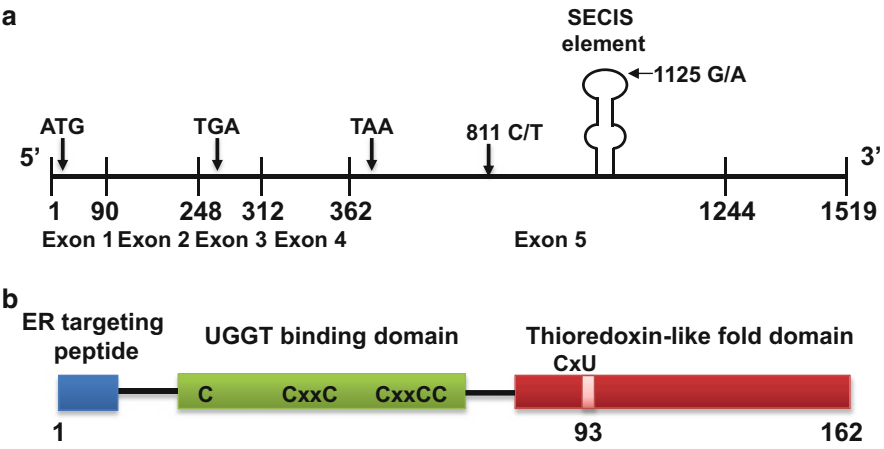

Fig. 19.1 Structure and domain organization of the human 15 kDa selenoprotein (*SEP15*). (**a**) The relative positions of the ATG initiation site, the TGA Sec codon, the TAA termination signal, and the detected polymorphisms (C/T at position 811, and G/A at position 1125 in the SECIS element) are shown. Alternative 3′-end sequences (position 1244 or 1519) are also indicated. (**b**) Protein domains (ER targeting, UGGT binding and thioredoxin-like fold) and location of the Sec codon (position 93) within *SEP15* are shown. The N-terminal domain contains six conserved cysteine residues, four of which form a pair of CxxC motifs important for mediating interaction with UGGT

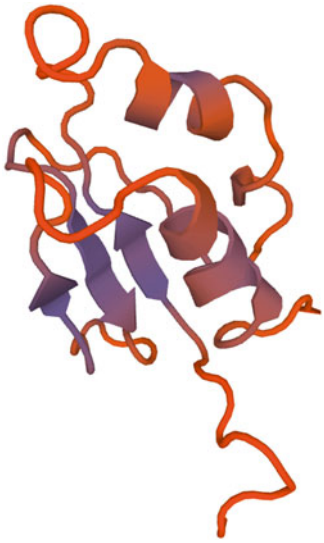

Fig. 19.2 Predicted NMR structure of human SEP15 with α-helices shown in *darker bands*, β-sheets in *purple* (modeled using SWISS-MODEL [47])

peptide in the N-terminal region (Fig. 19.1b) [11]. The structure of its thioredoxin-like domain, as shown by NMR spectroscopy of *Drosophila* SEP15, demonstrated that SEP15 consists of four-stranded β-sheet surrounded by three α-helices, comprising an α/β-fold common for thioredoxin-like oxidoreductases [2]. Human SEP15 is predicted to have a similar structure (Fig. 19.2). Although the thioredoxin domain in SEP15 (CxU) differs from that in thioredoxin and other functionally characterized oxidoreductases, the additional redox-active motifs in a loop between strand β1 and helix α1 further support the suggested oxidoreductase function of SEP15 [2, 12]. The redox potential and thus functional specificity of a protein is determined by the identity of residues in its redox motifs. The redox potential in *Drosophila* SEP15 was found to be −225 mV [9], which lies between known redox potentials of protein disulfide isomerase and the disulfide reductase thioredoxin. Therefore, SEP15 is thought to likely catalyze the reduction or isomerization of disulfide bonds in ER-localized or secretory proteins [9, 12].

Through its Cys-rich domain, SEP15 can form a strong 1:1 complex with UDP-glucose:glycoprotein glucosyltransferase (UGGT), a 150 kDa large ER chaperone enzyme that regulates the calnexin cycle [9], which is responsible for quality control in the ER [13]. Through calnexin, UGGT recognizes misfolded protein domains in the ER of eukaryotic cells and specifically glucosylates these proteins, allowing for another cycle of proper folding [9, 11, 14]. However, unlike its distant homolog, selenoprotein M (SELM), SEP15 lacks an ER retention signal, suggesting that its binding to UGGT is the reason it is retained in the ER [11]. The presence of a thioredoxin domain and its binding to UGGT suggest that SEP15 may contribute to UGGT's function via redox processes and assists in controlling folding or secretion of certain glycoproteins [2]. Thus far, SEP15 has only been found bound to UGGT [11], and the activity of both isoforms of UGGT was shown to be enhanced by the

complex with SEP15 [15, 16]. This suggests that SEP15 may serve as a functional and possibly structural extension of UGGT [17, 18]. Thus, UGGT, together with SEP15, may regulate the calnexin system, as UGGT senses the folding states of glycoproteins [18]. However, SEP15's function in ER quality control has been difficult to validate experimentally.

19.3 Biological Function of *SEP15*

Biological evidence for SEP15's involvement in the regulation of protein folding is observed with the reported prominent nuclear cataract development in eyes of *Sep15* knockout mice early in their life [1], presumably because of accumulation of misfolded proteins in the lens. Additional support for its possible role in protein folding in the lens is also supported by SEP15's co-localized expression with the ER chaperone calnexin and the ER-resident oxidoreductase ERp57, which are both major components of the quality control mechanism in neuronal cells within the ER [14].

Higher versus lower *SEP15* expression in human populations has been linked to altered cancer risks, such as the observation of a decrease of *SEP15* expression in lung cancer patients [19]. Low expression of *SEP15* also has been observed in malignant lung, breast, prostate and liver tissues [20], as well as in cell lines derived from malignant mesothelioma cells compared to normal lung cells [21]. In contrast, a recent global analysis of the hepatic selenoprotein expression showed *SEP15* mRNA to be upregulated in human liver cancer HepG2 and Huh7 cells compared to normal human hepatocytes [22]. Whereas earlier reports in lung, breast, prostate and also liver suggest a role of SEP15 in tumor suppression, these recent observations in liver cancer cell lines and our mouse in vivo studies suggest a role in colon tumor promotion [23].

Interestingly, increased SEP15 expression has been found in response to mild ER stressors such as the antibiotic drugs, tunicamycin and brefeldin A. Additionally, rapid proteasomal degradation of SEP15 has been reported in response to agents inducing a more robust ER stress, such as dithiothreitol [14]. However, decreased levels of SEP15 did not induce ER stress in these studies, indicating that there is a possible compensation mechanism for SEP15 function. Recent studies with a *Sep15* knockout mouse model further implicated its role in redox homeostasis [1]. Although *Sep15* knockout mice appeared normal and did not activate ER stress pathways systemically, parameters of oxidative stress were increased in livers of *Sep15* knockout mice [1]. In contrast, in a murine model of chemically-induced colon carcinogenesis, systemic knockout of *Sep15* decreased development of preneoplastic lesions [24], similar to what has been described in glutathione peroxidase 2 (*Gpx2*) knockout mice [25]. Therefore, even though much of its biological function remains to be elucidated and appears to display aspects of tissue specificity, the importance of SEP15 in health and disease seems supported.

In addition to the development of systemic *Sep15* knockout mice, targeted down-regulation of *Sep15* through RNA interference in mammalian cells in culture has been used to study the biological effects associated with decreased expression of this gene. Based on previous studies, much like TXNRD1, SEP15 may also harbor a split personality in that it has been linked to cancer-preventive [5, 19, 26] and cancer-promoting roles [23, 27], which is discussed in further detail in Chap. 37.

Mouse [27], as well as human colon cancer cells [28], containing shRNA constructs targeting *Sep15* displayed a reversal of the cancer phenotype, including decreased growth abilities compared to control cells. These observations in colon epithelia are in contrast to other published literature, where lowered expression of *SEP15* was reported in many malignant mesothelioma tumor specimens, and increased cell proliferation of mesothelioma cells in vitro [21]. Furthermore, a targeted down-regulation of *Sep15* in mouse Lewis lung carcinoma cells did not affect cell growth [27]. Combined, these results indicated a strong tissue specificity in the response of malignant cells to SEP15 expression.

In an attempt to determine the effect of diminished SEP15 expression in lens epithelium, a study was undertaken in human lens epithelial cells. Targeted down-regulation of *Sep15* alone in these cells did not result in apoptosis, yet it was found that tunicamycin-induced apoptosis and oxidative stress was enhanced in SEP15-deficient cells, while ER stress was not further elevated [29].

Inducible *Sep15* knockdown cell lines were used to examine the role of Sep15 in HeLa/Chang liver cells [30]. Loss of SEP15 was shown to lead to non-apoptotic membrane blebbing and reorganization of cytoskeletal proteins through a RhoA/ROCK/MLCK pathway. *Sep15* knockdown cells were arrested at the G1 phase and the cells also underwent a mild ER stress response. Inhibitors of the RhoA/ROCK pathway in cells with targeted downregulation of *Sep15* recovered the cells' migration and invasive ability, suggesting that SEP15 is involved in cell motility through the organization of cytoskeletal proteins [31].

Interestingly, further investigations of molecular targets affected by the loss of SEP15 in colon epithelium demonstrated a possible link to expression of interferon-γ-regulated inflammatory pathway genes and transcription factors [27, 32]. This finding was further supported by the observation in mice that their serum cytokine levels were affected by SEP15 expression [24]. Possible direct or indirect links between SEP15 expression and pro-inflammatory response continue to be elucidated. Cells regulate function and specific biological activity of cytokines, such as interferons and interleukins, through glycosylation, which may alter their biological activity and overall behavior [33, 34]. Proper glycosylation of cytokines is therefore important for their biological function, and it is possible that SEP15 indirectly affects inflammatory pathways through regulation of glycoproteins, including cytokines.

The interplay or compensatory mechanisms between SEP15 and other selenoproteins, such as TXNRD1, in cancer is less clear. Mouse colon adenocarcinoma CT26 cells have been used to elucidate the roles of both selenoenzymes through targeted downregulation of either *Sep15* or *Txnrd1*, or both [35]. Several of the typical

cancer properties such as cell proliferation and anchorage-independent growth were reversed in these colon cancer cells lacking either SEP15 or TXNRD1, as expected. However, instead of displaying an even stronger reversal of the cancer phenotype as anticipated based on observations with single-knockdowns, the combined down-regulation of SEP15 and TXNRD1 resulted in a recovery of the original malignant phenotype. Interestingly, components of the Wnt/β-catenin signaling pathway, which is frequently dysregulated in non-familial colon cancer, were up-regulated in cells lacking both SEP15 and TXNRD1, suggesting that these two selenoproteins participate in complex, and possibly interfering, specific regulatory pathways in colon cancer [35, 36].

19.4 Human *SEP15* Polymorphisms and Cancer

As with other selenoproteins, single nucleotide polymorphisms (SNP) have been described for *SEP15* (see Chaps. 13 and 29). Among several common SNPs for the *SEP15* gene, two have been reported to have functional consequences [7, 21]. These two SNPs are located in the 3′-untranslated region in the *SEP15* gene (rs5859 and rs5845), and result in C-T substitution at position 811 and a G-A substitution at position 1125, respectively (Fig. 19.1b). Via measurement of reporter gene activity, these SNPs were found to decrease the efficiency of the SECIS element at higher concentrations of selenium [7]. Interestingly, the haplotype with a T at position 811 and an A at 1125 is relatively rare, only occurring in 7 % of Caucasian-Americans, but in about 31 % of African-Americans [7]. The impact of these polymorphisms on the biological activity of SEP15 continue to be elucidated.

A number of epidemiological studies have suggested a relationship between *SEP15* SNPs and cancer risk or mortality, although the evidence remains controversial in many cases. Two previous studies have suggested a possible role of SEP15 in breast cancer. Using DNA obtained from breast tumors as well as from lymphocytes from cancer-free controls, a loss of heterozygosity and a statistically significant difference in allelic distribution for *SEP15* rs5859 in African-American women was reported [7]. A subsequent study supported a possible role of *SEP15* in breast cancer development among African-American women, and demonstrated a significant reduction of heterozygosity for the locus that was most tightly linked to *SEP15* [37]. Recently, the 811C/T (rs5845) *SEP15* polymorphism was further investigated in a Caucasian population of 83 non-familial breast cancer cases and 99 age-matched, healthy controls. The prevalence of the T allele in this Caucasian study population was relatively low, and the genotype variation in breast cancer patients and controls within the 3′-UTR of the *SEP15* gene showed no significant association with breast cancer risk or pathological parameters [38]. Thus, the supporting evidence of a possible role of SEP15 polymorphisms in breast cancer continues to be restricted to women of African-American descent.

For small-cell or non-small-cell lung cancer, an increased risk was found in a Polish population in those individuals with an AA genotype at position 1125 in *SEP15* and low basal selenium status. However, a decreased risk was observed among those with serum selenium levels above 80 ng/mL [19], indicating that those with the AA genotype benefitted from higher serum selenium concentrations. Intriguingly, in those with the GG or GA genotype, a higher selenium status suggested an increase in risk of lung cancer.

The effect of polymorphisms in *SEP15* on prostate and colorectal cancer appears less consistent. In a study conducted in New Zealand, the rs5845 minor T allele was associated with a higher risk for benign prostate disease compared to controls, but a lower risk of developing malignant disease compared to benign disease [39]. In contrast, in the Physician's Health Study, a nested case control study involving 1286 cases and 1267 controls, SNPs in *SEP15* (rs5859, rs479341, rs561104, rs527281, rs1407131) were not significantly associated with risk for prostate cancer [40]. However, prostate cancer-specific mortality was significantly associated with a recessive model of three *SEP15* variants (rs479341, rs1407131, rs561104), and variant rs561104 significantly modified the association of plasma selenium with prostate cancer survival [40]. Similarly, although *SEP15* polymorphism rs561104 in a population-based case-control study of men of European ancestry was initially significantly associated with local stage prostate cancer with an odds ratio of 1.28 for GG versus AA, this association did not remain significant after adjusting for multiple comparisons due to a relatively low sample size [41]. A recent study investigated the impact of genetic variants of *SEP15* and other selenoproteins in a cohort of 722 patients with localized or locally advanced prostate cancer. However, unlike SNPs in *TXNRD2* and selenium binding protein 1 (*SELENBP1*), genetic variants of *SEP15* were not associated with cancer aggressiveness at diagnosis or with plasma selenium levels [42].

No association was found between *SEP15* polymorphisms and colorectal cancer incidence in a case control study in the Czech Republic [43] or in a Korean patient population [20, 44]. However, a significant two-loci interaction between selenoprotein P and *SEP15* variant rs5859 was observed in colon cancer cases from the Czech Republic [43]. Additionally, a gender-specific increased rectal cancer risk in Korean men was associated with the minor alleles for rs5845 (GG-GA), rs5859 (CC-CT) and rs34713741 [20, 44]. Moreover, recent studies in the US also failed to find an association between these SNPs in *SEP15* and colorectal cancer [45]; however, there appeared to be an association between a different *SEP15* polymorphism (rs9433110) and survival after diagnosis with colon and rectal cancer [46].

It appears that the literature regarding the relationship between *SEP15* polymorphisms and cancer remains rather controversial. Gene (mRNA) expression analyses suggest that *SEP15* expression as a function of selenium availability varies among various polymorphic populations, at least in part, due to human polymorphisms in the *SEP15* gene. How these SNPs correlate with SEP15's biological activity, and how SEP15 protein expression and activity may influence cancer risk or mortality in various subpopulations defined by their ethnic/cultural background or otherwise, remains to be validated.

19.5 Concluding Remarks

SEP15 is an ER-resident selenoprotein thought to be involved in quality control of protein folding. Although its pairing with UGGT and the observations of altered inflammatory responses in mice suggests regulatory control over important glycoproteins such as cytokines, its specific biological function remains to be elucidated. A strong tissue specificity and split personality in terms of cancer initiation and promotion has been described for *SEP15*. Polymorphisms in the 3′-UTR lead to differential expression of *SEP15* among populations, and studies suggest a possible relationship to cancer risk. Further research is needed to evaluate the importance of this gene/protein in human health and disease.

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Chapter 20
Selenoprotein K and Protein Palmitoylation in Regulating Immune Cell Functions

Peter R. Hoffmann

Abstract While it has been recognized that dietary selenium (Se) levels can affect immune responses by modulating immune cell functions, mechanisms by which this occurs have not been fully understood. Our early work identified selenoprotein K (SELK) as a selenoprotein that is enriched in immune cells and its levels are influenced by dietary Se intake. Recently, experiments with mice and cell lines revealed that SELK functions as a partner for an enzyme, DHHC6 (where each letter represents an amino acid in the catalytic domain), to carry out the post-translational addition of palmitic acid to certain proteins. This palmitoylation reaction is an important post-translational modification that stabilizes the expression of proteins like the inositol-1,4,5-triphosphate receptor (IP3R), CD36, and likely others. This represents an important molecular mechanism by which dietary Se regulates immunity.

Keywords Calcium • Coenzyme • Immune cell • Immunity • Palmitoyl acyltransferase • Palmitoylation • Selenoprotein K

20.1 Introduction

Experiments in animals together with clinical studies have supported an important role for sufficient dietary selenium (Se) in maintaining robust immunity against infections [1–3], effective responses to vaccines [4, 5], and reducing pathology from chronic inflammatory disorders [6–8]. However, the mechanisms by which Se intake regulates immunity are still not well understood, in part due to the wide variety of cellular processes affected by this micronutrient [9–11]. The biological effects of dietary Se are exerted mainly through its incorporation into selenoproteins as the amino acid, selenocysteine (Sec). We have found that the endoplasmic reticulum (ER) transmembrane protein, selenoprotein K (SELK), is highly expressed in immune cells and its expression levels are sensitive to dietary Se in mice and Se

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levels in cell culture media [12]. Other studies have described roles for SELK, along with selenoprotein S (SELS), as binding partners for Derlin proteins during ER stress [13, 14]. A different study has shown redox potential for SELK and this may also reflect a biological role for this ER membrane protein [15]. Understanding SELK's function in immune cells is important for gaining insight into mechanisms by which Se influences immunity and may lead to a more selective therapeutic target for augmenting immune cell function with fewer side-effects compared to Se supplementation.

Several years ago, our laboratory set out with the goal to produce mechanistic insights into the role of SELK in regulating immune cell function and influencing immune responses. As described above, some studies in cell lines had demonstrated a role for SELK and another ER transmembrane protein, SELS, in ER associated degradation of misfolded proteins and regulating ER stress [13, 16]. In fact, publications have presented strong evidence that SELK is a member of protein complexes that facilitate the chaperoning of proteins from the ER to degradation machinery under certain conditions of ER stress. We initially investigated signs of in vivo ER stress in mice lacking SELK (i.e., *Selk*$^{-/-}$ mice) and immune cells from these mice when unstimulated or stimulated with inflammatory stimuli. We were not able to detect ER stress resulting from the lack of SELK [12]. This may be due to overlapping or redundant roles between SELK and SELS [13, 17]. Interestingly, there are data suggesting that cytosolic valosin-containing protein (p97(VCP)) is translocated to the ER membrane by binding to SELS in the ER membrane during endoplasmic reticulum-associated degradation, and this is required for SELK to associate with p97(VCP) [14]. The function of p97(VCP) in chaperoning misfolded protein to proteosomal degradation is consistent with SELK and SELS playing a role in ER stress-induced degradation, and some of these interactions may be cell-type specific as well.

Our initial experiments using cells from *Selk*$^{-/-}$ mice revealed that receptor mediated calcium (Ca^{2+}) flux was impaired in *Selk*$^{-/-}$ T cells, neutrophils, and macrophages. Ca^{2+} is mainly stored in the ER of immune cells and, upon engagement of specific receptors on the surface of leukocytes, the stores are released into the cytoplasm, which triggers a large influx of Ca^{2+} from extracellular spaces into the cytoplasm [18]. This process is termed store-operated Ca^{2+}entry and is critical for the full activation and function of these immune cells. The impaired Ca^{2+} flux observed in *Selk*$^{-/-}$ immune cells led to defects in a variety of functions including migration, proliferation, and oxidative burst that were associated with higher susceptibility to West Nile virus infection due to impaired viral clearance [12]. It was subsequently found that SELK function is regulated by proteolytic modulation through the calpain/calpastatin enzyme system in innate immune cells like macrophages, but not in T or B cells [19]. We then showed that SELK mediated Ca^{2+} flux was crucial for ERK activation and nitric oxide production required for phagocytosis of IgG-opsonized particles by macrophages [20].

In addition to IgG-opsonized particles, oxidized low density lipoprotein (LDL) uptake was impaired in *Selk*$^{-/-}$ macrophages, and this led to reduced foam cell formation and atherosclerosis [21]. The latter study provided a major breakthrough in understanding SELK's role in regulating immune cell functions due to our discovery that SELK deficiency led to reduced palmitoylation of the receptor for oxidized

LDL, CD36. This in turn led to other important discoveries regarding SELK and protein palmitoylation. Below, we will describe the current understanding of how SELK may act as a cofactor in the important post-translational modification, palmitoylation, and how this may affect not only immune cells but other cell types as well.

20.2 Protein Palmitoylation

Palmitoylation is a post-translational modification involving the reversible addition of the 16-carbon fatty acid, palmitate, to cysteine residues through a thioester bond [22], and this modification can facilitate membrane association of cytosolic proteins or stable expression of transmembrane proteins [23, 24]. There may be a requirement for reduction of oxidized cysteines during S-acylation and SELK may be involved in its reduction, although no evidence has yet been presented for this occurring in vivo. The active form of palmitic acid for this reaction is palmitoyl-coenzyme A (CoA), and CoA is released when the palmitoyl moiety is added via S-acylation to the target cysteine residue on the target protein.

The palmitoylation reaction is catalyzed by a family of enzymes called palmitoyl acyltransferases (PATs), which were only recently identified in yeast [25]. There are 23 mammalian PATs that each contain a common aspartic acid-histidine-histidine-cysteine (DHHC) motif within the catalytic domain [26]. The palmitoylation of target proteins on their cysteine residues is a two-step process. In the first step, a Cys residue within the PAT, presumably the conserved Cys within the DHHC catalytic domain, reacts with palmitoyl-CoA to form a thioester bond in a palmitoyl-PAT intermediate. In the second step, the palmitic acid is transferred from the PAT via a thioester bond to a Cys residue on the target protein. The first step is rapid, whereas the second is much slower. Thus, after the first step, there is the possibility that the unstable thioester bond between Cys on the PAT and the palmitic acid may hydrolyze prior to transfer of the palmitic acid to a target protein. There are examples of PATs interacting with cofactors to stabilize the palmitoylated PAT and thereby enable the second step of the reaction to occur in a more efficient manner [27, 28]. This is where we believe SELK enters the picture, and we will next describe some of the target proteins that require SELK for palmitoylation and our theories on how SELK makes this process more efficient.

20.3 The Role of SELK-Dependent Palmitoylation in Regulating the Stability and Function of CD36

CD36 is a transmembrane protein on the surface of cells including monocytes, macrophages, endothelial cells, and other cell types [29]. This molecule is of particular interest in regard to atherosclerosis due to its role in the uptake of oxidized low density lipoprotein (oxLDL). The early stages of atherogenesis involve conditions of high lipid deposition in the blood vessels, followed by a rapid influx of circulating

monocytes into the arterial intima [30, 31]. Recruited inflammatory monocytes differentiate into macrophages that express scavenger receptors like CD36 and scavenger receptor A (SR-A) that promote ingestion of the atherogenic modified cholesteryl ester-rich lipoproteins in the intima of the vessel wall [32].

When macrophages ingest excess lipids they develop into foam cells and experiments using these receptors to promote foam cell formation have revealed that CD36 is the principal receptor for ingesting oxLDL [33], whereas SR-A is mainly involved in uptake of acetylated LDL (acLDL) [34]. The accumulation of cholesterol-loaded macrophages in the arterial wall, termed foam cell formation, is a hallmark of early atherosclerotic lesions. Rupture of foam cells can lead to an increase in the necrotic core and progression of atherosclerotic plaques. LDL particles are internalized by macrophages in the arterial wall via the LDL receptor (LDLR) on the cell surface and LDL is hydrolyzed to free cholesterol within lysosomes [35]. Lipid uptake by macrophages is enhanced by inflammatory cytokines such as TNFα, which plays a key role in the formation of atherosclerotic plaques [36].

A key mouse model developed to study mechanisms that drive atherosclerosis involves the feeding of a high-fat diet (HFD) to mice lacking the LDL receptor (*Ldlr*$^{-/-}$ mice), which results in a well defined build up of lipid in the arteries [37]. This leads to atherogenesis and associated pathology that is dependent on the formation of foam cells. To investigate if SELK in immune cells is important for atherogenesis, we conducted studies in which lethally irradiated *Ldlr*$^{-/-}$ mice were reconstituted with bone marrow from *Selk*$^{-/-}$ mice and from wild type controls. These mice were maintained on a HFD and the development of atherosclerosis was investigated in detail [21]. We found that SELK deficiency in the immune system reduced the formation of atherosclerotic lesions with a concurrent decrease in foam cell formation. Molecular mechanisms were studied and it was found that SELK deficiency resulted in less CD36 expressed on the surface of macrophages, which led to lower uptake of oxLDL and lower foam cell formation. Because stable expression of CD36 was previously found to require its palmitoylation on both the C- and N-terminal regions, we tested whether SELK was important for palmitoylation of CD36. Indeed, it was found that CD36 palmitoylation required SELK but at this time we did not fully understand the link between protein palmitoylation and the requirement for SELK. This would be revealed with subsequent investigations into Ca^{2+} flux as described in detail below.

20.4 The Role of SELK-Dependent Palmitoylation in Regulating the Function of the IP3R

The requirement of SELK for the palmitoylation of CD36 and the impaired Ca^{2+} flux in SELK deficient immune cells led us to generate a new hypothesis as follows: the Ca^{2+} channel protein in the ER membrane that facilitates Ca^{2+} flux in stimulated immune cells, the inositol-1,4,5-triphosphate receptor (IP3R), is palmitoylated in a SELK-dependent manner and that this is required for its stable expression. There were no previous reports of IP3R being palmitoylated and we found that this large transmembrane protein in the ER membrane was indeed palmitoylated on multiple

cysteine residues [38]. This was required for stable assembly of the tetrameric Ca^{2+} channel in the ER membrane. This raised the question of how SELK is involved in palmitoylation. The answer turned out to be that SELK is not the enzyme that catalyzes palmitoylation, but is a cofactor that binds to the palmitoylating enzyme referred to as DHHC6. DHHC6 is a member of DHHC family of enzymes that catalyze protein palmitoylation as described above. DHHC6 contains a predicted Src-homology 3 (SH3) domain and DHHC6 is localized to the ER membrane. Since Selk is also an ER membrane protein and contains an SH3 binding domain, we conducted several studies and confirmed that these two proteins interact via the SH3/SH3 binding domain.

Importantly, the palmitoylation of the IP3R by DHHC6 was found to be impaired in both genetically and nutritionally induced SELK deficiency. Cells from *Selk*$^{-/-}$ T cells and macrophages were defective for DHHC6 leading to low IP3R palmitoylation and expression, and T cells maintained in low [Se] media had less SELK along with lower IP3R palmitoylation and expression. This study helped to explain our previous findings that low Se bioavailability to T cells led to lower Ca^{2+} flux when stimulated through the T cell receptor [39]. In addition, this provided mechanistic insight into the impaired immune cell functions that depend on Ca^{2+} flux in SELK-deficient cells. As shown in Fig. 20.1, under conditions of sufficient Se, there is abundant SELK that can bind to the DHHC6 enzyme and carry out efficient palmitoylation of CD36, IP3R, and likely other proteins.

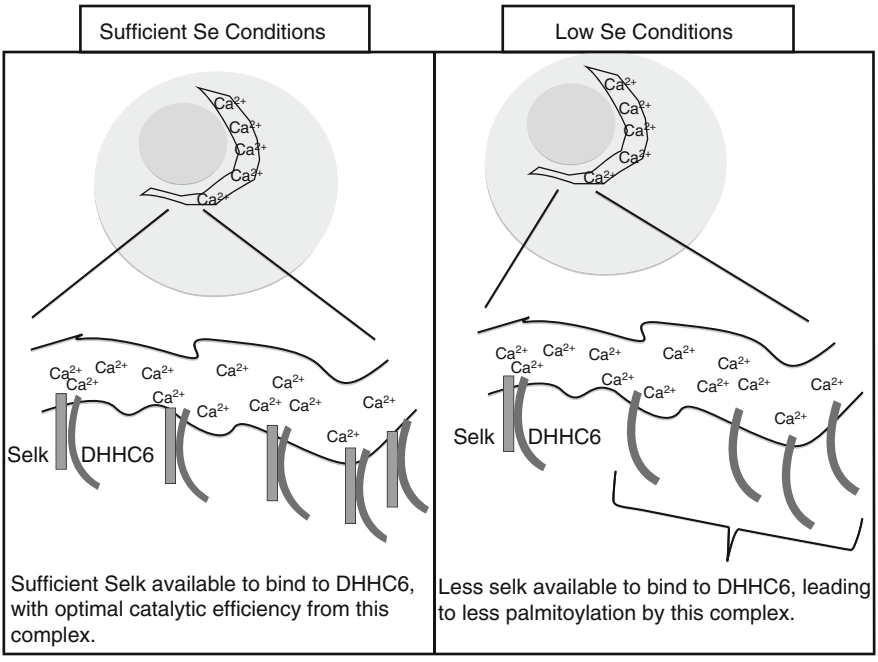

Fig. 20.1 Insufficient Se intake leads to lower levels of protein palmitoylation by DHHC6. The *left* panel shows the coupling of DHHC6 to SELK to promote efficient protein palmitoylation. The *right* panel indicates how low dietary Se reduces SELK protein levels in the ER membrane, which in turn leads to less efficient protein palmitoylation due to less coupling of SELK to DHHC6

20.5 How Does SELK Contribute to Protein Palmitoylation?

When initially considering the possible mechanisms by which SELK may regulate protein palmitoylation, the likelihood of SELK directly catalyzing the addition of palmitoyl fatty acid to target proteins seemed quite low due to the highly disordered nature of SELK and the lack of any conventional acyltransferase catalytic domain within this selenoprotein. It is more likely that SELK interacted with one or more of the members of the protein acyltransferase (PAT) family of enzymes that catalyze protein palmitoylation. In fact, there is another example in yeast of a PAT enzyme (the orthologue to mammalian DHHC9) interacting with a cofactor to carry out palmitoylation [27].

As described above, SELK interacts with DHHC6 in the ER membrane via SH3/SH3BD interactions. It is currently our hypothesis that SELK functions as a coenzyme for DHHC6 for efficient catalysis of the protein palmitoylation reaction. This is based on the notion that SELK binds to DHHC6 and somehow utilizes its Sec residue to reduce the energy required for the S-acylation of target proteins, which thereby increases the catalytic efficiency of the palmitoylation reaction (Fig. 20.2). We have previously proposed and described several possible molecular models to explain how SELK increases the efficiency of DHHC catalyzed palmitoylation [40].

It is our current hypothesis that the autopalmitoylation of DHHC6 on its Cys residue is not dependent on SELK. This is the rapid step in the two-step PAT reaction and likely occurs as the activated form of palmitate, palmitoyl-CoA, is available and comes into contact with the catalytic domain of DHHC6. It seems more likely that SELK bound to DHHC6 is involved in the slower second step carried out by PATs. In particular, SELK may somehow lower the energy of the second step in which the palmitate is transferred from the Cys residue in DHHC6 to the target protein's Cys residue. Whether this involves a Cys-Sec bridge between enzyme and

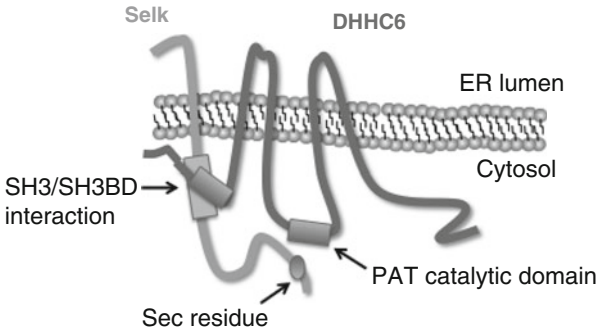

Fig. 20.2 A model of the interactions between SELK and DHHC6. Based on experimental evidence, the interaction between SELK and DHHC6 depends on the SH3 domain in DHHC6 binding to the SH3 binding domain (SH3BD) in SELK. Exactly how SELK promotes the catalysis of the palmitoylation reaction by DHHC6 is unknown, but we speculate that the catalytic domain of DHHC6 somehow acts in concert with the Sec residue of SELK to carry out this reaction

coenzyme or if the Sec of the SELK coenzyme actually forms a chemical bond with the palmitate to promote transfer of the lipid to the target protein is a topic current under investigation.

20.6 Concluding Remarks

The role of SELK in regulating immunity derives from a series of fundamental discoveries that have been made as described above. Our observation that Ca^{2+} flux was impaired in SELK deficient immune cells led us to our discovery that SELK interacts with the palmitoyl acyltransferase, DHHC6, in the ER membrane to palmitoylate the IP3R and stabilize its expression. This breakthrough reveals a major mechanism by which dietary Se, through the actions of SELK, modulates immunity. In addition to IP3R and CD36, other protein targets are likely palmitoylated by DHHC6/SELK and current studies are underway to identify these other proteins. Because IP3R and CD36 are expressed in cell types in addition to immune cells, it is likely that other tissue systems are affected by low SELK levels (e.g., brain for IP3R and endothelial cells for CD36).

To really understand how SELK regulates palmitoylation, an enzyme assay needs to be developed and our laboratory is currently undertaking this endeavor. Such an assay would allow enzyme kinetics to be measured and will determine the specific role of SELK as a cofactor or coenzyme. This includes answering questions about how SELK affects DHHC6 catalyzing efficiency, how Sec versus Cys residues in SELK may compare in increasing the efficiency, and how other conditions like low Se levels or calpain cleavage may also impinge upon the palmitoylation process.

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Chapter 21
Selenoprotein M: Structure, Expression and Functional Relevance

Ting Gong, Marla J. Berry, and Matthew W. Pitts

Abstract Selenoprotein M (SELM) is an ER-resident thiol-disulfide oxidoreductase that is highly expressed in the brain and bestows neuroprotective properties. Its active site consists of a selenocysteine-containing thioredoxin-like domain that is postulated to mediate thiol-disulfide exchange. Studies performed in cell culture indicate that SELM promotes intracellular calcium homeostasis and protects against oxidative stress. Additionally, *Selm* knockout mice display increased body weight, elevated white adipose tissue levels, and reduced hypothalamic leptin sensitivity relative to wild type counterparts, suggesting that SELM supports normal energy homeostasis. Finally, in the context of human disease, altered levels of SELM expression have been linked to both familial early onset of Alzheimer's disease and hepatocellular carcinoma. This review summarizes our current understanding of SELM with respect to structure, expression, and functional relevance.

Keywords Endoplasmic reticulum • Knockout mouse model • Neuroprotection • Selenoprotein M • Thiol-disulfide oxidoreductase

21.1 Discovery and Structure

Selenoprotein M (SELM) is a thiol-disulfide oxidoreductase localized to the endoplasmic reticulum (ER) that was first identified *in silico* by the Gladyshev laboratory in 2002 [1]. Comparative sequence analyses revealed that SELM is widely conserved among mammals and most closely related to the 15 kDa selenoprotein (SEP15), another ER-resident selenoprotein containing a thioredoxin-like domain [1–3], with

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which it shares structural similarities, suggesting a common evolutionary origin. Together, SELM and SEP15 form a distinct subfamily within the thioredoxin superfamily [4]. The distinguishing feature of the thioredoxin superfamily is an active site containing two reactive cysteine (Cys) residues that catalyze thiol/disulfide-like exchange reactions, of which thioredoxin is the classic example. SELM, along with several other selenoproteins (SEP15, SELH, SELO, SELT, SELV, SEPW1) contain thioredoxin-like domains, in which the second Cys residue is replaced by selenocysteine (Sec) [5].

In humans, the *SELM* gene is located on chromosome 22 (22q12.2) and consists of five exons spanning a total of three kilobases and four introns. This gene encodes a 145 amino acid protein, where Sec constitutes the 48th residue. The N-terminal 25 amino acids represent a signal peptide that appears to be necessary for localization to the ER [1]. The remainder of the protein consists of a central α/β domain, made up of three α-helices ($\alpha1$–$\alpha3$) and four antiparallel β-strands ($\beta1$–$\beta4$), and an ER retention tetrapeptide (H/R/K-x-D-L) in the C-terminal domain [2, 4].

The active site of SELM is a Sec-containing CxxU (U=Sec) thioredoxin-like domain that is located in the loop connecting the C-terminus of strand $\beta1$ and the N-terminus of helix $\alpha1$. The Sec-containing redox motif is thought to mediate thiol-disulfide exchange by forming reversible selenenylsulfide bonds during the cycle of oxidation and reduction. Three dimensional computer modeling revealed that the C-terminal region of SELM is highly flexible and does not assume a regular secondary structure [3, 4]. It has also been suggested that this C-terminal region may serve as a binding site for protein substrates serviced by SELM [4]. Moreover, bioinformatics analyses using NetPhos identified two putative phosphorylation sites, Y128, T139, in the C-terminal region of SELM that may influence protein-substrate interactions [3].

In addition, residue interaction network analysis identified three well conserved residues, F59, L82, L84, that serve as putative hub nodes for protein-protein interactions. Studies using the yeast two-hybrid system to screen for potential interaction partners determined that SELM interacts with Galectin-1, a lectin implicated in neuroprotection [6]. Recently published work investigating the global human protein interactome network identified roughly 14,000 binary protein-protein interactions [7], and among these, SELM was reported to interact with the Notch2 N-terminal like (NOTCH2NL) and microtubule associated tumor suppressor 2 (MTUS) proteins. A subsequent large scale systematic exploration of the human interactome also documented that SELM interacts with neudesin (NENF), a neurotrophic factor [8].

21.2 Expression

SELM is one of the selenoproteins that displays enriched expression in the brain. The initial report on the discovery of SELM analyzed its distribution in various tissues by Northern blot analysis and determined that levels were highest in brain, with lower levels detected in many additional organs [1]. We measured protein levels in mouse tissue by Western blotting and observed high SELM expression in the pancreas and

the pituitary, along with the brain [9]. Using data derived from the Allen Brain Atlas, Zhang and colleagues conducted comparative gene expression analysis for the entire selenoproteome and reported that *Gpx4*, *Selk*, *Selm*, *Sepp1*, *Sepw1*, and *Sep15* were the selenoprotein transcripts exhibiting highest expression in brain [10]. Within the brain, they found that *Selm* mRNA expression was most pronounced in the cerebellar cortex, the olfactory bulb, and the hippocampus. These researchers also performed immunohistochemistry (IHC) and Western blot analysis to evaluate regional expression of SELM in the mouse brain at the protein level. IHC confirmed that SELM is expressed in the hippocampus and the cerebellar cortex, whereas Western blot analysis showed high levels of SELM in whole brain and cerebellar cortex samples. Surprisingly, their Western blot data showed robust SELM expression in hypothalamus, which appeared higher than those samples derived from hippocampus, cortex, or olfactory bulb.

We performed Western blot analysis on dissected mouse brain tissue and found that, among the brain regions surveyed, SELM expression was highest in the cerebellum and olfactory bulb, lowest in the hippocampus and cerebral cortex, and expressed at intermediate levels in the hypothalamus and brain stem [9]. Our (IHC) analysis of the mouse brain revealed that SELM is highly expressed in several discrete brain regions, including cerebellar cortex, arcuate and paraventricular nuclei of the hypothalamus, medial septum, reticular thalamus, red nucleus, ventral tegmental area, various auditory brainstem nuclei (cochlear nucleus, lateral lemniscus, superior olive), and the CA2/CA3 region of the hippocampus. Moreover, in several regions, but not all, SELM exhibited an expression pattern that appeared to be predominantly localized to parvalbumin-expressing interneurons (M.W. Pitts, unpublished observations). Another study, using microarray analysis to identify genes with enriched expression in the inner ear, determined that *SELM* was one of 52 genes (out of 23,040) to display expression levels that were tenfold higher in the cochlea and vestibule when compared to that observed in other tissues, suggesting that SELM may contribute to cochlear development and hearing [11].

In addition to its characteristic regional pattern of expression, SELM is also regulated by a number of external factors. The *Selm* promoter contains a putative AP1 transcription factor binding site [12] and SELM has been documented to be upregulated by the ER-stress responsive transcriptional activator, XBP1s [13], and leptin [14]. *Selm* was also identified as one of the primary genes regulated by ATOH1, a neural basic helix-loop-helix transcription factor that is required for the formation of distinct neuronal subtypes of the proprioceptive pathway [15]. The aforementioned study observed *Selm* expression in multiple Atoh1-specified neuronal subtypes, including the granule cell layer of the cerebellum, hair cells of the inner ear, and Merkel cells of the vibrissae. Moreover, *Selm* mRNA levels were dramatically reduced in *Atoh1* knockout mice.

A final key influence upon SELM expression is the amount of dietary selenium (Se). A study examining the effect of Se supplementation upon selenoprotein mRNA expression in mouse kidney and liver found that *Selm* expression was moderately influenced by Se status, whereas *Gpx1*, *Selh*, and *Sepw1* were the most responsive selenoprotein transcripts [16]. Another report, investigating the consequence of

Se-deficiency on selenoprotein expression in the mouse colon, observed that *Gpx1*, *Selh*, *Sepw1*, and *Selm* were all significantly regulated by Se status [17]. These investigators also performed pathway analysis and identified protein biosynthesis, inflammation, Delta-Notch, and Wnt signaling pathways as being substantially affected by Se status. A more recent paper reported that SELM expression in chicken brain was significantly reduced at both the mRNA and protein levels in response to administration of a Se-deficient diet [18]. This study also found that Se-deficiency reduced the expression of SELM to a greater degree than that of SEPW1, which is surprising given that SEPW1 is considered one of the most sensitive biomarkers of Se status.

21.3 Role in Neuroprotection

Due to its antioxidant properties and high level of expression in brain, SELM has been of interest to researchers investigating neurodegenerative disorders. *Selm* was found to be significantly downregulated in transgenic mice overexpressing the human mutant presenilin-2 gene that causes early onset of Alzheimer's disease (FAD) in humans [19]. This group of researchers subsequently generated a transgenic rat overexpressing the human *SELM* gene [20], and reported that *SELM* overexpression resulted in increased activation of the ERK pathway and elevated activity of the antioxidant enzymes glutathione peroxidase and superoxide dismutase. Moreover, when combined with Se supplementation, *SELM* overexpression decreased γ-secretase activity, which drives production of Aβ42, the most pathogenic form of the Aβ peptide implicated in the development of FAD. Later studies on cortical tissue utilized two-dimensional electrophoresis to evaluate which proteins were most affected by *SELM* overexpression [21]. These analyses determined that five proteins, CKB, RING1, SYT15, LDHB, EIF4H, were upregulated and three proteins, PSMA3, CENPN, DPYSL2, were downregulated by *SELM* overexpression.

Further support for the involvement of SELM in neuroprotection comes from studies conducted in cell culture. SELM overexpression was observed to decrease superoxide production and reduce apoptotic cell death in response to oxidative challenge with hydrogen peroxide in mouse hippocampal HT22 cells [22]. Correspondingly, *Selm* knockdown increased reactive oxygen species production and reduced cell viability. In addition, overexpression of *SELM* decreased cytosolic calcium flux induced by hydrogen peroxide treatment, while *Selm* knockdown increased baseline cytosolic Ca^{2+} levels. These results suggest that SELM is involved in the regulation of Ca^{2+} homeostasis and the prevention of apoptosis. Dysregulation of Ca^{2+} homeostasis is a key feature of many neurodegenerative disorders, most prominently FAD [23]. In particular, mutations in the presenilin genes associated with FAD have been demonstrated to affect calcium signaling and redox balance [24, 25]. Interestingly, the disturbances in Ca^{2+} signaling resulting from FAD mutations are observed prior to onset of cognitive deficits or histopathological hallmarks in both humans [26] and transgenic Alzheimer's rodent models [27, 28].

21.4 Characterization of the *Selm* Knockout Mouse

Transgenic mice with a constitutive deletion of the *Selm* gene have been developed and phenotypically characterized when raised on standard lab chow containing approximately 0.25 ppm Se. Based upon the aforementioned studies demonstrating the neuroprotective role of *Selm*, it was hypothesized that *Selm* knockout mice would exhibit behavioral deficits. To our surprise, *Selm* knockout mice displayed normal brain morphology, comparable density of parvalbumin-expressing interneurons, and no impairments in motor coordination, anxiety-like behavior, or cognition relative to wild type controls [9]. Performance did not significantly differ from controls on the Barnes maze test for spatial learning, as well as for auditory and contextual fear conditioning. These results indicate that *Selm* knockout mice are able to hear and display no overt deficits in amygdalar and hippocampal function. However, it should be noted that these tests were conducted on young adult mice fed a Se-adequate diet. The possibility exists that *Selm* knockout mice may manifest behavioral deficits under certain conditions, such as when aged or challenged with a Se-deficient diet.

Whereas no evident cognitive deficits in *Selm* knockout mice were observed, these mice displayed increased weight gain, elevated white adipose tissue deposition, and higher serum leptin levels. Yet, no significant differences between *Selm* knockout mice and controls were observed for either glucose tolerance or hepatic insulin signaling. Further experiments determined that SELM is present in leptin receptor-expressing neurons of the arcuate hypothalamus and that *Selm* knockout mice display diminished hypothalamic STAT3 phosphorylation in response to challenge with leptin, indicative of leptin resistance. Of particular relevance, ER stress has been demonstrated to be a major contributing factor to obesity and leptin resistance [29, 30]. Moreover, reduced hypothalamic thioredoxin activity has also been linked to energy imbalance and impaired leptin sensing [31]. Results suggest that a lack of SELM may lead to increased ER stress and diminished hypothalamic sensitivity to leptin. Dysregulation of the hypothalamic-pituitary-adrenal (HPA) axis was also observed, as reduced morning baseline levels of corticosterone were apparent in *Selm* knockout mice.

Leptin has been shown to promote activation of the HPA axis by stimulating the release of corticotropin-releasing factor from the hypothalamus and adrenocorticotrophic hormone from the pituitary [32]. The fact that *Selm* knockout mice exhibited elevated levels of leptin in conjunction with diminished baseline corticosterone levels suggested impaired coupling between leptin signaling and the HPA axis.

Finally, there were apparent morphological differences in brown adipose tissue (BAT) (Fig. 21.1), suggestive of diminished activation and/or innervation of BAT in *Selm* knockout mice. BAT samples derived from these mice were observed to be paler and contain larger triglyceride deposits than those obtained from wild type controls. BAT activation promotes energy expenditure and reduces body fat by means of adaptive thermogenesis, a process in which BAT burns fatty acids and glucose to generate heat [33]. Defects in adaptive thermogenesis have been described in many rodent models of obesity and this process is controlled by the

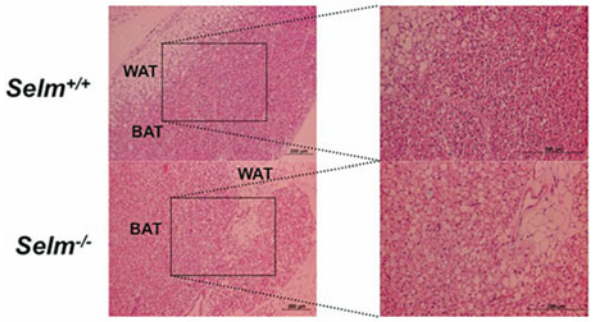

Fig. 21.1 Images of brown adipose tissue derived from adult male *Selm*$^{+/+}$ (*top row*) and *Selm*$^{-/-}$ (*bottom row*) mice fed a standard lab chow diet. Note that samples from *Selm* knockout mice are paler and contain larger triglyceride droplets. BAT = Brown adipose tissue; WAT = White adipose tissue. Scale bar = 200 μm

hypothalamus [31, 34]. Moreover, neurons in multiple hypothalamic regions have been identified that stimulate BAT activity, most notably in the dorsomedial [35, 36] and paraventricular nuclei [37]. Whereas the cause of BAT alterations in *Selm* knockout mice has not been clearly established, we speculate that it stems from upstream hypothalamic redox imbalance. In summary, the observed phenotype of the *Selm* knockout mouse indicates that SELM plays an important role in the regulation of body weight and energy metabolism.

21.5 Involvement in Cancer

SELM has also recently been documented to be upregulated in hepatocellular carcinoma (HCC). Guariniello and colleagues evaluated *SELM* expression in two human hepatoma cell lines, HepG2 and Huh7, by means of RT-PCR and Western blotting and found that SELM expression was significantly elevated at both the mRNA and protein levels [3]. These findings led the authors to suggest that SELM may be useful as a prognostic marker of HCC. IHC studies by the same group confirmed that SELM is upregulated in HCC and revealed that increased SELM expression is associated with higher grades of tumor malignancy [38]. Further experiments, using RT-PCR to quantify gene expression in the HepG2 and Huh7 HCC cell lines, determined that multiple other selenoprotein transcripts, GPX4, GPX7, SELK, SEPN1, SELT, SELV, SEPW1, SEP15, TXNRD1, in addition to SELM, were significantly upregulated in HCC [39]. Of potential relevance, earlier studies have shown that the leptin receptor is overexpressed in HCC and that leptin promotes hepatocellular carcinogenesis by activating the STAT3, AKT, and ERK signaling pathways [40].

21.6 Concluding Remarks

The current literature indicates that SELM is an antioxidant enzyme involved in the promotion of cell growth and survival in a variety of tissues, although the exact mechanisms remain to be fully elucidated. Evidence to date suggests that SELM positively regulates the ERK, NOTCH, and STAT3 signaling pathways. It also plays a role in mediating Ca^{2+} homeostasis, which is typically dysregulated by mutations that cause FAD in humans. At the present time, it is not mechanistically clear as to how SELM regulates Ca^{2+} levels, although interactions with the inositol triphosphate receptor or ryanodine receptor represent the most plausible scenarios. In addition, we have found that mice lacking *Selm* develop obesity and exhibit diminished hypothalamic activation of the STAT3 pathway in response to leptin. Conversely, SELM is overexpressed in HCC [3], a type of cancer in which STAT3 is often constitutively activated [41, 42]. In summary, SELM promotes redox balance in a variety of tissues and alterations of SELM expression have been linked to obesity, neurodegeneration, and cancer.

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Chapter 22
Selenoprotein P and Selenium Distribution in Mammals

Ulrich Schweizer, Lutz Schomburg, and Josef Köhrle

Abstract Selenoprotein P (Sepp) is a remarkable selenoprotein. Among all mammalian selenoproteins, it is the only one containing more than one selenocysteine, and ten in humans. Sepp is a plasma protein mainly originating in the liver, but it is also expressed in other organs, notably the brain, placenta, and the lactating mamma. The main function of Sepp is transport of selenium (Se) to target tissues. Hepatocytes thus convert nutritional selenocompounds into Sepp for transport and distribution, and the mother's milk contains Sepp as an essential gift to the offspring. The fact that about 25 % of all selenoprotein mRNAs in hepatocytes code for Sepp alone highlights its central position in the body's Se homeostasis. Selenium status of an individual is thus reflected by the serum concentration of Sepp. Endocytic receptors of the lipoprotein receptor-related protein (Lrp) family participate in targeting cell-specific Sepp uptake and retention. A Sepp-cycle exists in brain, testes, and kidney and appears to preserve tissue Se during times of poor nutritional supply, explaining the long known hierarchical differences in tissue-specific Se retention in deficiency. Individual genotype differences may modulate these processes exerting an influence on the relative expression levels of selenoproteins, response to Se intake, and individual risk for Se-dependent diseases.

Keywords ApoER2 • Lrp2 • Lrp8 • Megalin • Plasma • SelP • SePP • Sepp1 • Transport

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22.1 Introduction

Sepp was initially discovered as a plasma protein incorporating ^{75}Se in metabolically labeled rats [1]. In the same report, the authors demonstrated uptake of ^{75}Se-Sepp into extrahepatic organs and suggested a transport function for this protein. Later studies showed that Sepp contains up to 50 % of plasma Se in individuals with normal Se status [2]. When the cDNA for *Sepp* was cloned, it became apparent that it contains ten selenocysteine (Sec) codons within the open reading frame in both rodents and humans, and two separate **Sec**-**i**nsertion **s**equence (SECIS) elements in the 3′-untranslated regions of the transcripts [3]. This finding was important as it demonstrated that several UGA/Sec codons can share the same SECIS element in mammals, while in bacteria the SECIS elements are located immediately 3′ of the respective UGA codon [4]. Sepp carries one Sec within an N-terminal domain with a predicted similarity to thioredoxin and peroxidases, and a C-terminal Sec-rich domain with no known structure (Fig. 22.1). While peroxidase activity of the N-terminal part of Sepp has been demonstrated repeatedly, its physiological role remains unclear [5, 6].

22.2 Selenoprotein P (Sepp) Is a Plasma Selenium Transport Protein

Our current model of Sepp function is summarized as follows: Sepp is a plasma Se transport protein mainly secreted by liver and is taken up in target tissues by interaction with endocytic receptors. These key findings support our model: dietary Se intake determines plasma Sepp and plasma Se concentrations [7]. The unusual Sec-rich C-terminus of Sepp is partially conserved among species and its main property appears to be its content of many Sec residues [8]. Metabolic labeling studies indicated that dietary Se is rapidly taken up by the liver and incorporated into circulating Sepp which, slowly declining, gives rise to increasing kidney-derived plasma glutathione peroxidase (Gpx) concentrations [9]. Interestingly, Sepp constitutes the essential Se-containing component in serum-based cell culture media supporting growth and survival of primary neuronal cells [10, 11]. Transgenic mice with genetically disrupted Sepp biosynthesis (*Sepp*-knockout, *Sepp*$^{-/-}$) develop a number of Se-dependent phenotypes including growth defect, male infertility and neuronal abnormalities [12, 13] that can be rescued by liver-specific expression of human SEPP1 [14]. The identification of specific Sepp binding and uptake by members of the lipoprotein receptor-related protein (Lrp) family confirms transport by Sepp as a regulated and targeted process of Se supply to specific target tissues [15–17]. This concept has been integrated with the mechanisms of hierarchical Se retention and excretion [18].

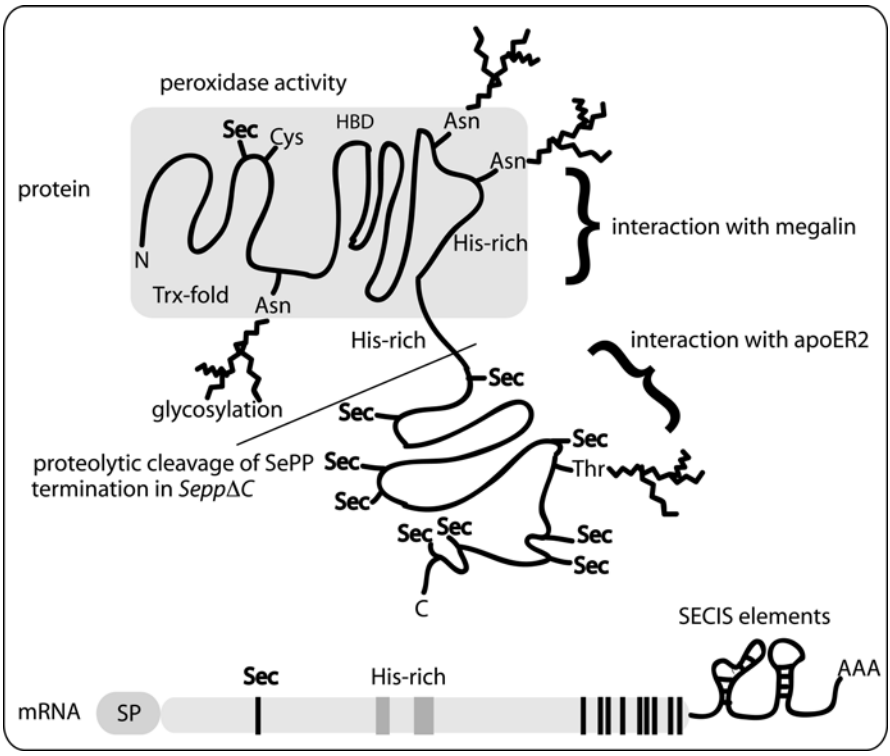

Fig. 22.1 Structural features of the two domains of selenoprotein P (Sepp). The N-terminal domain, predicted to adopt a thioredoxin (Trx)-fold, contains a Sec-X-X-Cys motif, showing weak peroxidase activity. The Sec-rich C-terminal domain is without homology to any known protein and likely serves a Se transport function. A classical N-terminal signal sequence (SP) directs Sepp biosynthesis into the ER lumen. The secreted protein carries three N-glycosylation sites (Asn) and one O-linked (Thr) glycosylation site. A heparin-binding-domain (HBD) has been identified along with two His-rich domains, which potentially mediate association to the extracellular matrix. Sepp isoforms may result from differential glycosylation, proteolytic cleavage or premature translational termination

22.3 Lipoprotein Receptor-Related Proteins as Endocytic Receptors Involved in Sepp Uptake

Lrps are endocytic receptors for lipoproteins, hormone- and vitamin-binding proteins, and morphogens [19–21]. These receptors usually are capable of binding many cargo proteins. Lrp2 (megalin or glycoprotein-330) is expressed along many epithelia, e.g., kidney proximal tubule cells, and binds vitamin D3/vitamin D binding globulin, progesterone/clara cell secretory protein, vitamin A/retinol binding protein, androgens and estrogens/sex-hormone binding globulin, thyroid hormones/transthyretin, vitamin B12/transcobalamin, and folate/soluble folate receptor [20, 22]. The physiological importance of Lrp2-mediated (re-)uptake and

internalization of hydrophobic ligands along with their high molecular weight carrier proteins is evident from their loss in a number of animal models and in patients with inherited deficiency in megalin/LRP2 [21, 23, 24]. The additional importance of Lrp2 for Se homeostasis by specific binding and re-uptake of Sepp came initially as a surprise to the field of Se biology [15, 25], but appears highly plausible as a logical addition to the list of functions for this internalizing system controlling the uptake of essential circulating serum factors such as nutrients, vitamins and hormones. Lrp-2 binds the N-terminus of Sepp and prevents the urinary loss of Se as found in *megalin*$^{-/-}$ mice [6, 25].

Lrp8 (Apolipoprotein E receptor 2, ApoER2) is highly homologous to the low density lipoprotein receptor (LDL-R) and the very low density lipoprotein-receptor (VLDL-R). Lipoprotein receptors can participate in signal transduction, e.g., Lrp8 is one of the cell-surface receptors involved in the reelin signaling pathway. Reelin is a large neuronal signaling molecule guiding neuronal cell migration during central nervous system development [26] and interacts with both Lrp8 and VLDL-R. Genetic inactivation of both receptors leads to neuronal migration deficits, tremor, and ataxia [27]. Mutations targeting the signaling function of Lrp8 do not affect Se metabolism [28]. However, inactivation of *Apoer2* leads to a similar neurological phenotype as observed in *Sepp*$^{-/-}$ mice when fed a low Se diet [17, 29]. This phenotypic similarity is explained by the interaction of Lrp8/Apoer2 with Sepp [16]. Accordingly, brain Se levels are strongly reduced in *Apoer2*$^{-/-}$ mice [17]. Lrp8 binds Sepp only if part of the Se-rich C-terminus is present [30]. Taken together, Sepp in combination with Lrp2 and Lrp8, respectively, constitute a Se-uptake and -delivery system essential for preferentially supplying Se to target organs and avoiding Sepp loss through the kidney.

22.4 Mouse Models of Modified Sepp, Lrp2 or Lrp8 Expression

22.4.1 Classical Gene Targeting

Genetic inactivation of *Sepp* profoundly disturbs Se metabolism in mice [12, 13]. As expected for the inactivation of the plasma Se transport protein, circulating Se concentrations were decreased in *Sepp*$^{-/-}$ mice. Moreover, Se content in brain and testes, organs normally preferentially supplied with Se, were strongly reduced. Male *Sepp*$^{-/-}$ mice were infertile and the appearance of sperm from *Sepp*$^{-/-}$ mice resembled sperm from *Apoer2*$^{-/-}$ mice [14, 31, 32]. A more detailed description of the role of Se in male fertility can be found in Chaps. 17 and 18.

Sepp$^{-/-}$ mice developed a neurological phenotype which was strictly dependent on Se content in their diet [29, 33]. The role of Se in brain is reviewed in Chap. 36. Sepp not only has a role in Se distribution, but can serve as a local Se storage device. It is not only expressed in the liver, but also in other organs, e.g., the brain. Provided

that Sepp is prevented from leaving the source organ (i.e., brain), it can be safely deposited outside of cells and taken up again, thus maintaining a stable Se level in the brain compartment during times of low nutritional Se supply. We called this mechanism a "Sepp-cycle" (Fig. 22.2) [18, 34].

Hepatic Sepp biosynthesis plays an important role in regulating whole body Se status [35]. *Sepp*$^{-/-}$ mice show increased loss of Se via the urine. In the absence of Sepp biosynthesis, increased hepatic Se becomes available for the production of small selenocompounds which are excreted via the urine, e.g., trimethylselenonium and selenosugars [18, 36]. Sepp also serves as the main source of Se in mouse and human milk [29, 37]. More recently it has been shown that glutathione peroxidase 3 (Gpx3) is also present in mouse milk. However, only Sepp contributed to Se transfer to offspring [38].

Lrp2$^{-/-}$ mice usually die perinatally [39], but *megalin*-deficient mice carrying a nonsense mutation on a different genetic background survive into adulthood [40]. We have used these mice as a model system for the analysis of the physiological functions of Lrp2 in adult mice, as discussed later, with respect to renal Sepp metabolism [25]. These results have recently been independently supported [6].

In contrast, *Lrp8*$^{-/-}$ mice are born at the expected Mendelian ratio and appear grossly normal. Reduced male fertility of these mice was associated with a reduced expression of the selenoenzyme Gpx4 in the initial segments of the epididymis [32]. Gpx4 is an important structural component of sperm [41]. Therefore, male infertility of *Lrp8*$^{-/-}$ mice appears secondary to decreased selenoprotein expression in sperm which in turn depends on Sepp-mediated Se supply [42]. Se deficiency also develops in the brains of *Lrp8*$^{-/-}$ mice and these could be rescued with increased dietary Se intake [17]. Selenite appears to be highly bioavailable to the brain, a finding which is of limited physiological importance as selenite is not a usual component of our diet, but needs to be considered in view of the supplementation of animal diets with sodium selenite or in rare diseases where normal Se transport is impaired.

22.4.2 Isoforms of Sepp

The structure of Sepp containing two clearly defined domains implies two separate functions for the protein, i.e., a peroxidase activity associated with the first Sec residue within the N-terminal thioredoxin-like domain and a Se supply function assigned to the C-terminus which harbors the remaining nine Sec residues [43]. Biochemical experiments have demonstrated that purified Sepp elicits phospholipid hydroperoxide peroxidase activity with glutathione [5], but thioredoxin was identified as a superior cofactor in vitro [44]. More recently, it was reported that the N-terminal domain of Sepp contains considerable peroxidase activity when coupled to thioredoxin reductase 1 [6]. In order to delineate the significance of the two domains in a physiological model, transgenic mice with a shortened Sepp isoform lacking the C-terminal Sec-rich domain were generated and compared to wild type

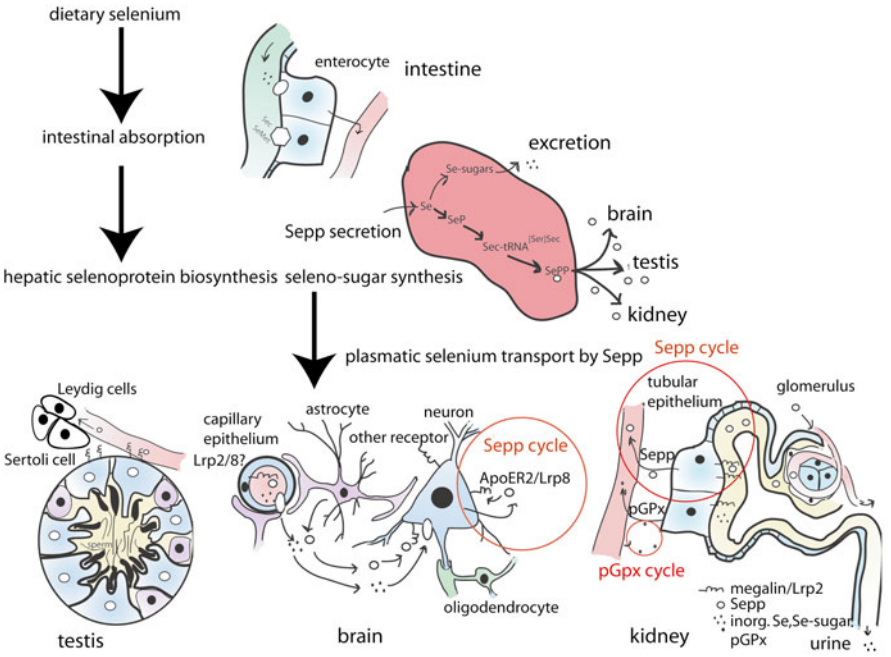

Fig. 22.2 Summary of Se transport processes described in vivo. Dietary Se is primarily taken up along the absorptive epithelium of the small bowel by transporters specific for inorganic divalent anions (e.g., the sodium-dependent sulfate transporter, NaSi-1) or transporters for amino acids and small peptides. Via portal circulation, different selenocompounds reach the liver where the selenium atom is converted into Sec on $tRNA^{[Ser]Sec}$ and subsequently translationally inserted into selenoproteins. Gpx1 has been proposed as a hepatic storage form for excess Se, which may also be converted into selenosugars or selenonium ions and excreted. The liver is central to Se metabolism as the major source of plasma Sepp, which transports Se to privileged target tissues, e.g., brain, testis, and kidney. Brain Se supply is complicated, since several cellular membranes must be crossed to finally reach the neurons. Megalin may be involved in Sepp uptake along the choroid plexus and ependymal epithelium, while ApoER2 is expressed by neurons. Astrocytes synthesize Sepp in vitro and may thus contribute to neuronal Se supply. Neurons express Sepp and may store excess Se outside the cell in the form of Sepp. Brain retains its privileged Se status during dietary restriction via reversible Sepp expression, extracellular deposition and re-uptake. We have termed neuronal Sepp synthesis and ApoER2-mediated Sepp re-uptake in brain as "Sepp-cycle". Testis function likewise depends on ApoER2-mediated Sepp uptake. Inactivation of either protein leads to decreased Gpx4 expression in maturing spermatozoa and infertility. Megalin expressed along the kidney tubular epithelium is involved in re-uptake of Sepp from the primary glomerular filtrate. Accordingly, inactivation of megalin leads to urinary loss of Sepp. Megalin-positive cells express the highest levels of Gpx1, Gpx3, and Sepp within the kidney, and inactivation of megalin decreases expression of all three proteins. Plasma Gpx3 originates from the kidney epithelium, but most Gpx3 is deposited locally within the kidney. Another "Sepp-cycle" can thus be proposed involving glomerular filtration, re-uptake, and renal re-synthesis of Sepp. Kidney insufficiency in patients is associated with low Se status. Tissues expressing selenoproteins, but not expressing ApoER2 and megalin likely operate by a still elusive Se uptake mechanism, which might rely on the poorly characterized selenocompounds from the gastrointestinal tract

and classical *Sepp*$^{-/-}$ mice [45]. Testis and brain Se concentrations were only slightly higher than in *Sepp*$^{-/-}$ mice. These findings corroborate that the C-terminus of Sepp is important for Se supply to the hierarchically preferred target tissues, which express ApoER2/Lrp8, the receptor for the Sepp C-terminus (Fig. 22.2). Apparently, evolution added a C-terminal extension to Sepp, whose only function is to make Se transport more efficient and targeted towards Lrp2 and Lrp8 expressing specific tissues. The peroxidase activity of the N-terminus of Sepp may be needed to limit tissue damage during infection with Trypanosomes [46].

22.4.3 Sepp in the Liver

Se organification, Se distribution, and Se metabolism/excretion are organized by the liver. Experimental studies have shown that liver is the organ converting dietary Se into circulating Sepp for supply of other tissues [9, 47]. Accordingly, liver disease leads to reduced serum Se and Sepp concentrations in patients [48]. Because Sepp is expressed in most tissues [49], cell type-specific gene targeting was used to delineate the role of hepatic Sepp expression for Se metabolism. Mice carrying a conditional allele of tRNA$^{[Ser]Sec}$ (*Trsp*$^{fl/fl}$) were crossed with a cell-specific Albumin-Cre strain abrogating selenoprotein biosynthesis specifically in hepatocytes [50]. The mice were viable and showed almost complete loss of hepatic *Trsp* at 3 weeks of age. As expected, circulating Se and Sepp concentrations were strongly decreased [50, 51]. In these mice, kidney Se and kidney-derived Gpx3 concentrations were also strongly reduced [51]. Testis and brain Se levels and brain selenoprotein expression were also lower in liver-specific *Trsp*-knockout mice indicating that liver-derived circulating Sepp is indeed the Se transfer protein bringing Se to Sepp-dependent tissues [51]. These studies were later independently supported by another group, who conditionally inactivated *Sepp* in hepatocytes [52]. Kidney and brain Se levels were decreased in liver-specific *Sepp*$^{-/-}$ mice and after feeding these mice for a prolonged time with a Se-deficient diet, the mice developed a neurological phenotype. The dependence of testis on circulating Sepp was further demonstrated by the morphological abnormalities of sperm from liver-specific *Sepp*$^{-/-}$ mice which were also described in classical *Sepp*$^{-/-}$ mice [52]. In addition, urinary Se excretion was increased, most likely because excess hepatic Se (not incorporated into Sepp and secreted into plasma) became available for hepatic selenosugar biosynthesis and excretion.

What role does hepatic Sepp play in the absence of *Sepp* expression in the rest of the body? This question was addressed in a complementary mouse model, in which hepatocyte-specific expression of a human *SEPP1* transgene was studied in a *Sepp*$^{-/-}$ background [14]. Compared to *Sepp*$^{-/-}$ mice, those with liver-specific expression of *SEPP1* had increased Se concentrations in most tissues, were less sensitive to Se restriction in terms of neurological dysfunction, and had restored male fertility [14]. Nevertheless, without locally expressed Sepp, the brain remained more sensitive to

dietary Se restriction despite hepatically-derived circulating SEPP1. These observations support the concept of a local "Sepp-cycle" in brain and possibly other organs (Fig. 22.2).

22.4.4 Sepp in the Brain

The brain depends on Se supply via circulating Sepp [13, 14, 33, 52]. SEPP is locally expressed in the human brain [53, 54]. Lrp8 is expressed on neurons and along the blood-brain-barrier and is of importance for Se uptake by neurons [17], although Lrp8 may not represent the only Sepp receptor in the brain (see Chap. 36 for a detailed discussion). Megalin contributes to Se uptake along the blood-brain-barrier [6, 25]. The model of a Sepp-cycle in brain has recently obtained additional support when it was shown that lower Se levels in the brain were tolerated as long as both Sepp and ApoER2 were expressed in the brain [55] (Fig. 22.2). The roles of various selenoproteins in brain development, function, and degeneration are treated in Chap. 36. Interestingly, a recent mouse study highlighted a potential competition between Sepp-mediated Se supply to testes versus to brain, where one tissue may profit from the other tissues in cases where uptake is tissue-specifically impaired [56].

22.4.5 Sepp in the Kidney

The importance of Sepp for kidney Se status and metabolism has not been addressed by tissue-specific knockout studies of *Lrp2*, *Trsp* or *Sepp*. Inactivation of selenoprotein expression (conditional targeting of *Trsp*) in kidney (using a *Pax8-Cre*, which is expressed in the developing kidney and thyroid) was lethal [57], possibly because loss of Gpx4 leads to ferroptosis in kidney tubules [58]. Instead, the physiological role of Sepp for kidney Se can be deduced from a number of findings in the aforementioned Sepp-specific mouse models. Kidney Se concentrations were strongly decreased in $Sepp^{-/-}$ mice. This finding implies that either hepatically-derived circulating Sepp transports Se to the kidneys or that renal Sepp biosynthesis itself is crucially important for controlling local tissue Se content [12, 13]. In order to solve this conundrum, hepatic or renal Sepp biosynthesis were specifically disrupted. Hepatocyte-specific inactivation of *Trsp* abrogated biosynthesis of all selenoproteins in hepatocytes and strongly reduced Sepp levels in plasma [51]. Kidney Se concentrations were decreased in this model and in hepatocyte-specific *Sepp*-deficient mice [52]. Reduced renal selenoprotein expression in $Sepp^{-/-}$ mice was rescued by hepatic expression of the human *SEPP1* transgene [14].

Lrp2 is abundantly expressed in the kidneys [59]. Lrp2/megalin has been implicated as a renal Sepp receptor [15], but $Lrp2^{-/-}$ mice analyzed in this pioneering

study did not survive birth, and thus a role of Lrp2 in renal Sepp uptake could not be directly demonstrated. We have taken advantage of a different *Lrp2* mutant mouse strain in which a significant fraction of *megalin*-deficient mice survive into adulthood. These mice carry a missense mutation in the extracellular domain of Lrp2 developing a less severe phenotype as compared to classical *Lrp2*$^{-/-}$ mice [40]. When Se metabolism was analyzed in these *Lrp2*-mutant mice, Se status was low, Gpx activities were decreased in kidney and serum, and Sepp concentrations were reduced in serum [25]. Movement coordination deteriorated in *Lrp2*-mutant mice when challenged by feeding a low Se diet. Interestingly, full-length Sepp was detected in the urine of these mice indicating, on the one hand, that Sepp is partially filtrated by the glomeruli into the primary urine and, on the other hand, that renal Lrp2/megalin recognizes, binds and removes Sepp from the primary urinary filtrate and prevents Sepp loss in wild type mice [25]. Based on this study, *megalin*$^{-/-}$ mice were extensively backcrossed on a genetic background which allows them to survive into adulthood and several of our initial observations have been supported by a more recent study [6]. Se metabolism in kidney is summarized in Fig. 22.2.

22.5 Regulation of Sepp Expression

Sepp gene expression has been studied in several cell types and experimental models. Proinflammatory cytokines such as interleukin-1α, TNFα, and interferon-γ suppress gene expression in cell lines involving activation of nitric oxide synthase-2 [60, 61], and TGFβ represses Sepp transcription by a SMAD-binding element in the proximal human promoter [62]. Similarly, interleukin-6 suppresses the Sepp promotor and down regulates Sepp biosynthesis in hepatocytes in culture [63]. Besides these cytokines, hypoxic conditions also negatively affect Sepp biosynthesis [64], which may collectively explain the particularly strong down-regulation of Sepp biosynthesis and Sepp serum concentrations in severe diseases like sepsis [65].

Recently, positive regulation of Sepp expression was also reported after activation of the forkhead box transcription factor, FoxO1a, and this effect was enhanced by overexpression of peroxisomal proliferator activated receptor-γ coactivator 1α (PGC-1α) [66]. These observations link Sepp expression to the glucose levels regulating hormones insulin and the adrenal glucocorticoids. In cultured hepatocytes, high glucose supported Sepp biosynthesis [67]. Accordingly, Sepp is proposed to serve as an insulin-antagonistic hepatokine, and higher Sepp concentrations were reported in diabetic subjects [68]. This study, unfortunately, has not addressed the established role of Sepp as a Se transport protein, and thus failed to consider Sepp-dependent stimulation of selenoprotein expression in insulin targeted tissues. The expression of selenoproteins in Sepp-target cells would have been important, since enhanced expression of Gpx1 is known to cause insulin resistance in mice [69]. Collectively, the role, if any, of Sepp in diabetes is a matter of current debate and research, and prospective clinical studies on its potential impact are missing.

22.6 Comparison of Experimental Concepts with Clinical Data

A systematic comparison of potential biomarkers of Se status in humans has been compiled and published [70]. In comparison to plasma or red blood cell Se concentrations or Gpx activities in serum, plasma or blood, SEPP concentration turned out as the more reliable and versatile biomarker of Se status [71–73]. However, due to its apparently saturated expression upon high Se intake, SEPP reliably reflects Se status in poorly or moderately supplied subjects only. In well supplied populations as found in the United States, SEPP concentrations do not correlate to Se intake or total serum Se concentrations, as maximal levels are already achieved in the majority of subjects [74].

Recent population-based studies on the associations between various cancer forms and SNPs of genes encoding for selenoproteins and enzymes involved in metabolism of ROS provided some evidence that *SEPP1* variants (Ala234Thr, rs3877899; G>A in 3′UTR, rs7579) affect Se availability to target tissues such as prostate or colon thereby modulating cancer risk in the context of other gene-gene (e.g., SOD2), gene-nutrient or lifestyle interactions [75–78]. Its direct contribution to colorectal cancer risk in moderately supplied subjects has just been determined in the European prospective investigation of cancer and nutrition cohort (EPIC), where higher SEPP concentrations were inversely associated with colorectal cancer risk, especially in women [79]. Decreased expression of *SEPP* mRNA and SEPP protein has been reported for several preneoplastic and cancerous lesions, e.g., of the respiratory and the GI tracts (gastric, colorectal and hepatic cancers) as well as for the prostate [80–83]. This down-regulation may limit Se loss from tumor cells causing an apparent Se accumulation in tumors. Accordingly, the first clinical study (SECAR) has tried to selectively poison tumors by high dose selenite treatment of cancer patients [84], and determined a maximally tolerated dose of around 10 mg Se/square meter body surface. It will be most interesting to monitor SEPP concentrations under such high dose supplementation attempts, and assess how far Se accumulation by tumor cells qualifies as a novel cancer treatment strategy.

Unfortunately, SEPP status has not been monitored in several other relevant and large cancer, cardiovascular or metabolic trials (e.g., SELECT, NPC, SU.VI.MAX, SETCAP, etc.) analyzing potential protective or therapeutic effects of supplementation with selenocompounds alone or in combination with other antioxidative compounds such as vitamin E [85–87]. The recent availability of several specific antibodies recognizing human SEPP helped to clarify the picture of systemic SEPP distribution vs. local production indicated by cellular expression of *SEPP* transcripts. These studies confirmed previous hints that SEPP reaches various tissues and cells via circulation, but also provided evidence for local SEPP production and secretion, e.g., within the cerebrospinal fluid (CSF) and in brain ependymal cells [53, 54]. SEPP expression and immunostaining showed specific spatial and temporal patterns during brain development and pathological alterations in brains from patients suffering from neurodegenerative diseases such as

Alzheimer's [88, 89]. Together, these observations suggest a strategic location of SEPP in brain potentially protecting cell types of high activity and functional relevance from Se deficiency, thereby ensuring regular development, differentiation and expression of Se-dependent antioxidative defense systems.

Impaired renal function and chronic hemodialysis markedly impact serum SEPP and Se homeostasis in patients with consequences on their thyroid hormone status [90]. Patients on chronic hemodialysis and apheresis develop Se deficiency and frequently receive Se supplements [91]. Currently, it is not clear, whether they lose SEPP or its (shorter) isoforms during the filtration process or whether their damaged renal tissue expresses insufficient Lrp2/megalin for adequate reabsorption of filtrated SEPP. Since a low Se status is a negative prognostic factor for long-term survival of chronically ill patients, it appears mandatory to control the trace element status of hemodialysis patients in order to avoid severe Se deficiency [92].

Besides cancer, chronic and degenerative diseases, the Se status and SEPP are implicated in male fertility, which is reviewed in detail in Chap. 18. An inherited defect causing impaired SEPP biosynthesis and low circulating SEPP concentrations has recently been described in humans. Individuals with certain mutations in *SECISBP2* display very low or undetectable levels of SEPP [93]. Interestingly, some of these subjects are reported with mental retardation and abnormal gait [94] or delayed neurological and motor skill milestones [95]. However, apparent SEPP-deficiency in patients carrying *SECISBP2* mutations does not lead to a phenotype as severe as in *Sepp*$^{-/-}$ mice, indicating that brain Se metabolism is not completely impaired in these individuals which is in line with some detectable, albeit considerably lower levels of circulating SEPP. In contrast, severe neurological symptoms involving brain atrophy and epilepsy were reported in patients carrying a mutation in another rate-limiting factor of selenoprotein biosynthesis, i.e., the Sec synthase gene, *SEPSECS* [96]. However, Se and SEPP status in the CSF have not been determined in *SEPSECS*-deficient patients, but are likely reduced.

22.7 Concluding Remarks

The hepatically-derived SEPP constitutes the Se transport form in blood circulation. Preferential supply to certain tissues like brain, testes, bone or muscles is achieved via SEPP recognition and specific uptake mediated by Sepp-receptors from the Lrp family, namely Lrp2 and Lrp8. This interaction not only ensures the hierarchical supply, but also an efficient retention of Se in prioritized tissues. Moreover, renal Lrp2 is essential for avoiding Se decline through Sepp loss via the urine. Pharmacological intervention of these endocytic receptor/Sepp interactions might become a therapeutic option in certain forms of male infertility, neurodegenerative disorders or in patients exposed to excess oxidative stress, e.g., during various forms of chemotherapy, systemic inflammation, bacterial or viral infections. However, no endogenous or synthetic modulators of Sepp/Sepp receptor interaction have been described so far.

One of the important experimental tasks in the future will thus be the molecular characterization of the different Sepp isoforms, their physiological functions, regulation and interaction with the different Sepp-receptors. Of particular interest is also the relation between Sepp-dependent delivery of Se to the kidneys in comparison to local recycling, biosynthesis and secretion of renal-derived Gpx3, which constitutes the second selenoprotein significantly contributing to circulating blood Se content. Our current knowledge is limited with regard to expression and regulation of Sepp during development, Se compartmentalization and supply to tissues and cells not depending on Sepp and devoid of the Lrp receptors. Nevertheless, with the identification of Sepp as the major transport, distribution and storage protein for Se, and the characterization of receptor-mediated tissue-specific uptake processes, a number of previous enigmatic findings are now explained by a plausible molecular pathway. SEPP's role as a functional biomarker reliably reflecting Se intake and Se status has become widely accepted. Now, both more experimental and clinical efforts are needed to apply this knowledge and the increasing number of available technologies for SEPP quantification to better understand its role in disease prevention and health support.

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Chapter 23
Selenoprotein T: From Discovery to Functional Studies Using Conditional Knockout Mice

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Abstract Selenoprotein T (SELT) is a thioredoxin-like enzyme that exerts an essential oxidoreductase activity in the endoplasmic reticulum during development and after tissue injury where its expression is highly induced. Disruption of the *Selt* gene is lethal during embryogenesis, and its conditional knockout in the brain causes the reduction of several cerebral structures and increases the vulnerability of mice to neurotoxin-induced neurodegeneration. While its expression is silenced in most tissues in the adult, SELT persists at high levels in endocrine tissues such as the pancreas where it controls hormone production. Thus, SELT could be involved in the redox circuits that control homeostasis and survival of cells with intense metabolic activity during development or in adult endocrine and lesioned cerebral tissues.

Keywords Brain • Endocrine tissues • Endoplasmic reticulum • Knockout mice • Oxidoreductase • Selenoprotein T • Thioredoxin-like

23.1 Introduction

Among the different selenoproteins with no known function that are currently in the spotlight, selenoprotein T (SELT) has gained a significant interest as a member of the thioredoxin-like family. SELT is among the first selenoproteins identified through a bioinformatics approach that enables the search for selenoprotein genes on the basis of their primary and secondary structures as well as free energy requirements of a stem-loop structure, which is only present in the 3′-untranslated region of the mRNAs encoding

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selenoproteins. This latter structure named the selenocysteine (Sec) insertion sequence (SECIS) element allows the decoding of an in frame UGA codon as a Sec in selenoproteins. The search for this sequence and the other characteristic features in available databases of human expressed sequence tags from various tissues allowed for the first time the identification of the two selenoproteins SELT and SELR [1]. The latter selenoprotein is currently known as methionine-R-sulfoxide reductase B1 [2], and has been linked to methionine sulfoxide redox control in different proteins [3]. Indeed, redox control of methionine oxidation has emerged as a novel post-translational mechanism that alters protein structure and function, as for example actin assembly/disassembly [4]. In contrast to the latter selenoprotein, very few studies have been performed to further characterize SELT since its discovery. Using a screen for genes involved in the neurotrophic effects of the pituitary adenylate cyclase-activating polypeptide (PACAP), a neuropeptide with prosurvival and differentiating effects in neuronal cells [5], we identified SELT as a PACAP-stimulated gene during PC12 cell differentiation [6]. Because PACAP exerts strong antioxidant activity, we postulated that SELT could play a major role in this effect of the neuropeptide through its Sec-containing redox center.

23.2 Structure, Subcellular Localization and Activity

Molecular cloning revealed that the *Selt* cDNA sequence encompasses 970 nucleotides encoding a protein of 195 amino acids with a calculated mass of 22.3 kDa [6]. Bioinformatic analyses predicted a 19 amino acid signal peptide and highly hydrophobic stretches of amino acids at positions 87–102 and 125–143 [6]. These hydrophobic amino acid sequences may represent transmembrane domains (Fig. 23.1) and may be required for anchoring SELT to the endoplasmic reticulum (ER), since their disruption resulted in a diffuse cytoplasmic labeling of a transfected protein in PC12 cells [6, 7]. Modeling studies suggest that these hydrophobic segments contain amphipathic helices that interface with the ER membrane allowing partial binding and insertion of SELT [8]. Studies using bioinformatics algorithms confirmed that SELT is a membrane protein [9]. Along with confocal and electron microscopy studies ([6, 10] and Hamieh et al., manuscript in preparation), these observations indicate

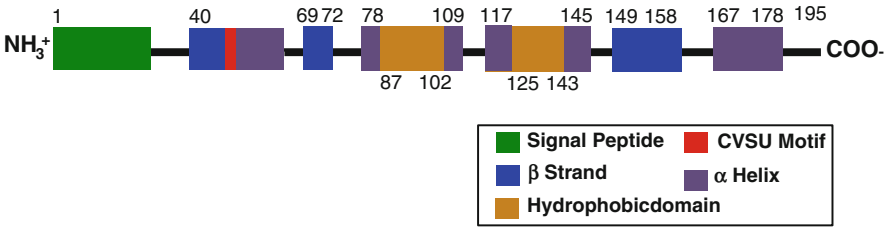

Fig. 23.1 Schematic structure of SELT. A bioinformatic analysis of the SELT amino acid sequence revealed the occurrence of several secondary structures and hydrophobic domains throughout the protein, in accordance with the structural organization of thioredoxin-like proteins [12]

that SELT is localized to the ER membrane where it can interact with and modify other thiol-containing proteins through its Sec active moiety. The Sec residue is located at position 49 in the N-terminal part of the protein and is separated from an upstream cysteine by two amino acids, thus forming a CVSU motif which represents a putative redox center as found in several other redox-active selenoproteins [11]. This redox site is comprised between predicted β-strand and α-helix secondary structures (Fig. 23.1), a topology also found in other redox proteins with a classical thioredoxin fold (CxxC), such as thioredoxins, glutaredoxins, and disulfide isomerases [12, 13]. Based on these structural properties, SELT, along with five other selenoproteins, i.e., SELM, SEP15, SELV, SELH and SEPW, which display a similar thioredoxin-like domain, have been grouped in a subclass of selenoproteins named redoxins [12, 14]. The CxxC or CxxU motifs are key for various functions of selenoproteins [11]. Thus, selenoproteins with a thioredoxin-like domain may exert glutathione peroxidase activity as demonstrated for SELH [15], or protein folding activity, as shown for SEPW and SEP15 [16]. Using a recombinant protein, we have recently shown that SELT exerts a thioredoxin reductase-like activity since it was able to reduce 5,5′-dithio-bis(2-dinitrobenzoic acid) (DTNB) to 5-thio-2-nitrobenzoic acid in the presence of NADPH. This activity was dependent on the redox center of SELT and was inhibited by aurothiomalate, a specific inhibitor of thioredoxin reductase activity [17]. In contrast, SELT did not display a glutathione peroxidase activity. This initial study established the oxidoreductase activity of SELT in vitro but its substrates and mode of action in vivo are still unknown.

Because the SELT amino acid sequence is extremely well conserved during evolution (Fig. 23.2), we speculate that this selenoprotein has an important enzymatic function. Of note, SELT is also present in protozoa and plants, but the Sec is replaced by a Cys in these organisms. Sequence alignment analysis of SELT with the other redoxins SEPW, SELH and SELV showed no significant homologies, albeit these proteins exhibiting a similar pattern of predicted secondary structures, with an additional central α-helix domain in SELT, indicating that redoxins are probably distant homologs [12].

23.3 Tissue-Distribution and Regulation of Gene Expression

Initial bioinformatics screens of EST clones from adult human tissues revealed a weak incidence of *Selt* clones, indicating a low expression level of this selenoprotein [1]. Only infant brain and placenta databases displayed an incidence of 1 per 10,000 for *Selt* ESTs. This is consistent with our initial studies which identified *Selt* in a mouse embryonic/placental cDNA library [5]. An in situ hybridization analysis performed on rat embryonic tissues confirmed the high and widespread expression of the *Selt* gene early during development [6]. This analysis at the level of gene expression was confirmed by immunohistochemistry and Western blotting, which revealed an abundance of SELT during embryogenesis [18]. In contrast, SELT levels were very low after birth (Fig. 23.3), except in endocrine tissues such as the pituitary, thyroid, testis or pancreas [10, 18]. In the adult brain, the overall expression is low but SELT is highly induced in some cells endowed with plasticity such as Bergmann

```
Mus musculus      MR-------LLLLLLVAASAVVRSEASANLGGVPSKRLKMQYATGPLLKFQICVSUGYRR 53
Rattus norvegicus MR-------LLLLLLVAASAVVRSEASANLGGVPSKRLKMQYATGPLLKFQICVSUGYRR 53
Homo sapiens      MR-------LLLLLLVAASAMVRSEASANLGGVPSKRLKMQYATGPLLKFQICVSUGYRR 53
Bos taurus        MR-------LLLLLLVAASAVVRSDASANLGGVPGKRLKMQYATGPLLKFQICVSUGYRR 53
Xenopus tropicalis MARSSG--PLCLLLLGGLVAGILSGASADGNGLPSKKLKMQYTAGPLLKFQICVSUGYRR 58
Gallus gallus     MRAAGLGLGIGLLLLAALAG---PGGSAEQGGVPAKKLRMAYATGPLLKFQICVSUGYRR 57
Danio rerio       MG------KMRWLPFSALLLWALCLHSASADNNGVKKMKMQFATGPLLKFQICVSUGYKR 54

Mus musculus      VFEEYMRVISQRYPDIRIEGENYLPQPIYRHIASFLSVFKLVLIGLIIVGKDPFAFFGMQ 113
Rattus norvegicus VFEEYMRVISQRYPDIRIEGENYLPQPIYRHIASFLSVFKLVLIGLIIVGKDPFAFFGMQ 113
Homo sapiens      VFEEYMRVISQRYPDIRIEGENYLPQPIYRHIASFLSVFKLVLIGLIIVGKDPFAFFGMQ 113
Bos taurus        VFEEYMRVISQRYPDIRIEGENYLPQPIYRHIASFLSVFKLVLIGLIIVGKDPFAFFGMQ 113
Xenopus tropicalis VFEDYMRVISQRYPDIRIEGENYLPHPIYRNIASFLSVFKLVLIGLIIAGKDPFAFFGMQ 118
Gallus gallus     VFEEYMRVISQRYPDIRIEGENYLPQPIYRHIASFLSVFKLVLIGLIIVGKDPFAFFGMQ 117
Danio rerio       VFEEYTQALYQRYPDIRIEGENYLPLPLYRHIASFLSMFKLLLIGVIILGKDPFALCGMQ 114

Mus musculus      APSIWQWGQENKVYACMMVFFLSNMIENQCMSTGAFEITLNDVPVWSKLESGHLPSMQQL 173
Rattus norvegicus APSIWQWGQENKVYACMMVFFLSNMIENQCMSTGAFEITLNDVPVWSKLESGHLPSMQQL 173
Homo sapiens      APSIWQWGQENKVYACMMVFFLSNMIENQCMSTGAFEITLNDVPVWSKLESGHLPSMQQL 173
Bos taurus        APSIWQWGQENKVYACMMVFFLSNMIENQCMSTGAFEITLNDVPVWSKLESGHLPSMQQL 173
Xenopus tropicalis APSVWQWGQENKVYACMMVFFVSNMIENQCMSTGAFEITLNDVPVWSKLESGHLPSVQQL 178
Gallus gallus     APSIWQWGQENKVYACMMVFFLSNMIENQCMSTGAFEITLNDVPVWSKLESGHLPSMQQL 177
Danio rerio       APGIWVWSQENKIYACMMVFFFSNMIENQCMSTGAFEITLNDVPVWSKLESGHLPSMQQL 174

Mus musculus      VQILDNEMKLNVHMDSIP-HHRS 195
Rattus norvegicus VQILDNEMKLNVHMDSIP-HHRS 195
Homo sapiens      VQILDNEMKLNVHMDSIP-HHRS 195
Bos taurus        VQILDNEMKLNVHMDSIP-HHRS 195
Xenopus tropicalis VQIIDNEMKLNVHMDAIPHHHRS 201
Gallus gallus     VQILDNEMKLNVHMESMP-HHRS 199
Danio rerio       VQILENEMKMSMHMDTLP-PHQS 196
```

Fig. 23.2 Sequence alignment of SELT in various species. Sources of sequences are indicated on the *left side* of the figure. The amino acid sequence of SELT is highly conserved during evolution. The putative redox center is *boxed*, and the amino acids are represented according to their nature (hydrophobic, in *red*; hydrophilic, in *green*; acidic, in *blue*; and basic, in *pink*)

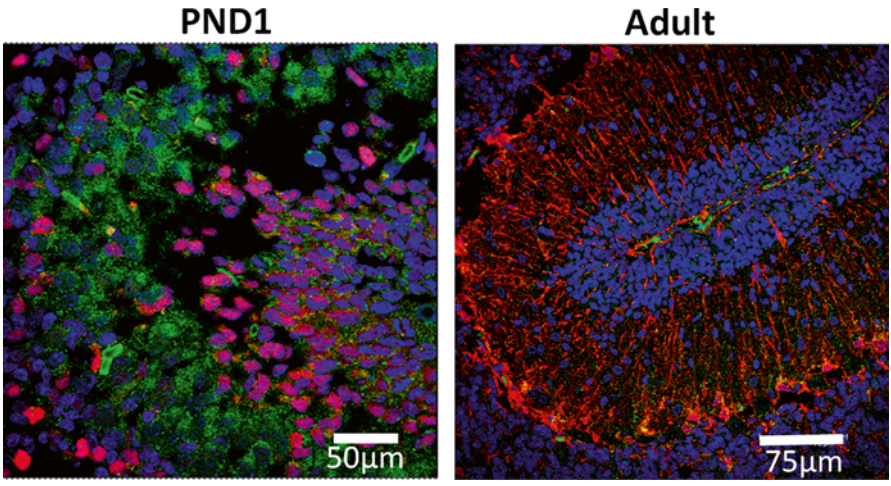

Fig. 23.3 SELT immunoreactivity in the cerebellum at postnatal day 1 (PND1) and adulthood. Note that SELT is very abundant in differentiating neurons at PND1, but is undetectable in adult cerebellar tissue. SELT labeling is in *green*, neuron labeling is in *red* (NeuN) and nuclei are in *blue* (DAPI)

glial cells in the cerebellum or the rostral migratory stream glial cells in the olfactory bulb [18]. In endocrine glands, SELT is strongly expressed in secretory cells as revealed by its localization with secretory markers, i.e., chromogranins or insulin [10, 18]. In the testis, SELT is found in the testosterone-producing Leydig cells, and also in the proliferating and differentiating spermatogenic cells. In support of a role of selenoproteins in endocrine tissues, high amounts of selenium have been found in secretory cells of the thyroid, and selenium supplementation has been shown to be protective for Leydig cells [19].

The mechanisms underlying the differential expression of the *Selt* gene in various tissues have not been elucidated. However, SELT expression was previously shown to be regulated by intracellular cAMP and Ca^{2+} levels, which are stimulated by the trophic peptide PACAP in neuroendocrine cells [6]. It is thus possible that *Selt* gene expression could be induced by regulatory cues in certain physiological or pathophysiological conditions in order to fulfill its roles. In general, the molecular mechanisms underlying the transcriptional regulation of selenoprotein genes in different tissues remain largely unknown. We have recently undertaken a study to elucidate the signaling pathways converging at the *Selt* gene promoter to regulate its transcription in response to PACAP and cAMP elevation. This study uncovered a hitherto unknown pathway linking cAMP and protein kinase A (PKA) to AMP-activated kinase and peroxisone proliferator-activated receptor gamma coactivator 1-alpha (PGC1-α), a major switch in metabolic regulations, which controls *Selt* gene transcription *via* nuclear respiratory factor 1 (NRF-1) binding sites present in the *Selt* gene promoter, in the pheochromocytoma (PC12) cell line derived from the rat adrenal gland (Fig. 23.4). Interestingly, PACAP and cAMP promote mitochondriogenesis in these cells through a common pathway, indicating that in neuroendocrine cells a common pathway couples mitochondrial biogenesis and the antioxidant response involving SELT (Abid et al., manuscript in preparation). Beside this original mechanism, other pathways could be at play in different cells or conditions, which remain to be elucidated. Indeed, we have also observed that *Selt* gene transcription could be induced in neurons and astrocytes upon neurotoxin treatment and oxidative stress condition but the molecular mechanisms involved in this up-regulation are unknown. Elucidating these mechanisms could help understand the underpinnings of the cell response to stressful conditions such as during high metabolic activity, and may have therapeutic implications for various diseases associated with cell stress.

23.4 Function

The subcellular localization of SELT determined by confocal and electron microscopy [5, 10] and the potent oxidoreductase activity of the recombinant protein [17] strongly suggest that SELT exerts a key redox function that controls protein processing in the ER, allowing cells to cope with oxidative stress and to ensure ER homeostasis. Akin to other selenoproteins in the ER, such as SELK, SELS or SEP15 [20–22], SELT may affect thiol redox circuits through its thioredoxin reductase-like activity, including other antioxidant enzymes such as peroxiredoxins or glutathione peroxidases, thus

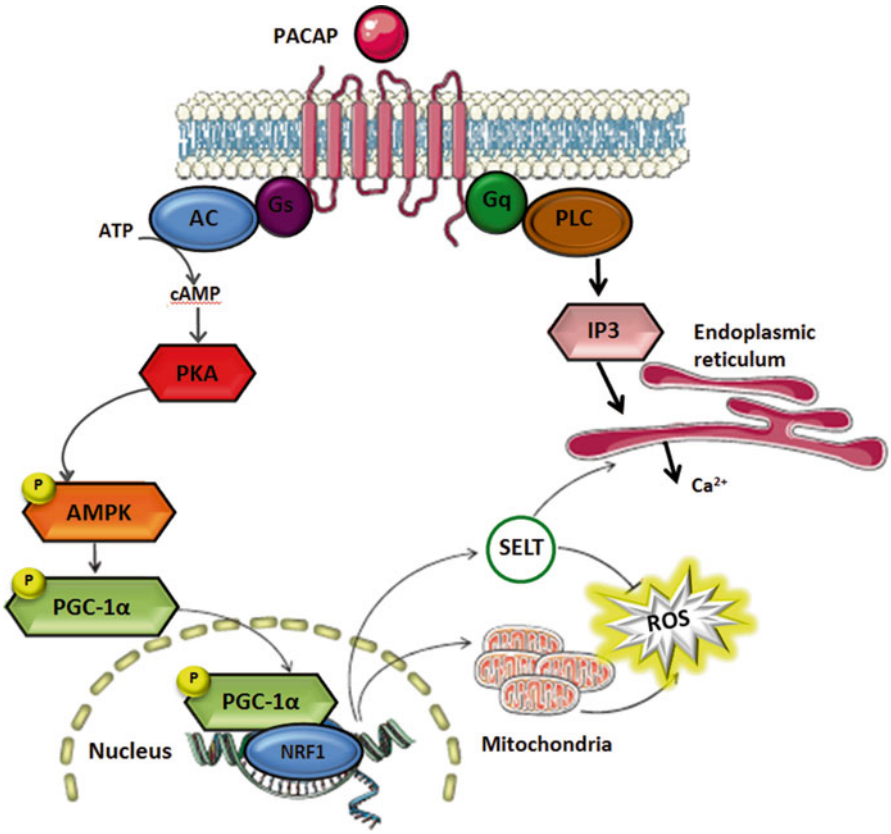

Fig. 23.4 Signaling pathways involved in PACAP- and cAMP-induced *Selt* gene transcription in PC12 cells. *Selt* gene transcription is coupled to mitochondriogenesis, ROS inhibition and Ca^{2+} mobilization from the ER during PACAP-promoted PC12 cell survival and differentiation

impacting various cellular processes including differentiation and survival. The fact that the *Selt* gene is well conserved during evolution and that its knockout leads to early embryonic lethality argues for a pivotal role of this selenoprotein [17]. It is interesting to note that so far four selenoproteins, i.e., two thioredoxin reductases, one glutathione peroxidase and SELT have been shown to be essential on the basis of gene knockout studies [23–25]. Although the mechanism of action of SELT is not known yet, recent findings highlight different effects of SELT in various physiological and pathophysiological conditions, during development and in adult, which are summarized below.

23.4.1 Role in Ca^{2+} Regulation and Cell Adhesion

Before the availability of knockout mice, the first studies on the role of SELT were performed in various cell lines, which suggested that this selenoprotein may exert important cellular functions. Indeed, we have identified *Selt* as a PACAP-stimulated

gene during PC12 cell differentiation, indicating that this selenoprotein could be involved in pro-differentiating and pro-survival effects exerted by the neurotrophic factor [5, 6]. Given the localization of SELT in the ER, a major source of intracellular Ca^{2+}, the involvement of this intracellular messenger in *Selt* gene expression, and the fact that Ca^{2+} is involved in the effects exerted by PACAP during neuronal cell differentiation [26], we reasoned that SELT may influence Ca^{2+} regulation in these cells. Using Ca^{2+} microfluorimetry analysis, SELT overexpression was shown to increase intracellular Ca^{2+} levels and, inversely, silencing the *Selt* gene impaired PACAP-induced Ca^{2+} mobilization from intracellular and extracellular sources. These results suggested that SELT may interact directly or indirectly with thiol groups of intracellular Ca^{2+} channels and pumps to regulate their activity through a redox mechanism [6]. This hypothesis is supported by the increasing evidence showing the involvement of different selenoproteins present in the ER such as SEPN1, SELK or SELM in the regulation of Ca^{2+} homeostasis and Ca^{2+} channel function [27–31].

In mouse fibroblast cells, *Selt* knockdown altered cell adhesion and the expression of genes involved in cell morphology and oxidative stress, suggesting the involvement of SELT in cell adhesion and the control of redox homeostasis [32]. It remains to be established whether free radicals and/or Ca^{2+} regulation are involved in these effects of SELT.

23.4.2 Role in Brain

The spatiotemporal distribution of SELT in the brain was determined by immunohistochemistry and Western blotting [18]. SELT was particularly abundant in the brain at different embryonic stages but was almost undetectable in the adult (Fig. 23.3). During brain development, SELT was present at high levels in both proliferating and differentiating neuronal precursors and its expression was maintained in immature neurogenic and gliogenic cells at different stages. SELT was particularly concentrated in the forebrain (neocortex and thalamus), the midbrain, and the hindbrain. For instance, a prominent immunopositive signal was observed in the olfactory bulb, particularly in the mitral cell layer, in the cerebral periventricular cells or in the cerebral neocortex. Interestingly, SELT expression was detected by Western blotting in the cerebellum at all the postnatal stages examined, including adult. SELT is expressed in neurons of the external germinative layer, particularly in Bergmann cells. An intense SELT immunoreactive signal was also observed in the developing Purkinje cell layer, but this expression declined in the adult differentiated Purkinje cells [18]. These data strongly suggest that SELT fulfills an important role during brain ontogenesis. To determine the physiological function of SELT in the developing and adult brain, we used a conditional knockout mouse line in which *Selt* was specifically disrupted in the nervous system. Morphometric analyses revealed that these mice exhibited reduced volumes of different brain structures, a phenomenon that was observed early during the first postnatal week [33]. This phenotype was associated with loss of immature neurons but not glial cells, which could be due to the elevated levels of intracellular reactive oxygen species

that were observed in immature neurons. However, developmental compensatory mechanisms may operate later during development, since brain volume alterations were attenuated in adult mice. Despite this morphological compensation, *Selt* knockout mice exhibited a hyperactive behavior, suggesting that SELT deficiency leads to cerebral malfunction in adulthood (see Chap. 36 on the function of other selenoproteins in brain). Thus, these data indicate that SELT is protecting neurons during their differentiation and is required later for proper behavior in adult, two phenomena that are probably related since alteration of neuronal differentiation may disrupt the circuits that control brain functions. These findings are reminiscent of the effects of PACAP which stimulate *Selt* expression [6, 18], suggesting a possible role of SELT in mediating, at least partially, the neurotrophic and behavioral effects of the neuropeptide [33].

The fact that SELT could be associated with oxidative stress control and neuronal survival prompted us to study its role in mouse models of Parkinson's disease (PD) [17]. In this neurodegenerative disease, oxidative stress is central to the process of dopaminergic neuron demise, and a role of selenoproteins in the protection of these neurons has been suggested [34, 35]. In the dopaminergic SH-SY5Y cell model, SELT promoted cell survival and inhibited oxidative stress provoked by the neurotoxin 1-methyl-4-phenylpyridinium (MPP^+) [17]. In wild type mice, treatment with PD-inducing neurotoxins such as 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) or rotenone triggered SELT expression in dopaminergic neurons and fibers, as well as astrocytes of the nigrostriatal pathway, indicating that SELT is probably mobilized in both cell types to prevent neurodegeneration. Treatment of brain *Selt* knockout mice with the same neurotoxins provoked rapid and severe parkinsonian-like motor impairment as compared to control wild type littermates, which was associated with marked oxidative stress and dopaminergic neurodegeneration (Fig. 23.5). These effects could be ascribed to the reduced tyrosine hydroxylase activity and dopamine levels observed in the nigrostriatal system after neurotoxin administration. These data showed that SELT plays an instrumental role in the protection of dopaminergic neurons against oxidative stress and cell death, thus preventing early and severe movement impairment in animal models of PD (Fig. 23.6). In a pilot study using PD patient brain specimens, an increase in *SELT* mRNA and protein levels was observed in the caudate putamen tissue, suggesting that this selenoprotein is involved in PD in human patients [17]. Further studies are required to determine the clinical value of SELT as a marker and/or a therapeutic target in this and other neurodegenerative diseases.

23.4.3 Role in Pancreas

In contrast to several tissues where SELT is undetectable after birth, tissue-distribution studies revealed the persistence of high expression of SELT in adult endocrine organs including pituitary, pancreas, testis, and thyroid [18]. Because SELT was particularly abundant in endocrine pancreatic cells, in particular insulin- and somatostatin-producing cells, conditional pancreatic β-cell-specific *Selt* knockout mice were generated [10] in order to elucidate the role of this selenoprotein in

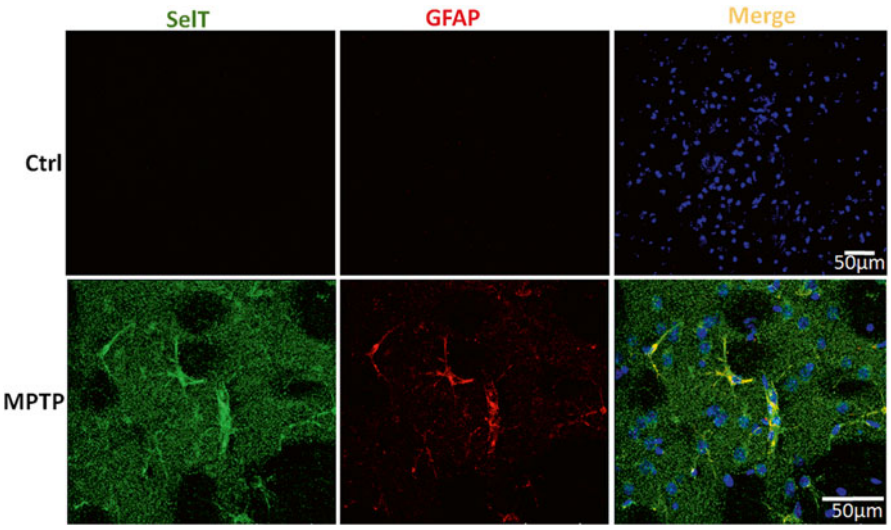

Fig. 23.5 SELT immunoreactivity in the striatum of control and MPTP-treated mice. Induction of SELT occurs in dopaminergic fibers and astrocytes. SELT immunoreactivity is in *green* and GFAP labeling of astrocytes is in *red*

the endocrine pancreas. These knockout mice displayed impaired glucose tolerance, which is likely due to altered insulin synthesis or release as revealed by the higher plasma glucose–to-insulin ratio in knockout mice compared to littermate controls. In contrast, SELT deficiency did not modify insulin sensitivity as indicated by similar levels of plasma glucose in mutant and control mice following insulin injection. SELT was not only found to be involved in glucose homeostasis, but also in the structural integrity of the endocrine pancreas, since islet morphology was altered and displayed a smaller size in knockout mice compared to their wild type littermates [10]. These results revealed for the first time the involvement of this thioredoxin-like protein in the regulation of insulin production and secretion, and hence, in the control of glucose homoeostasis. Our findings are consistent with the data reported by Labunskyy et al. [36], which showed that both overexpression and deficiency of selenoproteins can promote diabetes (see Chap. 49). Although several studies pointed to the role of thioredoxins in the control of β-cell function by protecting pancreatic cells against oxidative injury [37–39], the interplay of different thioredoxin-related proteins including SELT in the regulation of glucose homeostasis is not fully understood and deserves further investigation.

23.4.4 Role in Liver

Liver displays the particular characteristic of regeneration after partial hepatectomy. Hepatocytes, which make up 80 % of the liver parenchyma, proliferate after resection in order to regenerate the missing tissue. SELT was not detected in normal liver,

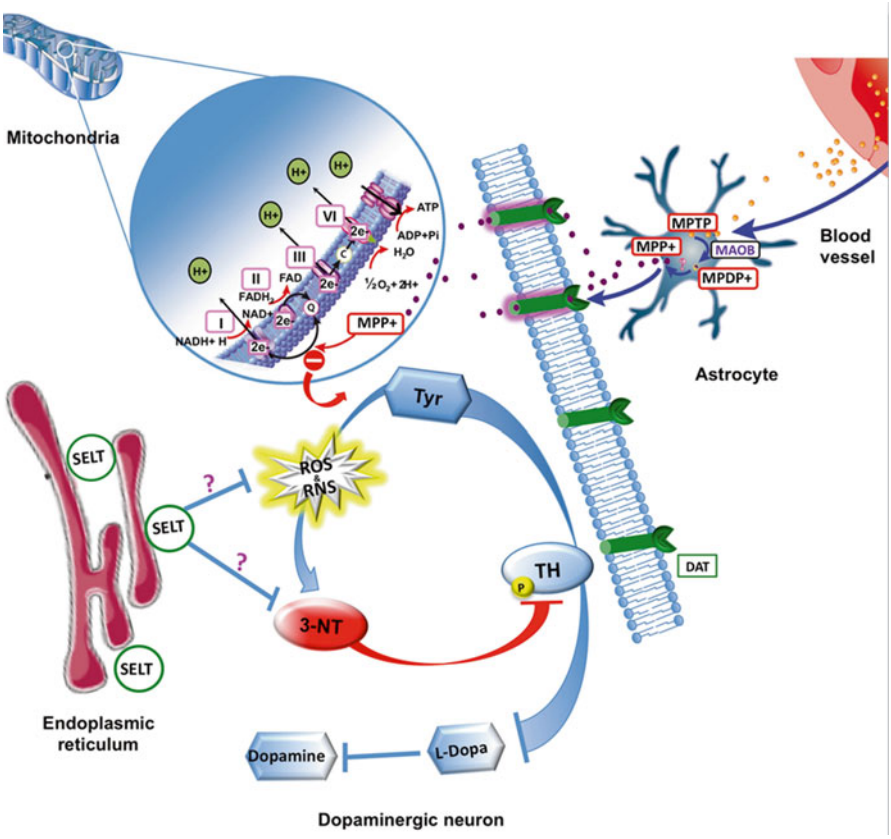

Fig. 23.6 Schematic representation of the effect of SELT in dopaminergic neurons following MPTP administration to mice. The neurotoxin is taken from blood to astrocytes where it is converted to MPP^+ by monoamine oxidase B (MAOB). MPP^+ is transported to neurons via dopamine transporters (DAT), where it blocks mitochondrial complex I, thus leading to oxidative stress. Free radicals which are inhibited by SELT provoke the accumulation of 3-nitrotyrosine and thus, tyrosine hydroxylase inactivation and dopamine decline. SELT protects dopaminergic neurons in these conditions [17]

but was strongly induced during the acute phase of liver regeneration, in line with the high expression of this selenoprotein in proliferating and differentiating cells [18]. This is reminiscent of the induction of the selenoenzyme deiodinase 3 in regenerating liver, which converts thyroid hormones into inactive metabolites effectively inhibiting their pro-differentiating activity and to facilitate thereby regeneration [40]. The role of SELT during regeneration remains to be elucidated, but the fact that its induction occurs mainly in Kupffer cells suggests that this selenoprotein could contribute actively to the production and secretion of paracrine factors from these cells such as the cytokines TNF-α, IL-6 or TGF-β1 which modulate hepatocyte proliferation during liver regeneration [41].

23.5 Concluding Remarks

Data available so far show that SELT is a redoxin with a thioredoxin-like fold, localized mainly in the ER and endowed with a thioredoxin reductase-like activity. Embryonic lethality in mice caused by knockout of the *Selt* gene suggests that SELT could be involved, through its thioredoxin-like domain, in different processes underlying cell homeostasis and survival. This activity of SELT could play an essential role in the various posttranslational modifications involving disulfide bridge formation in proteins in transit through the ER. Thus, SELT may affect thiol redox circuits that could impinge on the synthesis and modification of essential cues, whose impairment in the absence of SELT would lead to dysfunctional redox control, reactive oxygen species accumulation and cell death. High SELT expression in proliferating and differentiating cells during development, like in the brain, or in adult cells endowed with some plasticity such as endocrine cells implies that this selenoprotein is associated with intense cell metabolism and confers an advantage to these cells in order to prevent and/or to cope with oxidative stress associated with this state. In addition, the *Selt* gene is highly induced during dopaminergic neurodegeneration, liver regeneration or after brain hypoxia [42], indicating that SELT is required to protect injured tissues in the adult. It is interesting to note that *Selt* gene regulation in neuroendocrine cells recruits molecular signaling components, including AMP-activated kinase and PGC1-α, that control mitochondrial enzyme genes and mitochondriogenesis, indicating that SELT is integrated in cell metabolic pathways, which couple energy supply and the antioxidant response.

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Chapter 24
Biochemistry and Function of Methionine Sulfoxide Reductase

Byung Cheon Lee

Abstract Reactive oxygen species (ROS) oxidize methionine to a mixture of methionine-*S*-sulfoxide and methionine-*R*-sulfoxide. Methionine is also oxidized by various flavin-containing monooxygenases in a partially or fully stereospecific manner. Methionine sulfoxide can be reduced by methionine sulfoxide reductase (Msr) proteins in four different families. Both free and protein-based forms of methionine-*S*-sulfoxide are reduced by MsrA, whereas protein-based methionine-*R*-sulfoxide is reduced by MsrB. Among the three known mammalian MsrBs, only MsrB1 is a selenoprotein, containing a selenocysteine residue in place of the catalytic cysteine. Free methionine-*R*-sulfoxide reductase (fRMsr) reduces methionine-*R*-sulfoxide in the free, but not protein-based state, and is found only in unicellular organisms, whereas MsrA and MsrB are present in organisms in all three kingdoms of life. MsrP reduces both methionine-*S*-sulfoxide and methionine-*R*-sulfoxide, particularly in membrane-embedded proteins, with assistance from MsrQ, which is involved in electron transport in the mitochondrial respiratory chain. MsrP cannot utilize thioredoxin as a reducing agent. Msrs are oxidoreductases that protect against the effects of oxidative stress by increasing oxidative stress resistance and repairing damaged proteins via cyclic methionine oxidation/reduction. In addition to its two main functions, MsrB1 reversibly regulates actin assembly in conjunction with Mical, making methionine oxidation similar to other reversible posttranslational modifications. This finding highlights a new era in the understanding of Msr function and should facilitate further studies of the physiological role of Msrs.

Keywords Antioxidant • fRMsr • Methionine sulfoxide • Mical • MsrA • MsrB • MsrB1 • MsrPQ

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24.1 Introduction

Molecular oxygen is the second most common gas in the Earth's atmosphere and is essential to all aerobic organisms that engage in cellular respiration. Biologically, molecular oxygen is often associated with the generation of reactive oxygen species (ROS), which are harmful to organisms and often promote the development of many disorders, such as cancer, neurodegeneration, and acceleration of aging [1]. ROS are chemically reactive molecules, and their chemical reactivity can directly damage macromolecules such as DNA, lipids, and proteins. All amino acids in proteins are subject to ROS-induced oxidative damage, which can lead to structural and functional changes [2]. Methionine is one of the most oxidation-sensitive of the 20 amino acids commonly found in proteins due to the sulfur atom in the side chain. Thus, the sulfur atom in methionine is preferentially oxidized in the frontline of defense against oxidative stress [3]. A specific reductase enzyme known as methionine sulfoxide reductase (Msr) reverses the effect of methionine oxidation by reducing methionine sulfoxide back to methionine [4]. Interestingly, of the three known mammalian MsrBs, only MsrB1 is a selenoprotein containing a selenocysteine residue. Thus, selenium biology must be considered in order to more completely understand the function of Msr [5]. In this chapter, we will focus on the enzymatic system responsible for cyclic methionine oxidation and reduction and its biological role. Finally, we will briefly discuss the regulation of protein function by reversible methionine oxidation/reduction based upon recent research.

24.2 Methionine Oxidation

The sulfur atom of methionine is a prochiral center; thus, its oxidation leads to a mixture of two diastereomers, methionine-*S*-sulfoxide and methionine-*R*-sulfoxide (Fig. 24.1). Various ROS, such as hydrogen peroxide, hydroxyl radical, superoxide radical, and peroxynitrite, oxidize methionine to generate methionine sulfoxide non-stereospecifically [4]. Therefore, methionine-*S*-sulfoxide and methionine-*R*-sulfoxide may be generated at similar levels because ROS produced during mitochondrial respiration are responsible for the oxidation of amino acids in proteins, including methionine [1].

In contrast, some flavin-containing monooxygenases oxidize methionine in a stereospecific manner. Three flavin-containing monooxygenases (FMOs), FMO1, FMO2, and FMO3, are expressed in most mammalian tissues and catalyze the oxidation of methionine to methionine sulfoxide in a partially stereospecific manner based upon the substrate concentration in liver and kidney microsomes [6]. Recently identified Mical proteins have an FMO domain that is responsible for the stereospecific oxidation of two conserved methionine residues in actin [7, 8]. Interestingly, this is a completely stereospecific oxidation that converts the two methionine residues to methionine-*R*-sulfoxides.

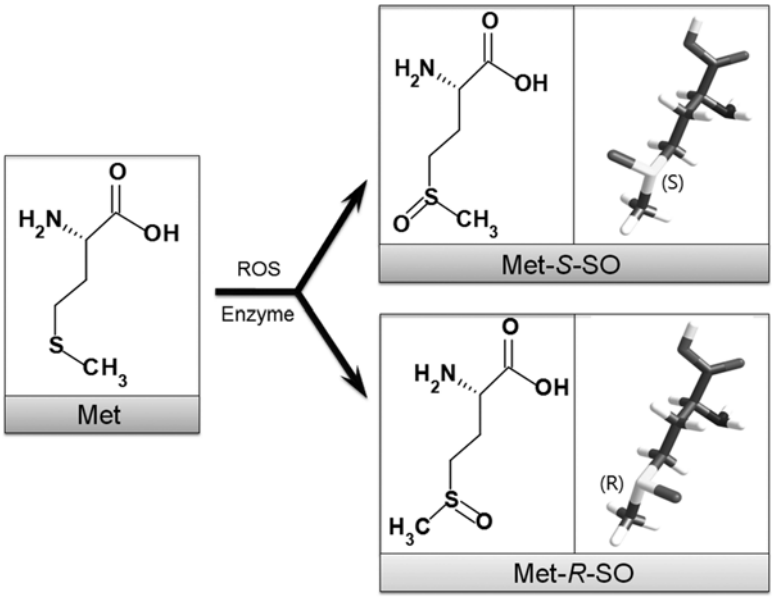

Fig. 24.1 Methionine oxidation. Methionine-*S*-sulfoxide and methionine-*R*-sulfoxide are formed by methionine oxidation

24.3 Methionine Sulfoxide Reductase Families

Methionine sulfoxide is reduced by the enzyme Msr, which is then recycled by thioredoxin, thioredoxin reductase, and NADPH. To date, four Msr families have been characterized based upon stereospecificity, substrate type, and other factors.

MsrA was first discovered around 35 years ago as the reductase that restores the function of oxidized ribosomal protein L12 in *Escherichia coli* [9]. Subsequent functional characterizations revealed that MsrA reduces both free and protein-based methionine-*S*-sulfoxide [10]. Interestingly, some lower organisms, such as green algae, contain MsrA as a form of selenoprotein in which selenocysteine takes the place of the catalytic cysteine, but the selenoprotein form of the enzyme is not found in higher organisms [11]. In mammals, MsrA is encoded by one gene and localizes in either the cytosol or mitochondria. Compared with other Msr families, MsrA has relatively broad substrate specificity; that is, it recognizes not only methionine-*S*-sulfoxide but also various other *S*-sulfoxide moieties, such as dimethyl sulfoxide, ethionine-*S*-sulfoxide, *S*-sulindac, and *S*-sulforaphane [12]. Although the physiological importance of this property is not clear, it was reported that certain xenobiotics containing methylsulfinyl groups are reduced by MsrA in vivo [13].

MsrB, which is alternatively described as SelR or SelX, was first identified and characterized in mice by Gladyshev and colleagues [14]. This enzyme reduces protein-based and free methionine-*R*-sulfoxide with very low efficiency. MsrB has a very narrow substrate specificity; methionine-*R*-sulfoxide is the only known

substrate. Like MsrA, MsrB is found in all three biological kingdoms, with the exception of some thermophilic bacteria. Three MsrBs have been identified in mammals, MsrB1, MsrB2, and MsrB3, which share the same substrate specificity for the reduction of methionine-*R*-sulfoxide [4]. MsrB1 is localized in the cytosol and nucleus, MsrB2 in the mitochondria, and MsrB3 in either the endoplasmic reticulum (ER) or mitochondria. Interestingly, MsrB1 is a selenoprotein in which a selenocysteine takes the place of the catalytic cysteine, which dramatically increases the catalytic efficiency due to the low pKa value and higher polarizability of selenocysteine. However, the expression level of MsrB1 is relatively low because the enzyme requires selenocysteine and additional translational machinery for insertion of the residue based on the in-frame UGA codon [15]. MsrB1 expression is highly dependent upon the availability of selenium and therefore declines dramatically in animals fed a selenium-deficient diet.

Free methionine-*R*-sulfoxide reductase (fRMsr) was first described in *E. coli* by Lowther and colleagues [16]. fRMsr reduces only free methionine-*R*-sulfoxide. Thus, protein-based methionine sulfoxide cannot be a substrate for this enzyme. fRMsr is found only in unicellular organisms [17]. The biological role of this enzyme remains unclear, but it is thought that fRMsr helps to provide methionine for rapidly proliferating cells by reducing oxidized methionine under conditions of increased oxidative stress.

MsrPQ was recently identified in *E. coli* and features some unique properties compared with other Msr families [18]. For example, MsrPQ is a periplasmic molybdopterin-containing enzyme that is conserved across gram-negative bacteria. This oxidoreductase is also non-stereospecific for the reduction of methionine sulfoxide, meaning it can reduce both methionine-*S*-sulfoxide and methionine-*R*-sulfoxide. In addition, recycling of MsrP is supported by the electron-transport chain via MsrQ, which is a heme-binding membrane protein. MsrPQ reportedly plays an important role in the maintenance of membrane integrity under HOCl-induced oxidative stress by reducing various structurally unrelated periplasmic proteins.

24.4 Functions of Methionine Sulfoxide Reductase

Msr is an antioxidant enzyme that supports resistance to oxidative stress via cyclic methionine oxidation and reduction. As mentioned above, methionine is highly susceptible to oxidation by ROS and therefore serves as an efficient ROS sink in cooperation with Msr enzymes (Fig. 24.2). The antioxidant properties of Msr enzymes offset the effects of aging and delay the onset of aging-associated diseases [19, 20]. For example, overexpression of MsrA contributes to lifespan extension in a diverse array of organisms, such as mice, fruit flies, yeast, and several other species [21].

Msrs also function in the repair of ROS-induced protein damage (Fig. 24.2). Methionine oxidation often interferes with protein function due to structural changes caused by the increased negative charge and size due to the addition of oxygen. Accordingly, Msr restores the functions of damaged proteins by reducing methionine sulfoxide back to methionine. For example, methionine oxidation at the N-terminus

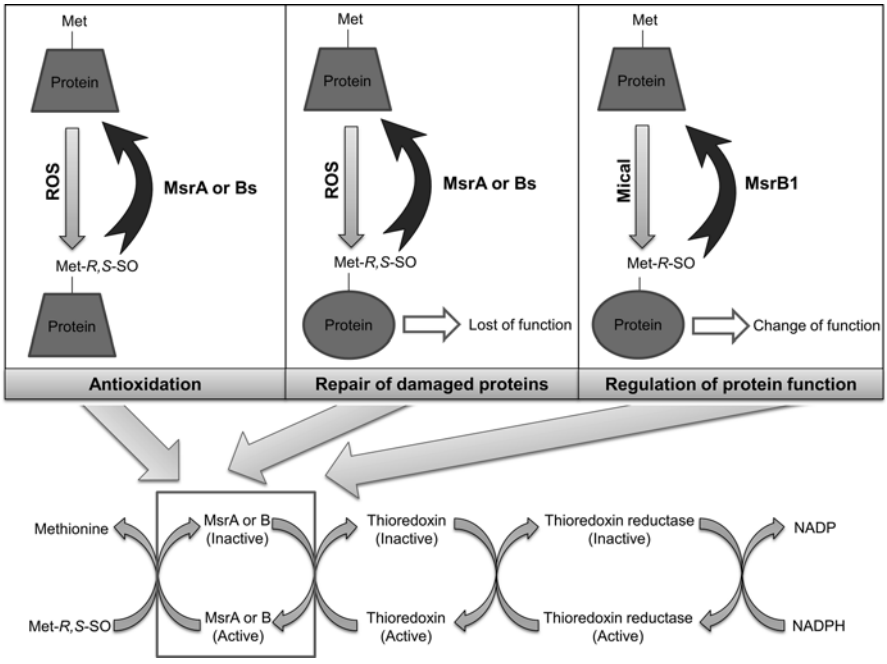

Fig. 24.2 Function and regeneration of Msr. Msr is recycled by thioredoxin/thioredoxin reductase/NADPH after reducing methionine-*R*,*S*-sulfoxide. Msr increases oxidative stress resistance, repairs damaged proteins, and regulates protein function, particularly that of MsrB1, in conjunction with Mical

of the ShC/B channel protein decreases the rate of K^+ channel inactivation, but the rate is restored following expression of MsrA [22]. HIV-2 protease is also inactivated by oxidation of Met76 and Met95, located at the dimer interface; and MsrA reactivates inactivated HIV-2 protease up to 40 % of its original activity [23]. HOCl disrupts the refolding capacity of the *E. coli* chaperone protein, GroEL, via methionine oxidation, but this function can be recovered by treating the cells with *Shewanella* MsrA/B [24]. CaMKII, a regulator of calcium flux, is activated without calmodulin binding by oxidation of two consecutive methionine residues, but this auto-activation is reversed by expression of MsrA [25]. Numerous reports have described the function recovery of damaged proteins by Msr treatment. However, the amount of oxidized methionine in all of the proteins in a given organism is very large. For example, it was reported that more than 4 % of the methionine residues in all proteins in mice are oxidized under normal conditions, indicating that the function of many proteins could be adversely affected by methionine oxidation.

Recently, a novel biological function of Msr in the regulation of actin polymerization in conjunction with Mical was discovered (Fig. 24.2). Mical contains a FMO domain, which can oxidize methionine [26]. Interestingly, Mical can convert two conserved methionine residues in actin to methionine-*R*-sulfoxides, resulting in actin disassembly due to disruption of the actin-actin interaction necessary for polymerization.

The selenoprotein MsrB1 reduces the methionine-R-sulfoxide residues in actin to methionines, restoring actin assembly for further biological processes that require actin polymers [7, 8]. Although the biological role of enzyme-based methionine oxidation remains poorly understood, the finding of cooperativity between Mical and MsrB1 in the regulation of actin assembly suggests that methionine sulfoxidation by Mical could be used to reversibly regulate the functions of proteins in conjunction with Msr. Such methionine oxidation thus represents a novel type of reversible post-translational modification, similar to acetylation, phosphorylation, and methylation, which could be exploited to regulate protein function and signal transduction. Although the co-regulation of actin assembly by Mical and MsrB1 has been well characterized through in vitro biochemical assays, further studies are needed before physiological applications are developed.

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Part III
Dietary Selenium and Its Impact on Human Health

Dietary selenium is an essential element to many life forms, and too little or too much in the diet can have severe consequences on health. The chapters in this section focus on the requirements for selenium in the diet and its many effects on health and development.

Chapter 25
Selenium: Dietary Sources, Human Nutritional Requirements and Intake Across Populations

Kristin M. Peters, Sarah E. Galinn, and Petra A. Tsuji

Abstract Selenium (Se) is an essential trace mineral found ubiquitously in the environment in geographically variable amounts. The bioavailability of Se compounds varies depending on the source and form of Se, with our primary source of Se intake through food, including grains, animal products and, increasingly recently, dietary supplements. Recommended dietary amounts vary slightly among countries due to differential average intakes and subsequent Se status across populations, and typically range from 40–45 μg/day for adults in Japan to 75 μg/day for adults in the United Kingdom. Controversies continue to prevail regarding adequate amounts of Se for health and disease prevention in the general population and for populations which are potentially more sensitive to changes in dietary Se. The biological response to dietary Se, much like that of other nutrients, likely follows a U-shaped curve, which may be modulated by a variety of factors, including gender, ethnicity, Se status, and background health.

Keywords Dietary sources • Geographic selenium distribution • Human selenium requirements • Recommended dietary allowance • Selenium deficiency • Selenium status • Selenosis

25.1 Introduction

In the environment, Se occurs as inorganic (elemental Se, selenide, selenite, and selenate), and organic chemical species, including methylated Se compounds and selenoamino acids (see Chap. 35). The complex metabolic role of Se nutriture [1–3] and its effects on human health are thought to be due to its incorporation in the form of selenocysteine, the 21st amino acid, into Se-containing proteins (selenoproteins) [1]. This chapter describes the sources, and compares average human consumption and requirements of this essential dietary nutrient across various geographical areas

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and populations. Information regarding potentially sensitive groups such as infants, pregnant or lactating women, and those with genetic predispositions or certain diseases, remains underrepresented in the literature, and thus difficult to attribute.

25.2 Geographic Distribution and Production

Inorganic Se species exist in the environment as elemental Se, selenide (Se^{-}), selenite (SeO_3^{2-}), and selenate (SeO_4^{2-}), and the amounts in which such Se species are found in the environment vary geographically, ranging from near zero to 1250 mg/kg [2–4]. Except in those areas, where burning fossil fuels, industrial settings of glass, and chemical or electronics manufacturing are substantial, the actual contribution of Se through inhalation is considered to be insignificant to human health [5, 6]. Se content in the soil appears highly variable. The average Se concentration is much higher in areas with sedimentary rocks, especially shales and coal, than in those with igneous rocks formed from the solidification of molten rock material, such as granite and basalt. Areas with unusually high soil Se concentrations include parts of Wyoming, North Dakota and South Dakota in the US, and parts of China, Ireland, Colombia and Venezuela. In such areas, specialized plants may accumulate up to 3 mg Se per gram plant matter and, therefore, though often not considered palatable, may potentially be toxic to livestock grazing in that geographic area [7]. In contrast, Finland (until 1984, before Se was added to agricultural fertilizers), central Serbia, and likely large parts of Africa, such as Congo [8], are known to be deficient in soil Se. However, strong regional differences also can be found within a country. Australia, as well as China, has both high and low Se soils. Most notably, a high Se region is found in Enshi County in China, in addition to a very low Se geographical belt that spans from northeast to southcentral China [9]. Additionally, anthropogenic influences may lead to addition of Se to top soil and ground water through intensive irrigation practices [10], industrial waste from metal smelters and steel plants [11], and the addition of Se to fertilizer and/or animal feed as a practice in areas where the soil is originally Se-poor [12, 13].

Soluble forms of Se in water are primarily inorganic selenate and selenite ions. Se is detectable in sea water in only relatively low amounts ranging from 0.09 to 0.11 μg/L [14], but may accumulate through the aquatic food chain. In contrast, Se levels in ground and surface waters are highly variable, and can range from 0.06 μg/L to over 400 μg/L, and in rare instances even up to 6000 μg/L [15]. In the US, federal standards allow up to 50 μg/L in drinking water which is regulated by the US Environmental Protection Agency under the Safe Drinking Water Act [16]. However, except for highly seliniferous geographic locations, the Se content in drinking water typically ranges from only 0.12 to 0.44 μg/L [17], thus contributing only minimally to the daily intake in the US [18]. Germany, and possibly other countries, also limit Se concentrations to 10 μg/L in bottled drinking water and tap water [19, 20].

25.3 Dietary Sources

Organic Se species, primarily found in biological systems of living matter, are represented by methylated Se compounds, selenoamino acids, and selenoproteins [21]. The bioavailability of Se compounds from food is highly varied [22, 23], depending on the source and form of Se consumed. In humans, the primary form of Se intake is through food [24]. The USDA National Nutrient Database for Standard Reference (http://ndb.nal.usda.gov//) provides a useful tool on nutrient information in the US, including Se content, in nearly 8800 food items (last modified, November 30, 2015). Analogous agencies, such as the Food Standards Agency (http://www.food.gov.uk/) and the Scientific Advisory Committee on Nutrition in the United Kingdom, provide similar information for other countries.

Due to geographical soil differences in terms of chemical Se species, as well as regional fertilization techniques, the difference in total Se content in fruits, vegetables, mushrooms and grains, can be substantial. For example, Se content has been reported to vary as much as 500-fold in wheat [25], in which selenomethionine is the most predominant Se species (~55 %). Although wheat product consumption, at least in the US, appears to have leveled out in recent years [26], wheat and wheat products are still consumed in large quantities in China, the European Union, India, Russia, and the US, and thus may contribute significantly to the Se intake of the population if the wheat consumed is grown in Se-rich soils. Typically, fruits and vegetables contain only smaller amounts of Se. However, plants in the genus *Allium*, which includes onions, leeks, scallions, and garlic, as well as those in the genus *Brassica*, which includes broccoli and cauliflower, are known to accumulate Se when grown under Se-enriched conditions [27]. In these plants, increased contents of Se-methylselenocysteine and γ-glutamyl-Se-methylselenocysteine have been reported [28–30]. Seeds from the South American tree *Bertholletia excelsa*, known as Brazil nuts, have a reported average Se content of 1470–1917 μg/100 g, with most of it present as selenomethionine [31].

Heterotrophic organisms, such as fungi, which include edible mushrooms and yeast, are also known to accumulate Se [32], so much that (non-edible) filamentous fungi even have been investigated for treatment of Se-contaminated aqueous media [33]. Frequently consumed are also medicinal mushrooms, including the oriental fungus, *Ganoderma lucidum*, which has been shown to accumulate over 44 mg Se/kg, when cultivated in Se-rich substrates [34]. Especially *Saccharomyces cerevisiae* (Baker's or Brewer's yeast), and *Candida utilis* (Torula yeast), may also be part of the human diet. Yeast cells bioaccumulate Se in the form of organic and inorganic compounds, and this enrichment may result in formation of selenoamino acids, including selenomethionine [35], which may be bioavailable to the organism.

Next to wheat and other grains, the human Se intake through meat consumption is another primary source of dietary Se, especially in the Western countries, including the US. The relatively high muscle and organ Se concentrations in animal products for human consumption depends on the animal's diet, which is not only a result of regional differences due to geographical variations in soil and subsequent plant

materials, but also because of the common practice of supplementing cattle, hogs and chickens with Se in their commercial feed [36]. Thus, the average Se content in beef and lamb typically varies between 20 and 35 μg/100 g, and for chicken between 10 and 24 μg/100 g, with predominant Se species thought to be selenomethionine and selenocysteine. Their ratio is determined by the type of Se supplementation provided (inorganic compared to organic Se forms) [37]. Organ meats are known to be enriched in Se and may contain from 60–80 μg/100 g poultry liver to 100–311 μg/100 g beef, lamb and pork kidneys. Whereas dairy products, such as cow's milk, typically provide only an estimated 3.7 μg/100 mL, the Se content in chicken eggs may be highly variable due to the supplementation practices in chicken feed [36, 38]. The ranges in Se content of fishes is rather large, with about 12 μg/100 g in freshwater catfish to well over 70 μg/100 g in canned tuna. However, the bioavailability of Se from fish may be modified by presence of heavy metals, which may decrease Se absorption via chelation and precipitation [39].

25.4 Human Se Requirements and Patterns of Consumption

The Food and Nutrition Board of the US National Academy of Sciences' Institute of Medicine and the equivalent organizations in other countries provide dietary reference intakes for essential nutrients. These intake values include the nutrient-specific Recommended Dietary Allowance (RDA), the adequate intake (AI) levels, and the Tolerable Upper Intake Level (UL). The RDA defines the average daily intake that meets the basic nutrient requirements of 97.5 % of the apparently healthy population. Because there continues to be insufficient information on Se to establish an RDA for children under the age of 1 year, AI levels are calculated based on the amount of Se consumed by healthy infants who are fed breast milk providing a level of assumed nutritional adequacy (see Table 25.1). The UL sets the level of maximum daily intake unlikely causing symptoms of adverse health effects [40]. The RDA is likely the most frequently used measure and also the most familiar to the consumer, and thus utilized herein. This chapter focuses on the normal adult Se status and requirements, whereas other chapters elaborate on Se in disease. Insufficient information continues to be available regarding possible differences in requirements for specific ethnic segments of the population based on cultural dietary patterns [41].

There is a fine line between the levels where specified criteria of basic human Se requirements are met allowing near-optimal functions of the many biological mechanisms in which Se is involved, and where the risks of deficit or excess are prevented [42]. In accordance with the World Health Organization, the RDA for Se was established in 1989 with 55 μg for women and 70 μg for men, reflecting corrections for body weight and subject variability in the population [39, 43]. These were based on extrapolations from animal studies as well as repletion studies in Se-deficient regions of China that found that approximately 40 μg Se per day achieved maximal activity of plasma glutathione peroxidase (GPX) [44], which is primarily reflecting

Table 25.1 Recommended dietary allowance (RDA), Tolerable Upper Intake Levels (UL), and Adequate Intake (AI) levels[a]

		Germany, Austria		Nordic countries		NZ, AUS		USA		UK	
Ages		RDA	UL	RDA	UL	RDI	UL	RDA	UL	RDA	UL
Infants[b] (age in months)	0–3	10		-			45	15	45	10	
	4–6	15						15		13	
	~7–12	15		15				20	60	10	
Children (age in years)	1–2	15		20		25	90	20	90	15	
	2–4	15		25		25	90	20	90	15	
	~4–8	20		30		30	150	30	150	20	
	~8–10	30		30		50	280	40	280	30	
	~10–13	43		40		50	280	40	280	45	
	~13–15	60		60		70	400	55	400	45	
Males	15–18	70				70	400	55	400	70	
	18–70	70	400	60	300	70	400	55	400	75	450
	>70	70	400	60	300	70	400	55	400	75	450
Females	15–18	60		50		60		55		60	
	19–70	60	400	50	300	60	400	55	400	60	450
	70+	60	400	50	300	60	400	55	400	60	450
Pregnancy		60		60		65	400	60	400		
Lactation		75		60		75	400	70	400	75	

[a]RDA, UL and AI in μg/day for Se as set forth by Germany, Austria and Switzerland [59], Nordic countries (Denmark, Finland, Iceland, Norway, Sweden, Faroe Islands, Greenland, Åland) [48], New Zealand (NZ) and Australia (AUS) [78], the USA [40], and the United Kingdom (UK) [79]
[b]There is insufficient information on the micronutrient Se to establish a RDA for children under the age of 1 year. Instead, an AI is calculated that is based on the amount of Se consumed by healthy infants who are fed breast milk [40]

activity of plasma GPX3. Other selenoproteins have also been suggested as useful biomarkers for assessing the selenium status, e.g., selenoprotein P (SEPP1) expression in plasma [45]. This is particularly interesting, as two Se supplementation studies in healthy Se-deficient Chinese subjects demonstrated that smaller doses and shorter Se supplementation times were needed for optimization of GPX activity compared to optimization of SEPP1 expression. Optimization, in this case, was defined as the point where the activity or expression level of the respective selenoprotein was not different from the average value of the subjects receiving larger supplements [45, 46]. The outcome of the second trial suggested that 75 μg Se per day in the form of Se-methionine on average would be sufficient to optimize SEPP1 expression [46]. Because other factors, including body mass index, inflammation status and gender may influence SEPP1 expression levels and thus contribute to variance in biomarkers of Se function, the possibility of SEPP1 expression as a biomarker for Se status likely needs to be further studied [47].

Since the year 2000, the National Academies of Science's Institute of Medicine has recommended an average nutritional intake of 55 μg Se per day for both

women and men in the US [40]. In Nordic countries, such as Denmark, Finland, Norway, and Sweden, current recommendations are set to 50 µg/day and 60 µg/day [48], 60 µg/day and 70 µg/day in Germany and Australia, and 60 µg/day and 75 µg/day for women and men in the United Kingdom, respectively (Table 25.1). Japan has much lower recommendations, 25 µg/day and 30 µg/day for women and men, respectively [49]. Gender-specific intake and recommendations are further discussed in Chap. 32. For an extensive list on estimated Se intakes and Se status, the reader is referred to the Combs' 2001 review [7].

A growing concern, especially in Western countries, is the use of dietary supplements, which can be a significant source of Se and other minerals or vitamins. In the US, the regular human consumption of dietary supplements has become very popular, and has grown into a large industry with very little regulation or oversight by federal authorities, such as the US Food and Drug Administration. The actual content of Se added to many multi-vitamin supplements, which may include selenomethionine, selenite and/or Se-enriched yeasts, varies between 10 and 200 µg/daily dose [50], and thus may be a significant source of Se. It should be noted that a dose of 200 µg/day constitutes nearly 50 % of the levels at which adverse health effects may be expected (see Table 25.1).

25.5 Availability, Absorption and Metabolism

The small intestine is the primary location for absorption of dietary Se [51], and initial absorption is estimated to be ~80 %, modulated by presence or absence of other trace elements as well as protein. Selenite is thought to passively diffuse across the membrane, whereas selenate may be absorbed via a sodium-mediated carrier mechanism [51, 52]. In contrast, selenomethionine appears to compete for the same uptake system as methionine [53]. Transport of Se in the body occurs mainly through binding to proteins in blood, as Se in plasma can be associated with serum albumin, and SEPP1 and GPX3. Overall, bioavailability has been reported to be around 14 % of the original Se content in ingested food, with 2–20 mg Se retained in an adult human body, and detectable in many tissues, and also urine, nails, and hair ([54], and Chap. 38). Excretion of Se can occur via the kidneys in urine, the gastrointestinal tract in feces, and the lungs through expired air.

25.6 Se Intake and Se Status

Determining the actual Se status of humans by measuring the consumption of dietary Se continues to be difficult [55, 56]. A dose-dependent physiological approach sensitively reflecting recent changes in dietary Se intake is provided by assaying selenoproteins in serum or whole blood [57, 58]. Thus, serum or plasma levels of GPX3, and especially SEPP1, which accounts for over 60 % of the Se in

human plasma based on its role in Se transport and metabolism, are highly informative biomarkers of Se status in humans [45, 59].

The National Health and Nutrition Examination Surveys [60] have assessed nutritional status across populations in the US, and have provided compelling evidence that over 99 % of the adult participants were Se replete, and routine Se supplementation does not appear warranted in many cases to meet the current US RDA needs of 55 μg/day [61]. Instead, Se intake levels above repletion status increases blood Se concentration through non-specific incorporation of selenomethionine into plasma proteins [40, 62], with hitherto controversial health effects. In the US population, high Se status has been linked with possible increased risks of diabetes and hypertension [63, 64]. It should also be considered that, like with many other nutrients, the biological response to Se likely depends on gender (see Chap. 36), single nucleotide polymorphisms in selenoprotein genes (see Chap. 34), Se status, age, and presence of disease, among other factors. Thus, it has been argued that a U-shaped curve best represents Se intake and negative health outcomes, suggesting that there is a profile for predicting the risk-benefit consequences of supplementation with dietary Se [65].

25.6.1 Se Status Across Geographic Locations

Given the geographical variations of Se in soil and subsequently plants and animals, it is not surprising that the Se intake varies accordingly across inhabitants of such locations. The physiological Se status, frequently measured in μg Se/L plasma or serum, reflects these differential intakes (Table 25.2). Lower Se intake and, thus, lower mean Se status is reported for Germany and many other European countries, whereas the US and Mexico have often reported higher mean Se status in their populations (reviewed in [7]). Of course, the Se intake can vastly differ within the population of a country, and China is usually presented as the prime example, because of its well-documented geographic areas with both extremely low and extremely high Se intakes and Se status. The average Se status in inhabitants of low Se areas in China not receiving any dietary supplements may be as low as 16 ± 4 μg/L, whereas the mean Se status in populations of high Se areas may be as high as 1438 ± 76.3 μg/L (Table 25.2). Such extremely low and extremely high Se status can result in adverse human health conditions further described below.

25.6.2 Se Deficiency

At least two diseases, Keshan disease and Kashin-Beck disease, have been linked with severe Se deficiency, as Se plays an important role in human development and immune function. Keshan disease, which results in congestive cardiomyopathy, has been linked to coxsackievirus B3 infections, and Se deficiency contributes to

Table 25.2 Se intake and average serum/plasma Se levels in select studies of healthy adults

Country	Se intake in μg/day (range or mean ± SD)	Serum or plasma Se in μg/L (range or mean ± SD)
Germany [80]	38–48	68 ± 32
Spain [81]	21–75	80.7 ± 10
Poland [7, 82]	11–94	78.0
Portugal [83, 84]	37	36–127
Sweden [85, 86]	31–38	75.6 ± 14.3
Denmark [87]	38–47	98.7 ± 19.8
Mexico [88, 89]	10–223	100 ± 18
UK [79]	48 ± 22	83.7 ± 14.2
Australia [90]	57–87	85.6 ± 0.5
USA [7, 62]	60–220	137.1
Japan [91, 92]	140–127	117 ± 6
China [93–95]		
Low Se area	62.0 ± 3.6	16 ± 4
High Se area	1438.2 ± 76.3	357 ± 36

the chronic myocarditis [66]. Kashin-Beck disease, a multifactorial bone and joint disease that results in osteoarthropathy and joint necrosis, may be a result of exposure to naturally occurring trichothecene mycotoxins, which are toxic mold byproducts of *Fusarium spp*. [67]. Previously reported from areas including Russia, central Africa, and China [68], currently, Kashin-Beck disease is generally restricted to children of Se-poor geographic areas in China. Both Keshan disease and Kashin-Beck disease respond to prophylactic oral Se administration (e.g., [69, 70]). Myxedematous endemic cretinism, which results in mental retardation, is now more thought to be associated with a combined iodine and Se deficiency, rather than Se deficiency alone [71].

25.6.3 Selenosis/Se Toxicity

Se toxicity, or selenosis, can result when Se is consumed in very high amounts over a long period of time, often resulting in blood Se levels greater that 100 μg/dL [72, 73]. Gastrointestinal upsets, hair loss, white blotchy nails, garlic breath odor (representing volatile (di-)methylselenide), fatigue, irritability, and mild nerve damage [74] are common symptoms described for selenosis, similar to what has been described by inhabitants in extremely high-Se areas of China. Reports of severe selenosis or even death from Se toxicity in humans, however, are only anecdotal [75], outside rare accidental industrial exposures or manufacturing errors of dietary supplements [76].

The UL for Se, which are below the lowest observed adverse effect levels of 910 μg/day [76], are set at 400 μg/day by organizations, such as the US Institute of

Medicine of the National Academy of Sciences and the German Nutrition Society, to avert possible selenosis in the general population. These UL vary, and range from 300 μg/day in Nordic countries (e.g., Sweden and Norway) to 450 μg/day in the United Kingdom (Table 25.1).

25.7 Concluding Remarks

The basic human requirements for dietary Se required for survival, and the amounts sufficient to maintain near-optimal functions of selenoproteins are in a delicate balance, and likely follow a U-shaped curve. These requirements are further modified by differential biological response depending on factors, such as gender, ethnicity, Se status, age, human gene polymorphisms in selenoprotein genes and background health. Thus, the established level of suggested Se intake for the general population continues to be controversial especially in light of subgroups potentially more sensitive to Se intake and status. Even a lower-dose chronic overexposure of Se as a result of indiscriminate use of Se supplementation is thought to contribute to adverse health outcomes rather than increased health benefits [77]. Thus, as we continue to collect information on Se status and dietary Se intake across populations and geographic locations, we hope to arrive at better general and individualized recommendations for Se intake and Se supplementation in the future.

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Chapter 26
Human Clinical Trials Involving Selenium

Lutz Schomburg

Abstract Since the first demonstration of its essentiality in rodents, scientists and clinicians have tried to elucidate the importance of selenium (Se) for human health and to modify the Se status of healthy subjects or patients to reduce disease risks and improve convalescence, respectively. There are two major approaches that have contributed to our current understanding of Se in human health: i) observational studies, which can be subdivided into descriptive and analytical studies that characterize Se status in specific diseases or aim to identify associations between Se status and disease risk in a pro- or retrospective design; and ii) intervention studies, which actively increase Se intake by providing supplements, and then monitor health risks, convalescence, or disease course in comparison to controls. It is important to appreciate the different study types and to highlight strengths and limitations in order to better understand what has been achieved and what remains to be studied or needs verification. This chapter highlights selected, particularly instructive and insightful studies from the areas of biomarker identification, chemoprevention and cancer treatment, sepsis, autoimmune thyroid disease, and other applications, with an emphasis on successful trials and positive study outcomes.

Keywords Biomarker • Cancer • Case report • Cross-sectional study • Randomized controlled trial • Sepsis • Supplement • Thyroid

26.1 Introduction

Elucidating the importance of selenium (Se) for human health has been a central research issue ever since the pioneering description of its essentiality for disease prevention in a vitamin E–deficient rat model of liver fibrosis [1]. Prior to that time, Se was generally known as an environmental toxin, similar to arsenic (As), capable of causing harm, cancer, or even death [2]. This problematic downside of Se still constitutes an important and relevant threat, and there are anecdotal case reports of

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selenosis associated with attempted suicide [3], homicide [4], overconsumption of Se-rich paranuts [5], or intoxication in the course of self-medication [6] or from wrongly formulated supplements [7]. The symptoms of acute selenosis are known and include unpleasant odor of exhalation, nausea, vomiting, hypotension, tachycardia, and gastrointestinal irritations with diarrhea and abdominal pain [8]. Chronic selenosis from a constant oversupply is less well defined and is a matter of debate. Besides obvious hair and nail loss due to severe over-dosage, slower-appearing side effects are difficult to quantify and prove. In this respect, intervention trials using different Se supplements are a valuable resource for understanding the thresholds of long-term supplementation and the symptoms of Se oversupply.

Currently, most clinical research on Se involves analyzing Se status in subpopulations, comparing patients and controls and trying to deduce associations of Se status with disease risks. Randomized controlled intervention trials (RCTs) are relatively few but are increasing in number. Given that the requirement of Se for prevention or treatment of certain diseases is not firmly established, it is hypothesized that many subjects are poorly supplied and would profit from moderate Se supplementation. Recently, some clinical studies also tested the direct or adjuvant effects of high dosages of Se in diseased patients, analyzing whether this strategy supports other therapeutic measures, positively contributing to disease course and convalescence. To date, there have been only a few such experimental trials involving a high-dose therapeutic intervention, and study designs and details (supplement, dosage, time period and patients) differ profoundly.

In comparison, there are numerous comparable observational studies evaluating Se status in healthy and diseased subjects and calculating the potential risk attributable to Se deficiency for certain disease incidences. By nature, these studies cannot distinguish cause and consequence. Especially in view of Se as a negative acute-phase reactant [9], a low Se status may develop during disease or may have preexisted, potentially increasing disease risk and/or disease incidence, which remains unanswered by descriptive studies [10].

When comparing study designs, descriptive studies yield basic hints regarding the importance of Se in certain diseases but are not aimed at testing causal relationships (Fig. 26.1). In comparison, analytical studies often involve several time points in a pro- or retrospective design. Therefore, some evidence of the importance of Se with respect to the risk and course of a disease can be obtained. This approach has been widely used in the areas of cancer, autoimmune diseases, infection, and disease-related mortality. When sufficient evidence is accumulated, experimental studies are initiated, involving an active supplementation, testing directly whether Se affects disease risks and convalescence, modulates side effects of pharmacotherapy or surgical interventions, or prolongs life expectancy. The highest-quality evidence is obtained when such intervention trials are conducted with a sufficiently large number of participants, optimally in multiple clinical centers in parallel, using a placebo as a control [11]. However, these RCTs are expensive, tedious, time-consuming, and complex. The growth of knowledge from clinical studies in a particular medical area, therefore, often follows a certain series of study types and combines different lines of evidence before large RCTs are finally conducted. Often, the starting point is cross-sectional data, case reports, and descriptive comparisons

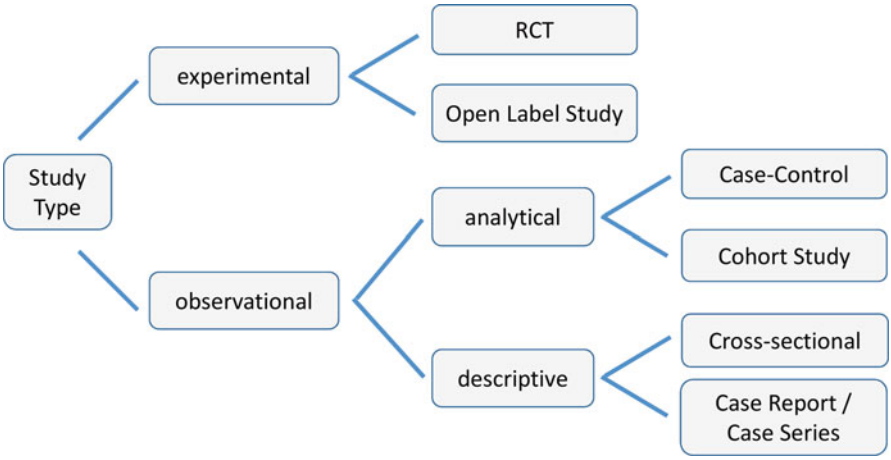

Fig. 26.1 Overview of study designs in research on the role of Se in human health and disease. The studies are either experimental or observational, depending on whether or not a supplement is given. Observational studies can be of a descriptive or analytical nature. The descriptive studies provide information on the characteristics of the disease. The analytical studies aim to identify associations between Se and a disease by comparing a set of cases and controls, or a population or cohort, in a pro- or retrospective design. Experimental trials involve active Se supplementation either as an open-label study or as a randomized controlled trial (RCT). The latter provide the highest-quality evidence of causal relationships

between affected and control subjects. Observational retrospective or prospective studies analyze larger case-control cohorts or sub-populations. The transition to active intervention often starts by supplementing a limited number of participants. Here, different dosage regimens and readouts can be compared and analyzed. These results may then constitute a solid basis on which large-scale RCTs can be designed for testing specific hypotheses ("end points"). All of these types of studies have been conducted in Se research, especially in the areas of biomarker identification, chemoprevention, sepsis, and thyroid disease. The latter probably represents the most intensively studied and thus a most useful example of how different approaches have converged and contribute to our current understanding (see Table 26.1). Additional examples from all of these areas are presented below and are discussed in order to provide some overview on this fascinating issue.

26.2 Successful Prevention of Endemic Diseases Linked to Se Deficiency

There are three well-established and intensively studied diseases that are associated with endemic Se deficiency: myxedematous cretinism [12], Kashin-Beck osteoarthropathy [13], and Keshan disease [14]. In each of these diseases, Se deficiency has been identified as an important risk factor by observational analyses, and successful supplementations aiming at disease prevention have been conducted in the

Table 26.1 Examples of clinical studies assessing the importance of Se for the thyroid axis

Study type	Year	Intervention	Major results	Ref.
Open label	1993	50 µg SeMet per day, 2 months	Se aggravates hypothyroidism in iodine- and Se-deficient children	[96]
RCT	2002	200 µg selenite per day, 3 months	Reduction of TPO autoantibody levels in patients with Hashimoto's thyroiditis	[97]
Cross-sectional	2003	None	Association between low Se status and enlarged thyroid volume, esp. in women	[78]
RCT	2004	125–500 µg selenite per day	Lack of effects of Se on the low T3 syndrome in critically ill patients	[98]
Case report	2005	None	Mutations in SECISBP2 cause resistance to thyroid hormone in children	[76]
RCT and cross sectional	2005	None, or 10–200 µg SeMet per day	No association between Se status or Se supplementation and thyroid hormone	[99]
RCT	2007	200 µg SeMet per day, >12 months	Prevention of postpartum thyroiditis and hypothyroidism in pregnant women by Se	[88]
Case control	2009	100–400 µg SeMet or selenite per day	No effect of Se on thyroid hormones in children with SECIBP2 mutations	[100]
RCT	2011	2× 200 µg selenite per day, 6 months	Reduction in proptosis, improved quality of life in endocrine orbitopathy	[86]
Case control	2014	None	Association between selenoprotein S genotype and risk of Hashimoto's thyroiditis	[101]
Cross-sectional	2015	None	Association between thyroid disease prevalence and Se soil concentration and Se intake	[79]

form of open-label studies or RCTs. The myxedematous form of cretinism was found to be due both to a severe Se and iodine deficiency. A supplementation trial with 50 µg selenomethionine (SeMet) per day for 2 months significantly affected the thyroid hormone pattern in children, verifying the importance of Se in disease development [15]. The importance of Se in Kashin-Beck disease appears more complex. A double-blind RCT tested Se supplementation in iodine-supplemented children (N = 324) with Kashin-Beck disease [16]. The children first received supplementation with iodine and then 100 µg/day of sodium selenate or placebo for 11 months. At the end of supplementation, no effects of Se on growth or thyroid function were noted. This finding emphasizes that Se supplementation alone is not suitable as a treatment for Kashin-Beck disease, despite its role in reducing disease risk. A respective meta-analysis of five RCTs and ten non-RCTs supports the concept that Se supplementation constitutes a preventive measure against Kashin-Beck disease [17].

In comparison, the importance of Se for preventing Keshan disease can be considered firmly proven. The incidence of this disease was reduced in China from about 1 to 0.1 % between 1974 and 1980 by Se supplementation (0.5–1.0 mg selenite/week) [18]. This positive effect was reproduced on an epidemiologic level with more than one million supplemented subjects and 0.6 million controls [19]. Nevertheless, in areas with low soil Se concentrations, Keshan disease still constitutes an endemic problem and enduring challenge in need of an efficient supplementation and monitoring program [20].

26.3 Clinical Trials for Biomarker Identification of Se Status

A detailed discussion on "biomarkers of Se status" is beyond the scope of this chapter and the reader is referred to some recent excellent publications (see [21–23] and Chaps. 25, 27 and 31). Briefly, "Se status" is not a clearly defined term, even though it is well understood as a concept of Se supply (i.e., a parameter describing how close a subject has come to reaching a sufficiently high Se intake for optimal health). Currently, the expression level of selenoproteins is used as a surrogate marker, assuming that maximal concentrations of selenoproteins confer health benefits as compared with lower concentrations [24].

It is difficult to estimate nutritional Se intake, as the Se content of nutrients varies with origin of production, and a precise monitoring of a person's diet is only possible in well-controlled experimental setups (e.g., in clinics). For these reasons, Se status is usually determined from biosamples. Hair and nails are rarely chosen for reasons of quality, comparability, and technical aspects. Blood, serum, or plasma are the matrices of choice [25]. In addition to the determination of total Se in these liquid biosamples, two selenoproteins are measured: GPX (either from serum or plasma in the form of GPX3, or from blood cells in the form of GPX1) and selenoprotein P (SePP) [26, 27].

Several independent studies have compared the suitability of these biomarkers for Se status, especially in supplementation trials of poorly supplied subjects [28]. An RCT with different amounts of supplemental SeMet (six dosages ranging from 21 to 125 μg/day for 40 weeks) was conducted with healthy Chinese subjects who had a basal intake of 14 μg Se/day [29]. Comparing plasma Se, GPX activities, and SePP concentrations, the latter turned out to best reflect Se intake and to respond over a large range to Se with increasing expression, becoming optimal at an intake of 50–75 μg Se/day (depending on body weight). These results are in agreement with a similar prior analysis comparing selenite and SeMet as alternative supplements [30]. A low intake of less than 30 μg Se/day was also reported to occur in New Zealand. A similar RCT compared the effects of supplemental SeMet intakes of 10–40 μg/day over a time course of 20 weeks. The results confirmed that both circulating selenoproteins reflect Se intake in deficient subjects, and recommended lower and upper intake levels of 39 μg Se/day and 90 μg Se/day were calculated, respectively [31].

As these studies were conducted with Se-deficient subjects, follow-up trials tested the biomarkers in better-supplied individuals. A study in the UK supplied Se-rich yeast (50, 100, or 200 μg Se/day) or placebo to healthy adults [32]. A total Se intake of 105 μg/day was determined as necessary for full expression of SePP [32]. The study verified that once a threshold intake is exceeded, no further response of SePP or GPX3 to higher intake occurs. This notion was also supported by a trial with healthy US subjects with a basal Se intake of 109 μg/day; supplementation of SeMet (0, 50, 100, or 200 μg/day) for 12 months failed to increase GPX3 or SePP [33]. Only the non-selenoprotein fraction increased, stressing the need for biomarkers of Se excess [25].

Collectively, these studies indicate that below a regular intake of 40–50 μg Se/day, all three biomarkers (i.e., total Se, GPX3, or SePP) are suitable for quantifying Se deficit. At an intake level, at which GPX3 expression is maximized while SePP is not yet maximally expressed, the choice of marker determines whether a subject is classified as Se-deficient or not [34]. The monitoring of Se status in already well-supplied subjects in which supplemental Se does not further increase circulating selenoprotein expression remains an open research issue. Under presumably replete conditions, as decided by the biomarkers in blood, the expression of intracellular selenoproteins might still increase, albeit unnoticed for lack of accessibility. In this case, health effects independent of selenoproteins may also be exerted by Se supplements, for which suitable biomarkers are unavailable.

26.4 Se and Cancer Prevention

The association between Se and cancer is one of the initial and dominant health issues in the multifaceted history of Se research. The first studies involving rodents treated with high amounts of Se yielded hints of a carcinogenic potential for Se, especially with respect to Se-sulfide–caused hepatocarcinoma in rats and female mice [35]. However, the relevance of these findings is questionable, as humans do not consume Se-sulfide. A contrary view of Se as an anti-carcinogenic element emerged when cross-sectional analyses compared Se concentrations in crops with cancer death rates in different states of the US [36]. From these observational analyses, it was extrapolated that a high Se status may confer cancer protection [37]. This notion was supported in follow-up studies in which dietary Se intakes were estimated from food consumption patterns in 27 countries and shown to be inversely correlated with cancer-related mortalities [38]. In a number of smaller, prospective and case-control studies, the interaction was further verified, and most of the studies reported a strong inverse association (reviewed in [39–41]). However, null results were also obtained, and the issue remains unresolved.

A Cochrane meta-analysis came to a positive conclusion based on the observational studies, supporting a link between cancer prevention and higher dietary Se intake, but the analysis was critical of the intervention trials, which did not seem to support a positive health claim [42]. The meta-analysis on prevention was able to

pool data from a total of 55 observational studies and from more than one million participants, concluding that cancer incidence is lower with higher Se exposure (odds ratio [95 % confidence interval] = 0.69 [0.53, 0.91]) [42]. However, the meta-analysis of supplementation was based on only two large RCTs, because the inclusion criteria were strict. This is a problematic aspect, as some studies were excluded despite their large size and clear results (e.g., the Qidong study, which documented a clear, chemopreventive effect of selenized table salt in a county in China, where hepatitis B virus infection was widespread and caused a high incidence of liver cancer [43]).

Moreover, it is the nature of the Cochrane approach to rely solely on statistical methodology; accordingly, this type of rigorous data analysis fails to integrate important biological knowledge regarding selenoproteins, selenocompounds, and Se-related health effects. This becomes obvious when comparing the two RCTs analyzed, which used non-identical selenocompounds and differed profoundly in the basal Se status of the participants. In the Nutritional Prevention of Cancer (NPC) trial, a total of 1312 patients received a daily supplement of 0.5 g high-Se brewer's yeast (equivalent to 200 μg Se, mainly as SeMet) or placebo for a mean of 4.5 years. During this time, the primary end point (recurrence of squamous cell carcinoma) was not affected, whereas the incidences of lung, prostate, and colorectal cancer were markedly reduced upon Se supplementation [44]. A re-analysis highlighted that Se was selectively effective in those individuals who displayed the lowest baseline Se concentrations at enrollment [45].

The Selenium and Vitamin E Cancer Prevention Trial (SELECT) was a follow-up study, enrolling >35,000 men from over 400 sites in the US, Puerto Rico, and Canada between 2001 and 2004 [46]. The primary aim was to test Se and vitamin E, alone and together, on prostate cancer incidence. No effect was detected in an intermediate analysis, and the trial was discontinued in 2008 [47]. In hindsight, this decision appears appropriate, as later analyses indicated that vitamin E alone or with Se tends to increase prostate cancer risk [48]. Initial indications of a higher type 2 diabetes mellitus incidence in the Se group proved incorrect in follow-up analyses. Importantly, the baseline Se status differed markedly (i.e., Se status was above saturating levels for full expression of selenoproteins in SELECT, and thus decisively higher than in the NPC trial [49–51]). It is thus not possible to easily compare and pool the data from these two RCTs into a single meta-analysis, hoping it will yield meaningful insights.

Nevertheless, the expensive SELECT is providing additional options and a valuable set of biorepository data and samples and constitutes an excellent example of an RCT turning into an observational cohort study, as many participants are still monitored by their study centers [52]. One example of an important outcome is the recent analysis of the potential role of Se supplementation in preventing cataract formation, which proved not to be supported by the data collected from the SELECT participants [53].

Only a few studies have analyzed the functional biomarker SePP in cancer research. In a retrospective study, SePP at time of diagnosis was associated with higher survival odds in renal cell cancer; a >80 % 5-year survival rate was observed

in the highest SePP tertile, which contrasted drastically with a <20 % 5-year survival rate in the lowest tertile [54]. In a nested case-control study of 966 colorectal cancer patients and controls, an inverse association between Se and SePP and cancer risk was observed, especially in women [55]. Similarly, in a prospective study comparing adult Americans, low SePP concentrations were associated with a higher lung cancer risk among black, but not among white, US adults [56]. These studies indicate that Se status, sex, and race constitute modifying factors in the relationship between Se and cancer and that careless extrapolations across these modifiers should be avoided.

26.5 Se and Cancer Treatment

Se is both a toxic and essential trace element, with harmful and beneficial effects [57]. Adjuvant Se supplementation during radiotherapy of gynecologic tumors has been evaluated in an RCT in Germany, in which reduced frequency and severity of radiotherapy-induced side effects were observed [58]. Similar positive effects were reported regarding chemotherapy [59] and chemoradiation of patients with non–small cell lung cancer [60]. However, null results were reported from a similar study of head and neck squamous cell cancer [61]. A recent RCT tested Se supplementation for the amelioration of side effects of chemotherapy in children and reported that the prevalence of fatigue, nausea, and impaired physical fitness were reduced and renal and liver functions improved [62]. Further RCTs evaluating Se during chemo- or radiotherapy are thus warranted, especially in view of the lack of side effects.

As some selenocompounds are selectively or preferentially taken up by cancer cells, high-dose Se is being tested as an adjuvant treatment option in cancer [63]. The toxicity of selenite was determined in a small, phase 1 trial (SECAR) [64]. The maximum tolerated dose was determined by dose-escalation and was reached at around 10 mg/m^2. A median plasma half-life of 18.25 h was calculated. Given an average surface area of 2 m^2 of an adult male (180 cm, 80 kg), an astonishing 20 mg selenite/day may be applied as a chemotherapeutic dosage over the course of several days without signs of acute selenosis.

26.6 Se in Sepsis

Severe sepsis causes the majority of deaths in intensive care units (ICUs). An observational study analyzed serum Se at time of admission and during ICU stay and documented low initial concentrations and reduced survival odds in patients who showed a further decline [65]. Accordingly, active Se supplementation to correct the developing Se deficit was discussed as an adjuvant treatment option [66]. This hypothesis was supported by the results of the Se in Intensive Care (SIC) study, a

multicenter RCT in which Se supplementation reduced the mortality rate of severe sepsis and septic shock [67]. The effect was pronounced for male patients [68]. However, a recent large, retrospective study failed to observe a different survival rate between selenite-supplemented and control ICU patients [69]. This ambiguous picture is supported by meta-analyses [70]. Again, basic characteristics of the patients, as well as dosage and form of the supplement, may underlie these discrepancies in study outcome [71]. Additional studies are needed. Surgery is another severe risk factor for developing sepsis in the clinic and triggers a decline in Se concentration [9, 72]. Low Se is associated with post-surgery complications, and positive effects of Se supplementation in patients undergoing elective cardiac surgery have been reported [73]. Whether these promising results are a blueprint for other surgical interventions remains to be tested.

26.7 Se in Thyroid Disease

With the identification of the deiodinase (DIO) family, a direct molecular link between Se and the thyroid gland was established. The relevance of this link for health was identified based on a combined deficiency in Se and iodine, causing myxedematous cretinism [74]. Several subsequent observational and intervention studies tested the direct effect of Se on thyroid hormone concentrations, which apparently are not related in healthy subjects. Even in severe diseases, in which a dysfunction of thyroid hormone feedback regulation occurs (the "low T3 syndrome"), thyroid hormones were found to be unrelated to Se status [75]. Nevertheless, the essential and non-redundant role of DIO in thyroid hormone metabolism was finally established by case reports describing children with inherited defects in *SECISBP2*, who display some resistance to thyroid hormone due to low DIO expression [76].

Autoimmune diseases of the thyroid gland are prevalent, affecting 3–7.5 % of the adult female population [77]. Consequently, many studies have examined this issue (Table 26.1). Large epidemiologic analyses documented an inverse relationship between Se status and thyroid gland size [78] and reported an increased risk of thyroid diseases with poor Se intake [79]. Notably, most of these studies were carried out in Europe or China, where there is suboptimal Se supply. Similarly, all of the RCTs mentioned below were carried out in areas of Se deficiency, usually in or near mainland Europe.

Hashimoto's thyroiditis (HT) results from lymphocytic infiltration and destruction of the thyroid gland. RCTs involving HT patients have yielded ambiguous results, with the initial trials reporting a reduction in autoantibody titers and improvements in quality-of-life [80], whereas some later trials failed to observe positive effects of Se (summarized in [75, 81]). In view of this ambiguity, larger studies are needed, and at least one multicenter RCT is under way (CATALYST trial; NCT02013479) [82].

Similarly, Graves' disease (GD) is a prevalent autoimmune thyroid disease characterized by stimulating autoantibodies against the thyrotropin receptor of thyrocytes. In GD, the thyroid gland becomes chronically activated, irrespective of increased thyroid hormone status, leading to symptoms of hyperthyroidism, including weight loss, tachycardia, tremors, sweating, and nervousness. Many GD patients develop Graves' orbitopathy (GO), which involves protrusion of the eyeballs, impairing vision and severely reducing quality of life [83]. The Se concentrations in patients with GD and GO are relatively low [84, 85]. A multicenter RCT involving patients with mild GO reported diminished proptosis, reduced inflammation, and significant improvements in quality of life in the Se-supplemented patients [86]. Albeit, a replication of these important findings is missing, and the potential benefit of Se in GD is discussed in reviews rather than reports of original research. As with HT, at least one RCT is under way (GRASS trial; NCT01611896) [87].

Another important RCT, which also has not been replicated, showed a protective effect of 200 μg SeMet/day on the incidence of postpartum thyroiditis, with an incidence of 20.3 % in the placebo group and only 11.7 % in the Se group [88]. Similarly, postpartum depression was reduced in another RCT examining Se supplementation [89], an important finding that also awaits independent verification.

26.8 Viral Infections

An observational study analyzed the prevalence of hantavirus-induced hemorrhagic fever with renal syndrome in different areas of China and found a strong association between Se deficiency and the infection rate in both rodents and humans [90]. An RCT testing high Se-yeast supplementation (200 μg/day) for 9 months in HIV-positive patients in the US reported a decrease in HIV-1 viral load and an increase in CD4 T-cell count in supplemented patients [91]. However, these patients were already receiving antiretroviral therapy (ART); thus, it remains unclear whether Se was effective alone or exhibited an effect as an adjuvant to ART. In a similar supplementation study in Botswana conducted in 878 HIV-positive patients not receiving ART (2004–2009), Se supplementation (200 μg/day) for 24 months proved to be safe and significantly reduced the risk of immune cell decline and morbidity [92]. These findings are promising (see also Chap. 28).

26.9 Pregnancy

During pregnancy, pre-eclampsia constitutes a health risk for mothers and unborn children. In a nested case-control study, Se deficiency was identified as a risk factor [93]. An RCT (60 μg Se/day, as Se-enriched yeast) was conducted in the UK to evaluate the effect in reducing the risk of pre-eclampsia in women from 12 to 14 weeks of gestation until delivery. Serum soluble vascular endothelial growth factor

receptor-1 (sFlt-1), a biomarker of pre-eclampsia risk, decreased upon supplementation, indicating a potential benefit of Se (see also Chap. 30). Notably, although Se increased in supplemented females, it decreased in the control group, further supporting the importance of Se for both mother and embryo in pregnancy [94]. In another related RCT, Se supplementation (100 μg/day) in pregnant women decreased the rate of pre-labor rupture of the membrane [95].

26.10 Concluding Remarks

The number of clinical studies (and importantly, the number of sufficiently powered RCTs) evaluating Se for positively reducing health risks and improving convalescence and therapeutic interventions is constantly increasing, collectively supporting the notion that Se deficiency constitutes an easily avoidable risk factor for disease incidence and poor health. The thorough monitoring of participants in such trials and the stratifying analyses of supplementation effects with respect to confounding variables such as sex, age, weight, disease characteristics, and basal Se status are important for improving our knowledge regarding who would likely profit from supplemental Se and who would not. However, it is critical to note that a uniform picture has not been obtained; many surprisingly positive findings from focused RCTs have yet to be replicated, and many contradictory findings have been reported. In particular, precise monitoring of Se status before, during, and at the end of supplementation is needed for a better understanding of trial results and for stratifying patients into poorly and well-supplied groups, likely or unlikely to experience a positive clinical response to a Se-containing supplement.

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Chapter 27
Status of Dietary Selenium in Cancer Prevention

Gerald F. Combs Jr. and Lin Yan

Abstract An abundance of data shows that selenium (Se) can be antitumorigenic. Hundreds of studies using a wide variety of animal tumor models, as well as a much smaller number of clinical studies, demonstrate that treatment with Se in non-Se-deficient subjects can reduce cancer risk. Effective doses are substantially greater than those required for supporting maximal selenoprotein expression, which implies independent mechanisms of antitumorigenic action. We proposed a model for Se-anticarcinogenesis that links effects on gene expression, DNA damage/repair, cell signaling, cell proliferation, apoptosis, neo-angiogenesis and metastasis, with underlying metabolic activities of Se-metabolites in redox cycling, modifying protein-thiols, and mimicking methionine in metabolism. That these effects appear physiologically consequential only at supranutritional Se exposures follows from the apparently low levels of free Se-metabolites normally present in tissues. However, it is not clear whether the same mechanisms might have adverse effects including promotion of tumor growth. While present evidence suggests that supra-nutritional intakes of Se may benefit Se-deficient individuals and many who are Se-adequate, further research is needed to rationalize the mixed results of human clinical trials with the compelling results of animal models. Seven questions are posed to guide that research agenda.

Keywords Cancer • Carcinogenesis • Methylselenol • Selenide • Selenite • Selenium • Selenomethionine • Selenoprotein • Tumorigenesis

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27.1 Evidence for a Selenium-Mediated Antitumorigenesis

27.1.1 Emergence of Evidence

That Se might protect against cancer was first suggested in the late 1960s based on empirical observations of inverse relationships of cancer mortality rates and forage crop Se contents in the United States. Subsequent evidence has demonstrated protective effects of Se in reducing/delaying tumorigenesis in virtually all animal and cell models studied, including models of the spread of malignant cells to distant organs [1, 2]. This body of research has shown that both organic and inorganic Se-compounds can reduce both primary and secondary tumorigenesis in animal models when administered at levels (≥1.5 mg/kg diet) at least an order of magnitude greater than those required to support maximal expression of selenoenzymes (0.2–0.3 mg/kg diet). This would imply that supranutritional intakes of Se can benefit Se-adequate as well as Se-deficient individuals.

27.1.2 Clinical Trial Results

A dozen randomized clinical trials have been conducted to test the hypothesis that supplemental Se can reduce cancer risk in non-Se-deficient subjects (see reviews [3–6]). These yielded inconsistent results suggesting protection by selenite-enriched table salt against primary liver cancer, and by Se-containing, multi-agent supplements against esophageal cancer, precancerous oral lesions, and prostate cancer [3, 6]. A systematic review concluded that the evidence for supplemental Se reducing cancer risk is not convincing [3]. However, two others concluded that Se may be effective in cancer prevention for individuals with low-to-adequate Se status [4, 5]. The more recent Cochrane Review [6] found evidence of cancer risk reduction by Se for cancers of the stomach, bladder and prostate in 55 observational studies; however, it found no clear evidence of risk reductions in four clinical trials.

At the heart of the confusion over the antitumorigenic efficacy of Se in humans are the apparently discrepant results of two major trials, the Nutritional Prevention of Cancer Trial (NPC) [7] and the Selenium and Vitamin E Cancer Prevention Trial (SELECT) [8]. The NPC Trial [7, 9–11] tested the hypothesis that a daily oral dose of Se (200 μg/day as Se-yeast) could reduce the rate of recurrent non-melanoma skin cancer in a high-risk group of 1312 older Americans. Results showed Se-treatment not to affect risk to either squamous cell or basal cell carcinoma (BCC), although it appeared to delay diagnosis of the first BCC. It also found Se-supplementation to reduce risks of total carcinomas, total cancer mortality, and cancers of the prostate and colon-rectum by as much as 65 %. Selenium appeared to be protective only in early stages of tumorigenesis, as it reduced prostate cancer risk only in men with plasma prostate specific antigen levels <4 ng/mL. Greatest risk reduction occurred among subjects with baseline plasma Se levels in the lowest tertile of the cohort

(<106 ng/mL, 86 % risk reduction), with a lesser level in the middle tertile (107–123 ng/mL, 61 % reduction) and none in the highest tertile (>123 ng/mL).

SELECT tested the hypothesis that Se and/or vitamin E could reduce prostate cancer risk. It involved 35,553 men randomized to four treatments including Se (200 μg/day as L-selenomethionine [SeMet]), vitamin E (400 IU/day) in a factorial design. After 7+ years of follow-up, results showed no effects of Se-supplementation on prostate cancer risk, which were presented as negating those of NPC [8]. In fact, the results of these trials are not discrepant. This is because the subjects in SELECT were of relatively high baseline plasma Se level (136 ng/mL), i.e., similar to those of the upper tertile of NPC subjects who showed no reduction in prostate cancer risk [12]. In fact, subjects with the greatest prostate cancer risk reduction in NPC, those with baseline plasma Se <106 ng/mL, were underrepresented in SELECT. Thus, the totality of clinical evidence suggests that Se may reduce cancer risk for individuals of low-to-adequate Se status defined by plasma Se <106 ng/mL.

27.2 Mechanisms of Selenium Antitumorigenicity

It is clear that Se intake in *excess* of required levels can inhibit tumorigenesis in animals. In tumor models, antitumorigenically effective Se-exposures (e.g., of ≥1.5 mg/kg diet) exceed those needed for maximal selenoprotein expression (0.1–0.3 mg/kg diet). This implies a selenoprotein-independent mechanism of Se-antitumorigenesis.

27.2.1 Roles of Selenocysteine-Containing Selenoproteins

It is reasonable to expect selenoenzymes to have antitumorigenic roles in as much as the etiologies of some cancers appear to involve oxidative stress. The glutathione peroxidases (GPXs) and thioredoxin reductases (TXNRDs) would be expected to have anti-carcinogenic impacts by removing DNA-damaging H_2O_2 and lipid hydroperoxides, blocking production of reactive oxygen species (ROS), and regulating the redox signaling system critical to growth of many cancers. Accordingly, Se has been shown to modulate p53 activity by redox modification of cys275, 277 mediated by Ref-1, enhancing repair of DNA damage [13–16]. As p53 suppresses vascular endothelial growth factor [17] and induces angiogenesis-suppressing thrombospondin-1 [18], Se-mediated increases of p53 could play a role in switching off angiogenesis in early lesions. Knock-out of TXNRD1 in the liver of mice was found to enhance chemically induced hepato-carcinogenesis [19].

Selenoprotein involvement may also be expected on the basis of their differential expression in tumors and normal tissues. Tumors of the colon [20] and prostate [21] show lower levels of selenoprotein P (SEPP1) than adjacent tissues. Lung cancer patients have shown lower amounts of 15 kDa selenoprotein (SEP15) than healthy controls [22], and lower amounts of SEP15 mRNA were found in leukocytes of

male bladder cancer patients than controls, with patients with high-grade, poorly differentiated tumors showing greater amounts than those with lower-grade tumors [23]. SEP15 mRNA is also reduced in cancers of the liver and prostate [24]. Reduced expression of SEP15 by mouse colon cancer cells was associated with reduced expression of pathways involved in cell growth and proliferation [25]. Methionine sulfoxide reductase A is down-regulated in a number of human breast cancers [26], resulting in increased tumor aggressiveness and de-repression of the phosphoinositide proliferation pathway due to decreased levels of phosphatase and tensin homolog tumor suppressor protein.

Additional evidence points to selenoproteins having antitumorigenic roles. GPX2 is thought to protect gastrointestinal epithelial cells from hydroperoxide-mediated DNA damage [27]. Its knockout in prostate cancer cells has been found to increase intracellular production of ROS [28]. In the mouse, deletion of GPX2 resulted in spontaneous ileocolitis and colon tumorigenesis [29], and reduced induction of preneoplastic lesions and colonic tumors by azoxymethame [30]. The role of GPX2 may depend on the stage of tumorigenesis, as its expression is down-regulated in nuclear factor (erythroid-derived 2)-like 2 (NFE2L2 or Nrf2)-knockout mice [31] and up-regulated by Nrf2 activators [29]. Knockdown of TXNRD1 in liver increased expression of GPX2 and Nrf2 in mouse hepatocytes [19], and activation of Nrf2-like transcription factor increased expression of both TXNRD1 and GPX2 [32].

27.2.2 Roles of other Selenium-Associated Proteins

The Se-binding protein SBP1 has been related to prostate cancer risk [33]. Its level in the nucleus of prostatic cells was inversely related with prostate tumor grade in patients, and its expression was associated with increased phosphorylation of the p53 tumor suppressor.

27.2.3 Roles of Selenium-Metabolites

It would appear that most antitumorigenic effects of supranutritional exposures to Se are more likely to be related to the actions of Se-metabolites than those of selenoproteins or Se-binding proteins. Indeed, antitumorigenic activities have been demonstrated for several intermediary metabolites of Se; selenodiglutathione, the reductive metabolite of oxidized inorganic salts (selenite, selenate); hydrogen selenide, the common intermediate of that reductive pathway and of the catabolism of selenoamino acids including SeMet, a methionine analog and dominant food form of Se; and methylated metabolites of selenide (methylselenol, dimethylselenide, trimethylselenonium). These metabolites appear to execute functions underlying Se-antitumorigenesis [34].

27.2.3.1 Effects of Selenium-Metabolites on Tumor Cells

High Se exposures can arrest the cell cycle in different ways. Selenite can inhibit cells in S-phase leading to caspase-independent apoptosis, while methylated Se can inhibit in G1-phase leading to caspase-mediated apoptosis [35]. The methylselenol precursor, Se-methylselenocysteine, arrests cells in the G_1 or early S phase, inducing apoptosis in a caspase-dependent manner involving mitochondrial cytochrome C release, poly(ADP-ribose) cleavage, and nucleosomal DNA fragmentation [36]. In cell lines that lack functional p53, the pro-apoptotic action of methyl-Se is caspase-dependent [37]. In contrast, SeMet transiently activates Akt before inactivating it in a PTEN-dependent fashion [38]. Selenite can activate a p53-dependent pathway, increasing p21 and phosphorylated p53, as well as a p38 pathway leading to accumulation of Bax [39]. The product of the thiol-dependent reduction of selenite, oxidized glutathione, can inhibit the DNA-binding of transcription factor activator protein-1 [40], inhibit cell proliferation [41], and enhance apoptosis [42]. Methylated Se-compounds can produce transient upregulation of p21/CIP1 and p27/KIP1 in G1-arrested endothelial cells [43], indicating a link between cell cycle and Se-mediated antiangiogenesis. Mammary carcinoma cells, and other cancer cells, are more sensitive to methylated Se-induced apoptosis than normal mammary epithelial cells [44]; this involves down-regulated expression of two anti-apoptosis proteins, Bcl-XL and survivin [37].

Both selenite and SeMet can inhibit growth of secondary tumors in animal models. This involves altered collagen gene expression by methylated Se [45]. SeMet has been shown to reduce tumor cell invasion by decreasing ROS and blunting Akt-dependent matrix metalloproteinase (MMP) secretion [46]. Precursors of CH_3SeH have been shown to inhibit expression of MMP-2 in vascular endothelial cells and of VEGF in plasma [47] and cancer cells [36, 43, 48], suggesting that methylated Se inhibits cellular proliferation and survival of activated endothelial cells by inhibiting neo-angiogenesis. Selenium treatment has been found to impair microvascular development of tumors, which was related to the effects of methylated Se in reducing microvessel density in tumors by inducing cell cycle arrest in microvascular endothelial cells [37]. In fact, methylated Se was more effective than selenite in this regard [49].

Selenium was capable of disrupting sex-hormone signaling pathways by altering the expression of androgen and estrogen receptors. Methylselenol, produced by the action of methionase on SeMet, and methylseleninic acid inhibit the expression of androgen receptors and consequent signaling to the prostate specific antigen [50]. Methylseleninic acid can also inhibit estrogen receptor signaling in breast and endometrial cancer cells [51] and suppress aromatase expression by markedly down-regulating its promoters [52].

Because supranutritional intakes of Se have been shown to reduce colon carcinogenesis in both animal models and humans, it is of interest to know whether Se, which is not quantitatively absorbed in the small intestine, may affect the microbiota of the hind gut, which has long been thought to contribute to colon tumorigenesis. Studies have shown differences in the diversity of the microbiota of patients with colon cancer compared to healthy controls [53]. We found that supranutritional intakes of Se

increased fermentation by the microbiota of both rat colon and bovine rumen [54, 55], slightly increasing production of butyrate at the expense of acetate. Butyrate, a principal energy source for colonocytes, plays roles in preventing colonic tumorigenesis by reducing the number and size of aberrant cryptic foci [56], by reducing expression of cyclin D1 and c-myc [57], and by reducing NF-kB-activated expression of pro-inflammatory cytokines [58]. Selenium is thought to be required by several methanogenic Archaea and gram-positive enteric bacteria [59], on the basis of their having selenoenzymes [formylmethanofuran and formate dehydrogenases] that catalyze the first committed steps in methanogenesis. Therefore, it is reasonable to propose that Se supply to the hindgut may affect the abundance of Se-requiring bacterial species, thus affecting the net output of the hindgut microbiota, particularly of methanogens which depend on constant sources of hydrogen obtained by interspecies transfer.

27.2.3.2 Mechanisms Underlying Selenium-Antitumorigenesis

Redox Cycling

Redox cycling and covalent protein-thiol modification appear to be competing pathways available to Se. The disposition of Se-metabolites through these pathways would appear to determine their biological effects. Selenite, diselenides and the oxidation product of hydrogen selenide, Se dioxide, can each be reduced by GSH to produce selenolate ion and oxidized glutathione [60]. In the presence of molecular oxygen, these species can redox cycle to deplete GSH and produce ROS [61]. Thus, it is possible that selenite elicits biological effects through ROS-initiated cell damage responses leading to DNA damage and thiol modification [62], which appears to be the basis of caspase-independent apoptosis in selenite-treated cells [63]. While CH_3SeH can also redox cycle, the antitumorigenic effects of CH_3SeH-precursors are qualitatively different from those of H_2Se-precursors, suggesting that CH_3SeH functions by a different mechanism(s).

Both free and peptide-bound forms of SeMet can scavenge ROS and are regenerated non-enzymatically by GSH. Thus, the SeMet/Se(O)Met couple may serve as a cellular defense mechanism. It is known that SeMet is more readily oxidized than Met [64]; that oxidation can alter protein activity [65]; therefore, it is possible that the Met → SeMet substitution may sensitize proteins to ROS.

Modification of Protein-Thiols

It is possible that the presence of Se-metabolites can alter protein-thiol redox status, affecting cell-signaling mechanisms [66]. Products derived from both H_2Se and MeSeH can form covalent adducts with protein-thiols. Similarly, oxidized Se species can react with thiols in cell surface proteins to render them cross-linked [67]. Mixed selenosulfides of GSH can act through protein-thiol modification. For example, treatment with SeMet has been found to affect expression of redox-sensitive proteins in prostate cancer cells [68], and treatment with CH_3SeH-precursors has

been shown to decrease CDK2 kinase activity [69] and inhibit the expression of vascular endothelial MMPs and VEGF [70]. Such reactions are likely the bases of the inhibition by Se of ribonuclease [71], NaK-ATPase [72], protein kinase C [73] and, perhaps, other enzymes to trigger such effects as cell cycle arrest, apoptosis and angiogenic switch regulation.

Methionine Mimicry

SeMet competes with Met in general metabolism including protein synthesis, charging $tRNA^{Met}$ at rates similar to that of Met; thus, it is incorporated into proteins according to their respective Met contents [74]. In most cases, Met → SeMet substitution does not appear to affect the kinetic characteristics of enzymes [75], and non-specific SeMet incorporation is regarded as having no physiological import other than to serve as a means for building tissue stores of Se. While such stores account for most of Se in the tissues (e.g., 60 % of the Se in plasma of individuals fed SeMet [76]), it is still a small fraction (<1 %) of the potential number of Met residues even in individuals fed supranutritional levels of SeMet.

Selenomethionine can also be metabolized to analogues of Met-metabolites by the pathway used for the transsulfuration of Met to cysteine. An effective substrate for the Met-adenosyl transferase, SeMet is activated to adenosyl-SeMet [77], which serves as a methyl donor for methyltransferases involved in the methylation of RNA, phosphotidyl lipids, creatine, histamine and thiols; and play roles in gene silencing, repair of damaged proteins and activation of oncogenes. In fact, it is more effective than Met as a substrate for Met-adenosyl transferase [78], forming Se-adenosylselenomethionine, which is also a better methyltransferase substrate than S-adenosylmethionine [79].

27.3 Evidence for Tumorigenic Actions of Selenoproteins

Selenoprotein involvement in tumorigenesis is suggested by associations of their allelic variations and cancer risk responses to supplemental Se (see Chap. 29). A single nucleotide polymorphism (SNP) at codon 198 of human *GPX1*, resulting in a leucine-for-proline substitution, has been associated with increased risks of cancers of the lung [80], breast [81], head and neck [82], bladder [83] and prostate [22]. The 198-leucine genotype may also be less responsive to Se than the 198-proline genotype [84], suggesting that increased cancer susceptibility of individuals with that allele may involve their reduced ability to utilize Se for selenoprotein expression. A SNP (25191) of *SEPP1* predicts increase in plasma Se level with Se-supplementation [80], half of which is associated with SEPP1. Lung cancer risk has been related to SNPs of *SEP15* [22]; individuals with the 1125AA genotype appeared to benefit most from being of higher Se status. Risk to high-grade prostate cancer has been associated with SNPs in the human *TXNRD1* gene; and risk of recurrent prostate cancer in men with non-metastatic disease has been associated with multiple SNPs in *TXNRD1*,*2*, *GPX3* and *SEP15* [85].

Cases have been reported in which functions of at least some selenoenzymes are associated with enhanced tumor cell growth. GPX2 is known to be a target of the Wnt pathway [86], which is associated with cell proliferation; its up-regulation would be expected to promote tumor growth (see Chap. 17). Overexpression of GPX2 in MCF7 breast cancer cells reduced their sensitivity to H_2O_2-induced cell death [87], presumably by enhancing their antioxidant protective capacity. Suppressed expression of Sep15 in colon carcinoma cells (by transfection with *Sep15*-targeted shRNA) greatly reduced their metastasis and development of solid tumors when injected into mice [25]. Studies by Tsuji et al. showed that Sep15 increased cancer progression, possibly through affecting cell cycle regulation and/or through interferon-γ-regulated inflammation (see [88, 89] and Chaps. 19 and 37). Many types of cancer cells have been found to express high levels of TXNRD1, and tumorigenesis has been shown to be reduced by specific inhibitors of TXNRD1 [90]. Mice injected with TXNRD1-knockdown lung carcinoma cells showed much less primary tumor progression and metastasis compared to mice injected with TXNRD1-expressing carcinoma cells (see [91] and Chaps. 19 and 37).

27.4 Risks of Supranutritional Se Intakes

That high but apparently non-toxic Se status may be associated with metabolic risks was suggested by Waters et al. [92], who noted increases in canine prostatic DNA damage in animals of either low or high Se status. Their data showed a clearly U-shaped dose-response relationship, implying at least two different metabolic actions of Se in non-deficient individuals: one related to antitumorigenesis and operative over the low-adequate range of Se status; another related to adverse metabolic effects and operative over the high-adequate range. Consistent with this model are findings in different randomized trials suggestive of increased risk of prostate cancer among non-deficient men supplemented with supranutritional levels of Se [93], and of type 2 diabetes in non-deficient subjects treated with Se [94].

27.5 Concluding Remarks

Se-compounds and at least some selenoproteins appear to play roles in inhibiting and/or delaying tumorigenesis. Results of mechanistic studies have demonstrated roles of several selenoproteins as antitumorigenic metabolic effectors, which would imply that dietary deprivation of Se would enhance tumorigenesis by limiting their expression. Support for that hypothesis is weak; only two animal studies have provided such evidence [95, 96]. It is clear, however, that tumorigenesis can be reduced and/or delayed by intakes of Se well above those required for maximal expression of most, if not all, selenoenzymes. These effects appear to be discharged by

Se-metabolites through three mechanisms: ROS production from redox cycling; protein impairment by thiol modification; and increased susceptibility to oxidation due to Met → SeMet substitution. Provocative data have been presented for a possible role of one selenoprotein, selenoprotein W (SEPW), which has been reported to be up-regulated by supranutritional intakes of Se [97]. It has been found to suppress the ubiquitination and degradation of the epidermal growth factor (EGF) receptor, inhibiting the EGF-dependent cell cycle in prostate cancer cells [98].

A few reports have raised possibilities that other selenoproteins may have cellular functions that enhance tumorigenesis. Might such results indicate vital functions that, when discharged in malignant cells, are deleterious to the host? If so, then dietary deprivation of Se, which is known to limit the expression of these selenoproteins, would be expected to reduce tumorigenesis. There are no reports of such findings in any animal tumor model.

Therefore, the collective evidence suggests that supplemental Se offers the potential of reducing cancer risk in both Se-deficient and possibly non-deficient individuals. Human data suggest that individuals with plasma Se levels as great as 106 ng/mL may benefit; that level that would include many US Americans, most Europeans, and many other adults in other countries. However, that conclusion rests on a narrow evidence base, which demands expansion through additional human studies for Se-supplementation to be considered as a strategy for reducing cancer risk. For further human trials to be considered, seven key research questions must be addressed:

1. *How applicable are the animal tumor model data to the consideration of human Se-supplementation for reducing cancer risk*? A metabolically sensible explanation is needed to rationalize the apparent difference in the protective effects of modest supplemental levels of Se reported in some human trials (e.g., fivefold required amounts) with the need for much greater intakes (e.g., 15-fold required amounts) to reduce tumorigenesis observed in animal models.
2. *What is the degree of equivalency of SeMet and selenite for antitumorigenesis*? A metabolically robust rationale is needed to understand the implications of antitumorigenic effects of selenite with those of organic Se-compounds including SeMet. It would appear that antitumorigenesis by most Se-compounds may be due mostly to redox cycling, while SeMet may have additional effects including Met mimicry and protein modification. It is also likely that differences in efficacy of SeMet and other Se-compounds may be significant only over the relatively short durations of animal studies, before the establishment of steady-state equilibria for SeMet incorporation into and release from tissue proteins. This limitation of most animal studies may thus limit their informative value to the long-term dietary patterns and practices of humans.
3. *What selenoproteins are induced under conditions of supranutritional Se intake*? Further investigation of SEPW, expression of which appears to be up-regulated by high-level Se exposure, is needed to determine whether its expression is up-regulated by supranutritional levels of Se in animal models, what role it may have in Se-antitumorigenesis, and whether any other selenoproteins may also respond to high-level Se exposure.

4. *What are the effects of Se and its metabolites on the gut microbiome*? While Se-compounds are not quantitatively absorbed in the small intestine, and are excreted in the bile, the fate of Se in the hind gut is a grossly under-studied area. Research is needed to determine the extent to which Se from selenite and/or SeMet at supranutritional intakes is accessible to the microbiome, and whether such exposure can affect the taxonomic composition and metabolite output of major human enterotypes.
5. *What biomarkers are most informative regarding the relationship of Se status and cancer risk*? Ample data are available to support the use of total Se and selenoproteins in plasma/tissue for the assessment of nutritional Se status in individuals with Se intakes of known amounts and composition. However, it is not clear that such biomarkers have any functional relevance to antitumorigenesis. Biomarkers with such relevance are needed to improve the informative value of epidemiological investigations, to identify subjects likely to benefit from Se-supplementation, and to evaluate the efficacy of Se-supplements.
6. *What are the risks of supranutritional Se intakes*? Research is needed to address the possibility that high, but not overtly toxic doses of Se may have metabolic risks, such as DNA damage, cancer promotion, and type 2 diabetes risk. Such knowledge is needed to define better the widow of safety for Se-compounds.
7. *Does Se-deficiency increase tumorigenesis*? This prospect has been suggested by epidemiological studies, but has not been well researched using animal tumor models. It has implication to large numbers of people of low-to-deficient Se status.

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Chapter 28
Selenium in HIV/AIDS

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Abstract Selenium (Se) supplementation in people living with HIV (PLWH) has demonstrated benefits in terms of HIV disease progression, morbidity, and mortality. The HIV pandemic continues to be a major health priority worldwide, and the life span of PLWH increases due to the life-prolonging effects of antiretroviral therapy (ART). Subsequently, nutritional interventions become critical as adjuvant therapies. Nutritional interventions assist in the recuperation of the immune system, preventing nutritional deficiencies, and limiting oxidative stress damage, especially in an aging population on chronic ART. Se status influences HIV disease progression through its role in modulating cytokine signaling for the activation of the immune system and through its antioxidant activity. In addition, this chapter reviews other aspects of the relationship between HIV/AIDS and Se status and Se supplementation, such as associations with vaginal HIV shedding, mitochondrial damage, and HIV transmission.

Keywords Antioxidant • HIV • Immunity • Nutrition • Nutrition deficiency • Oxidative stress • Selenium • Selenium supplementation

28.1 Introduction

The Centers for Disease Control and Prevention (CDC) reported an incidence of approximately 50,000 new cases of HIV infection in the United States in 2015. Currently there are approximately 1.2 million people, aged 13 and older, living with HIV/AIDS in this country [1]. In countries with access to ART and behavioral prevention interventions, the incidence of adult cases has decreased. The introduction of successful interventions during pregnancy, such as universal maternal HIV testing, perinatal and post-natal care, have dramatically reduced the incidence of pediatric HIV/AIDS worldwide [2].

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The HIV pandemic, however, is still a major public health concern worldwide. This is especially evident in limited-resource countries in Sub-Saharan Africa, where the HIV epidemic is superimposed on the high proportion of severe malnutrition in regions where this condition is prevalent [3]. In 2013, the World Health Organization (WHO) reported 2.1 million new infections in low- and middle-income countries, a reduction compared with previous years. By the end of 2013, an estimated 35 million people were living with HIV worldwide; and of those, 37 % were receiving ART. The WHO goal of 15 million in treatment by the end of 2015 has been surpassed, and 15.8 million PLWH were receiving ART globally as of mid-2015 [4]. Poor nutritional status affects immune function independent of HIV infection [5, 6], and the mortality rate is increased among PLWH who are malnourished, even among those initiated on ART [7, 8]. Multiple research reports have shown that nutritional deficiencies are widespread among PLWH and accelerate HIV disease progression and increase mortality [9–18], while supplementation with multivitamins and Se delays progression of the disease [19]. In addition, nutrient deficits interfere with the effectiveness of ART by delaying the recuperation of the immune system and aggravating side-effects, such as inflammation and oxidative damage [20–24].

28.2 Se and Immunity

Se deficiency produces changes in metabolic functions such as depressing the immune response and decreased protection from reactive oxygen species (ROS) [25, 26]. Among several potential mechanisms of action, Se influences the immune response by modulating cytokine signaling [25]. In an in vitro model, the addition of Se enhanced the production of interleukin-2 through increased expansion of high-affinity cytokine receptors in a dose-dependent manner [26]. In animal models, phagocytic neutrophils and macrophages exposed to Se-deficiency had a reduced ability to destroy antigens. Se's immune-stimulatory properties have been reported in animal supplementation studies [27], in supplementation of older adults [28], and in patients with chronic uremia, psoriatic lesions, and gastrointestinal failure syndrome [29–31].

In PLWH, plasma Se levels have been associated with immune parameters. Plasma Se levels were positively related with CD4 cell counts and CD4/CD8 ratios, and inversely correlated with β_2-microglobulin levels, a marker of cellular immune system activation and inflammation, and with thymidine-kinase activity, which has a role in nucleoside analogs activation and toxicity [32]. Low Se status is also associated with the production of TNF-α, a cytokine related to anorexia, wasting, and Kaposi's sarcoma [33]. Plasma Se levels were reported to be inversely associated with TNF type II receptors in PLWH [32]. Other findings suggested that the effect of Se supplementation on reducing viral replications was via the synthesis of selenoproteins in the glutathione and thioredoxin systems [34]. Several in vitro and in vivo reports provided evidence that adequate Se status decreases neuropathogenesis by

reduction of interleukin-induced HIV-1 replication, neuronal apoptosis, and blood brain barrier damage, and by the potential interactions between Se and cytokine signaling [32–35].

28.3 HIV, Antiretroviral Treatment, Oxidative Stress and Se

Increased oxidative stress has been one of the early features recognized in HIV-infection [36–39]. Potentially as a consequence of higher antioxidant demands, a decrease in serum levels of major antioxidant nutrients, such as vitamins E and C, carotenoids, zinc, and Se has been reported in PLWH [40–44]. HIV-infection causes chronic immune activation which contributes to an increase in the production of ROS. In vitro, ROS are continually produced as a consequence of substrate oxidation, aerobic respiration, and immune activation [45, 46]. The production of ROS is useful to several cellular processes, including cell growth, apoptosis, immunity, and microbial defense [46–49]. HIV-infection, however, generates an excessive amount of oxidative products, which may be damaging to tissues and cells. To quench these excessive circulating ROS, multiple enzymatic and non-enzymatic antioxidant defense systems exist to prevent damage by oxygen radicals [50, 51].

Se is a critical component of the body's antioxidant system due to its role in the production of glutathione peroxidases and other selenoproteins [52]. Supplementation with several forms of Se has been studied for the prevention and treatment of cancer in order to increase the effectiveness of the enzymatic antioxidant defense systems [53, 54]. Investigations from early studies on Se status at different stages of HIV progression reported that total GSH levels declined as HIV advanced to AIDS [55]. Alterations in antioxidant defense enzymes, including superoxide dismutase (SOD), catalase, and glutathione peroxidase have been reported in PLWH [37, 38, 42]. Compared with HIV-negative patients, PLWH have increased SOD activity, reduced GSH and total glutathione, and increased plasma levels of malondialdehyde (MDA), which is a marker of lipid peroxidation, and lymphocyte DNA fragmentation. Total antioxidant markers in PLWH were also diminished compared with that of their HIV-negative counterparts [39].

Supplementing antioxidants to reduce oxidative stress has been a strategy used in several investigations. An in vitro study showed that addition of Se increased total glutathione activity in latently HIV-infected T-lymphocytes [35]. Total glutathione activity increased in PLWH, who were supplemented with 100 μg Se daily for 1 year [54]. Rather than diminishing the importance of antioxidant supplementation, however, provision of ART created new challenges for the role of Se in controlling oxidative stress in HIV-infection. Some types of ART have been associated with increased oxidative stress and oxidative damage [56–59]. In contrast, some human studies have detected increased antioxidant capacity and repair of DNA damage with the use of ART, especially in combinations containing the new families of antiretrovirals [60–62]. Although the effect of different types of antiretroviral drugs on oxidative stress varies, protease inhibitors have generally been found to increase the production of ROS including peroxides, which are associated with endothelial dysfunction

and dyslipidemias leading to increased cardiovascular risk [55, 63]. Nucleotide reverse transcriptase inhibitors have a well-established adverse effect on mitochondria, which results in increased oxidative damage including lipid peroxidation products, protein carbonyls and mitochondrial damage [56–59, 64, 65]. Eighty-five PLWH who were either ART-naïve or on three different ART regimens, were included in a study that examined lipid and protein peroxidation, compared to 90 healthy controls. There was an increase in overall oxidative stress in those on ART compared with the ART-naïve group, and in these two HIV+ groups compared with healthy controls [66].

Cardiovascular disease (CVD) has become a major cause of non-HIV related deaths among PLWH [67]. Oxidative stress represented by increased generation of ROS in aortic endothelial cells in those exposed to ART has become a concern [68]. Lipodystrophy, hyperlipidemias and insulin resistance in patients receiving HIV protease inhibitors [69] may increase the long-term risk of oxidative damage associated with development of atherosclerosis and coronary heart disease [67, 70]. Increased oxidative stress due to mitochondrial toxicity, produced either by the HIV disease or ART, may amplify the pathophysiology of HIV disease and the cellular damage seen in AIDS [71]. Thus, an important goal is to prevent or attenuate these effects of ART and improve HIV disease management.

28.4 Observational Studies of Se Deficiency and HIV

Se deficiency has been associated with HIV disease progression and mortality in numerous studies of patients prior to initiating ART as well as those who were on ART [16, 17, 72–74]. Lower levels of Se in pregnant women appeared to predict a high risk for intrapartum HIV transmission, and fetal and child death [78]. Se deficiency has been found to be highly prevalent in the general population in Sub-Saharan Africa, but lower rates of Se deficiency were reported in studies of PLWH in the United States. Overall, the reported prevalence of Se deficiency has been 7–33 % among various HIV-1 infected cohorts at the baseline and Se levels progressively decreased as the disease advanced to AIDS [44, 72]. In addition, in a model of simian immunodeficiency virus infection, levels of plasma and cellular Se also decreased as the infection advanced [75].

In a study of PLWH who were chronic drug users, Se deficiency was reported to be an independent predictor of mortality after controlling for CD4 cell count and other nutritional deficiencies [16]. When these investigators conducted a study in a cohort of HIV-infected men who had sex with men (MSM), the odds ratio (OR) was 7.2 for mortality in those with low plasma Se compared to those with normal Se levels, after controlling for age, race, and CD4 cell count <200 cells/μL at baseline [44]. Participants with Se deficiency lived 31.4 months, compared with 57.4 months for those with normal plasma Se levels after controlling for CD4 cell counts, viral load and antiretroviral medications. McDermid et al. [76] found that adequate dietary Se intake was strongly associated with reduced measures of oxidative stress in PLWH.

Se deficiency has been associated with advanced immune-deficiency [77] and mortality in HIV-infected children [72]. In agreement with the previous findings, a 2-year study of 610 children born to HIV-infected women in Tanzania showed that the children's plasma Se levels were inversely associated with risk of mortality [17]. In addition, depressed maternal plasma Se levels significantly predicted risks of fetal death, child death, and intrapartum HIV transmission, but were not associated with risks of delivering children small in size for gestational age [17].

Hileman et al. [78] examined the association of plasma Se with markers of inflammation, immune activation, and subclinical vascular disease, as CVD has become a major risk for non-HIV related deaths in this population on ART. Among 147 PLWH on ART, those receiving protease inhibitors were found to have higher plasma Se concentrations. Se deficiencies were not found in this group and levels of Se were not associated with subclinical vascular disease; however, higher levels of Se were associated with statin use [78]. In contrast, Se deficiency was the most frequently found nutritional deficit (53.2 %) among a subset of 270 participants from the ACTG Prospective Evaluation of Antiretrovirals in Resource Limited Settings (PEARLS) cohort [83]. The participants were selected randomly from the 1571 participants who were not receiving ART at the PEARLS study baseline. They represented nine countries, among them the United States. More than half of the group (57 %) had more than one nutritional deficiency. After 48 weeks of receiving ART, Se deficiency remained high (50 %), suggesting that ART alone may not be able to correct Se deficiency, and that supplementation may be needed, especially in settings where Se deficiency is prevalent, such as Sub-Saharan Africa. In contrast, other investigators have found that similar or longer exposure to ART for more than 2 years appeared to be able to reverse Se deficiency [79–81]. In the same subset from the PEARLS cohort, Se deficiency was not associated with clinical or virologic failure [87]. However, higher levels of plasma Se were associated with an increased hazard ratio (HR = 3.5, 95 % CI: 1.30–9.42) for WHO stages 3, 4 or death, but not for controlling viral load under detectable levels. Based on this finding, the investigators advised caution when supplementing Se to PLWH with adequate or unknown levels of plasma Se at baseline.

A relationship between Se deficiency and increased vaginal HIV-RNA shedding was suggested in an observational study of Kenyan women, reporting a threefold higher likelihood of genital mucosal shedding of HIV infected cells. Kupka et al. [82], however, found an association of higher plasma Se levels (≥114 μg/L) with an increased risk of genital shedding of HIV-RNA in African HIV+ pregnant women, a relationship that was strengthened by excluding women with diagnosed genital infections.

Se deficiency has been shown to be associated with increased risk of herpes and candida infections [83], and a higher risk for mycobacterial disease, both tuberculosis (TB) and mycobacterium avium (RR = 3, $P = 0.015$), adjusting for ART and CD4 cell count in PLWH who reside in Miami [84]. Low serum Se concentration among PLWH in Nigeria were also associated with pruritic papular eruption, xeroderma, fluffy hair, blue-black nail hyper pigmentation, and oral candidiasis [85].

The significant association of Se status with HIV-related morbidity and mortality may be related not only to Se's role in maintaining immune competence, but also to

its activity in modulating viral expression and protection against the oxidative damage caused by chronic infection and its treatment [86–90].

28.5 Se Supplementation in HIV

Early in the history of HIV/AIDS, Kavanaugh-McHugh et al. [91] described signs and symptoms similar to those in Keshan Disease, which itself is associated with Se deficiency [92]. These deficiency symptoms improved after administration with Se (4 μg/kg) [91]. Several studies have reported health improvements in PLWH after daily supplementation with Se [43, 93, 94], without adverse effects [43, 93]. Early findings on the relationship of Se deficiency with mortality in an observational study underline the importance of maintaining adequate Se status in PLWH [16].

Several clinical trials were conducted supplementing Se in PLWH, reporting largely beneficial effects in terms of slowing HIV disease progression. In two early reports from a 12-month study [54, 95], benefits in supplementing PLWH with 100 μg of Se daily, compared to 30 mg of β-carotene twice daily and to a control group without supplementation were reported. At the end of the 12-month supplementation, the control group had more endothelial damage, while the groups who received antioxidant nutrients, Se and β-carotene, were unchanged over time [95]. Glutathione peroxidase activity increased significantly in the group who received Se within 3–6 months, compared to the other two groups [54].

In a cohort that included PLWH with and without ART, 186 adults were randomized into receiving 200 μg of selenomethionine or placebo in a double-blind, placebo-controlled study. Those supplemented with Se were 60 % less likely to be hospitalized during the 24-month follow-up period [96]. Furthermore, those supplemented with 200 μg of Se for 9 months, significantly suppressed HIV viral load, and increased CD4 cell count over time [97]. An increase in plasma Se concentration predicted a decrease in viral load, and Se levels affected CD4 cell count through its effect on viral load.

In a large randomized trial of supplementation with Se (200 μg in the form of selenomethionine), 915 HIV-infected pregnant women in Tanzania were supplemented from the 12th to the 27th week of gestation until 6 months after delivery. Kupka et al. [98, 99] reported a reduction of diarrhea by 40 % without significantly increasing the risk for anemia in women, and a reduction in risk of child mortality after 6 weeks of delivery.

In a multicenter, randomized double-blinded clinical trial, the effect of Se supplementation compared to placebo on CD4 cell counts was investigated. A 24-month Se supplementation significantly reduced the decline of CD4 cell counts among ART-naïve participants, confirming early results in a large trial of Se and multivitamins supplementation [19].

28.6 Clinical Trials of Supplementation in HIV-Positive Patients that Included Se in the Experimental Formula

Se at different doses has been used in numerous randomized double-blinded clinical trials in PLWH. Trials of Se alone [54, 95–99] or in combination with other antioxidants, vitamins, and minerals have provided evidence of beneficial outcomes [100–104]. In those trials in which Se has been part of a formula in combination with other antioxidants and micronutrients, it has not been possible to separate the benefits of Se from those of the other components. The interaction, however, might be desirable, because the benefits may be magnified by the synergistic effects of micronutrients.

Early in the HIV epidemic, Kelly et al. [100] randomized 106 PLWH into Albendazole with placebo or Albendazole with a micronutrient formula with vitamins A, C and E, zinc and Se. Participants were ART-naïve and had diarrhea-related wasting. Addition of the nutrient formula to Albendazole did not improve outcomes after 2 weeks of supplementation, which is probably a very short period to observe significant changes.

In a recent pilot study in Nigeria, 32 HIV-positive, ART-naïve patients were supplemented with 200 μg of Se, vitamin A, B-complex, C and D. Twenty-three patients were randomized into the multivitamin/mineral formula group with 300 mg of aspirin 4–6 times daily, and a second group comprised of nine patients into the multivitamin/mineral formula without aspirin. After 6 months of supplementation, the post-therapy mean weight was significantly higher in the arm receiving the micronutrient formula with aspirin compared to the micronutrient formula alone, and CD4 cell count increased by an average of 36.2 cells/μL, showing a trend towards improvement [105].

In the United States, supplementation of 40 HIV-positive adults on stable ART, which have a controlled viral load, with a combined formula of antioxidants, minerals, and vitamins that contained 200 μg of Se for 12 weeks resulted in significant improvement of CD4 cell counts. Supplementation with a complex formula containing 400 μg of Se daily resulted in improved survival in those with advanced HIV disease [102].

HIV and tuberculosis (TB) co-infection is one of the main causes of mortality in resource-limited countries [106, 107]. In a randomized controlled clinical trial in Tanzania, Villamor et al. [103] randomized 887 TB patients, of whom 471 were also HIV-positive, into a micronutrient formula containing Se or placebo. Supplementation increased CD3 and CD4 cell counts, decreased the incidence of extra-pulmonary TB and genital ulcers in those who were HIV-negative, and reduced peripheral neuropathy by 57% irrespective of HIV status [103]. In addition, participants in a cohort study, that included patients infected with TB alone and patients co-infected with HIV and TB, were provided with a multivitamin/mineral supplement which contained 200 μg of Se in their daily dose. This trial reported significant reduction in mortality in those supplemented who were co-infected with HIV and TB [103].

McClelland et al. [108], in randomized clinical trial in Kenya, supplemented multivitamins with 200 μg of Se or placebo for 6 weeks, and reported that disease progression, evidenced by CD4 (+23 cells/μL, $P=0.03$) and CD8 cell counts (+74 cells/μL, $P=0.005$) improved with supplementation when compared to placebo, which had no effect on viral load. In addition to this beneficial results, these investigators found an increase in genital HIV shedding with Se supplementation (OR = 2.5, 95% CI: 1.4–4.4, $P=0.001$), adjusting for baseline $\log_{10}$ vaginal HIV-1 RNA, and body mass index. This study, however, did not determine the Se status of the participants. Thus, it appears that there may be a range of Se supplementation levels that improves immunity, while higher levels of supplementation increase HIV shedding of HIV-infected cells. There is also a possibility that Se supplementation to those with Se deficiency is beneficial, while supplementing participants who are Se sufficient may not provide benefit. Further studies are needed to clarify which patients benefit and the range of Se that is not harmful. In a large trial of supplementation with 878 PLWH, who were ART-naïve, Baum et al. [19] randomized participants into four groups of daily supplements: multivitamins (B vitamins and vitamins C and E) alone, Se alone, or multivitamins with Se vs placebo in a factorial design for 24 months. The investigators concluded that the supplement containing multivitamins and Se was safe, especially in a population with a very high rate (97%) of Se deficiency and that Se significantly reduced the risk of immune decline and morbidity. Participants receiving the combined supplement of multivitamins plus Se had a lower risk than the placebo group of reaching CD4 cell count ≤250 cells/μL (adjusted hazard ratio = 0.46; 95% CI, 0.25–0.85; $P=0.01$). In addition, the investigators observed that micronutrient supplementation is more effective when started in the early stages of HIV disease.

28.7 Concluding Remarks

Randomized clinical trials involving Se and PLWH wherein Se was given alone or with micronutrient formulas, demonstrated benefits on parameters of disease progression, morbidity, and mortality. The adverse role of Se in other outcomes such as HIV shedding, clinical failure and HIV transmission needs further investigation to optimize the levels and modes of Se supplementation in order to provide the well-established benefits for PLWH that also minimize adverse consequences.

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Chapter 29
Genetic Variations in the Genes for Selenoproteins Implicate the Encoded Proteins in Cancer Etiology

Rama Saad and Alan M. Diamond

Abstract Genetic variations in the genes for selenium-containing proteins have been investigated in human epidemiological studies with regard to their association with the risk and/or outcome of a variety of cancer types. For several of these, including those known to be involved in anti-oxidant functions and selenium transport, positive associations of specific polymorphisms within their corresponding genes have revealed significant associations as well as interactions with intrinsic and extrinsic effect modifiers. These studies provide evidence for a contributing role of specific selenoproteins in the prevention of cancer and contribute to the understanding of the mechanisms involved.

Keywords Anti-oxidants • Cancer • Genetic variations • Peroxidase • Polymorphisms • Selenoproteins

29.1 Introduction

The impact of selenium on biological processes and human health has been investigated for decades. Perhaps the greatest focus of these efforts has been the role selenium may play in cancer risk and outcome. This area of research emerged from epidemiological data published in 1983 indicating that there was an inverse association between the amount of selenium in the soil of a particular region and the overall cancer incidence in that area [1]. In subsequent years, additional studies have looked more specifically into either dietary selenium intake or the selenium levels in representative tissues in individuals. While the results have not always been consistent, there are now numerous reports supporting the relevancy of selenium in reducing the morbidity and mortality associated with cancer.

While the mechanisms behind the putative benefits of higher selenium intake are controversial, the consequences of selenium availability on the levels and activities of

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selenium-containing proteins remain among the strongest possibilities. The human genome contains the information for 25 proteins in which selenium is present in the form of selenocysteine (Sec) [2]. These proteins are encoded by one or more in-frame UGA codons that direct the co-translational incorporation of Sec, so designated due to the presence of a regulatory sequence termed the SECIS (Selenocysteine Insertion Sequence) element located in the 3′-untranslated sequence of the corresponding mRNA [3]. There are also a small number of less characterized proteins that contain selenium as a covalently bound moiety, but not Sec [4]. The responsiveness of many of these proteins to the levels of available selenium makes them reasonable candidates as the mediators of the chemoprotective effects of selenium. Moreover, the presence of Sec at the active sites of many of these proteins make them particularly well suited to function in redox reactions and serve as cellular anti-oxidants.

By virtue of being able to participate in the detoxification of reactive oxygen species (ROS), many selenoproteins were considered candidates, alone or as a group, as the mediators of the chemoprotective effects of selenium. And while this may in part be true, the action of anti-oxidants as a means to reduce cancer seems much more complicated than originally thought, as evidenced by the general failure of anti-oxidants provided as a supplement to reduce cancer risk or attenuate disease progression [5]. Selenoproteins have also been implicated in cancer etiology due to significant differences between their levels in tumors and corresponding healthy tissues. However, these observations cannot distinguish whether those differences contributed to the disease process or were consequences of the altered regulation that often accompany neoplastic development.

Genetic variations among naturally-occurring alleles may impact a host of phenotypes as well as susceptibility to diseases such as cancer. Polymorphisms and other sequence variations in the genes for selenoproteins have been used to look for associations between these differences and cancer risk and/or outcome, therefore implicating those genes as effectors of the carcinogenic process. Several recent comprehensive reviews have catalogued these variations and described the cancers that they are associated with [6, 7], including a list of the most studied selenoprotein genes with polymorphisms linked to cancer risk or outcome presented in Table 29.1. In this chapter, the focus will be on the functional consequences of these variations as arguments for their direct involvement in the disease process and modifiers of their penetrance, such as interaction with other gene products or extrinsic factors as, for instance, diet.

29.2 The Types of Genetic Variations to Consider

Broadly speaking, allelic differences that can impact a selenoprotein's role in cancer can be quantitative, ultimately affecting that protein's levels by a variety of mechanisms. Such differences can include polymorphisms in transcriptional or translational start sites, or other regulatory domains, or variations that alter the stability of the selenoprotein or mRNA. An additional relevant region that is unique to selenoproteins containing Sec that can have a profound effect on selenoprotein levels is

Table 29.1 Selenoprotein polymorphisms associated with cancer

Gene	Chromosome location	RS#	Nucleotide identity/ location	Amino acid identity
GPX1	3p21.3	*rs1050450*	C/T, codon 198	*Pro/Leu*
SEPP1	5P12	*rs3877899*	G/A, codon 234	*Ala/Thr*
		rs7579	A/G, 3′ UTR	
SEP15	1p31	*rs5845*	A/G, 3′ UTR, position 811	–
		rs5859	C/T, 3′ UTR, position 1125	
GPX4	19p13.3	*rs713041*	C/T, 3′UTR, position 718	–
		rs3746162	A/G, 3′ UTR	
		rs2074452	C/T, 3′ UTR	
		rs3746165	C/T, 2 kb upstream	
TXNRD1	12q23.3	rs4964778	C/G, intronic	–
		rs4523760	T/C, intronic	
		rs17202060	C/T, intronic	
TXNRD2	22q11.21	rs2073750	G/A, intronic	–
		rs3788314	G/A, intronic	
		rs756661	T/C, intronic	
SELS	15q26.3	rs34713741	G/A, 2 kb upstream	–

the SECIS element, the component of the mRNA which determines whether the in-frame UGA codon is recognized as Sec or a stop codon, and at what rate [8]. Qualitative variations can typically be single nucleotide polymorphisms that alter the identity of a single amino acid altering the primary sequence of the encoded protein. Consequences to these types of variations can include direct changes in enzymology, altered interaction with substrates and binding partners as well as changes to that protein's distribution in cellular compartments.

29.3 Glutathione Peroxidase 1 (GPX1)

GPX1 is member of the GPx family of proteins that reduce hydrogen and/or lipid peroxides to water using reducing equivalents from glutathione. Of the members of this family, GPX1 was the first to be discovered and remains the best characterized ([9] and Chap. 17 for comprehensive reviews). It has often been considered a prime candidate for a protein relevant to cancer given its broad function as an anti-oxidant and the general concept that oxidative damage to DNA can be mutagenic and the damage incurred can be oncogenic. Variations in the levels of GPX1 in tumors compared to the corresponding normal tissues have often been reported. However, it is

difficult to distinguish whether such observations indicate that the different levels are contributing to tumorigenesis or the consequence. Alternatively, germ line variations in the *GPX1* gene sequence that are associated with cancer incidence provide an indication of cause, unless that polymorphism is genetically linked to a consequential neighboring variation.

Considerable attention has been given to a single nucleotide polymorphism first described in 1999 that results in a leucine (*leu*) or proline (*pro*) at codon 198 of the *GPX1* sequence, sometimes referred to codon 199 [10]. Numerous studies have revealed an association between the leu198pro polymorphism and cancer risk. Most studies are consistent in indicating the *leu* expressing allele is the "at-risk" polymorphism, although other studies have indicated the proline-expressing *pro* allele to be associated with risk, while yet others have reported no associations at all [6, 7, 11]. The list of cancers for which an association between *GPX1* genotype and cancer risk was found include some of the most common types, including those of the colon, prostate and breast [6]. Another common variation in the *GPX1* gene is a variable number of alanine repeat codons resulting in five, six or seven alanines in the amino terminus of the encoded protein [12]. Few studies have searched for associations between this variation and cancer risk, although associations between the number of repeats and cancer have been reported for cancers of the breast [13], prostate [14] and those of the head and neck [15]; these studies did not reveal a consistency among the number of repeats associated with risk.

Clues to the molecular consequences of the GPX1 variations emerged using human cells cultured in the laboratory. The human MCF-7 breast cancer cell line is unusual in that it expresses negligible levels of GPX1 [16], allowing the modification of these cells by transfection of distinct *GPX1* expression constructs, resulting in the exclusive expression of specific *GPX1* alleles [17, 18]. GPX1 protein levels are particularly sensitive to the availability of selenium, and exposing the allele-specific transfectants to varying levels of selenium revealed a differential response to selenium supplementation. The increase in GPX1 levels in cells expressing the allele containing five alanine repeats and 198^{leu} was less than what was observed for cells expressing other combinations of these two genetic variations. One possibility raised by these data is that individuals whose genome contains one or two copies of the *GPX1 leu* allele may require a higher intake of selenium to maximize GPX1 levels and thus the benefits of its expression. Several studies have shown an association between lower GPX1 enzyme activity and the *leu* allele as a function of selenium status of people, consistent with the in vitro observations [19–28]. It is therefore noteworthy that a study investigating the relationships between selenium status, polymorphisms in the genes for selenoproteins, and prostate cancer revealed that a statistically significant interaction occurred between selenium serum concentration, the codon 198 polymorphism, for both total prostate cancer ($p = 0.03$), and much more importantly, high grade prostate cancer ($p < 0.001$) [22]. Other factors reported to interact with *GPX1* genotype to impact cancer risk include smoking, gender, age, alcohol intake and vitamin use [27, 29–31].

29.4 The 15 kDa Selenoprotein (SEP15)

SEP15 was originally identified as a human T cell 15 kDa protein that was labeled with Se^{75} and was expressed at high levels in the prostate [32, 33]. Like GPX1, SEP15 belongs to the family of selenium-containing proteins in which selenium is inserted co-translationally in response to a UGA codon in the corresponding mRNA [34]. As for other selenoproteins, the molecular decision to insert Sec is determined by sequences in the 3′-untranslated region of the mRNA referred to as a SECIS element [3, 35]. The *SEP15* gene is polymorphic in the SECIS sequence at positions 811 and 1125 [32]. These genetic variations are functional and contribute to determining the amount of SEP15 protein generated as a function of selenium availability [33, 36]. Using these polymorphisms to investigate genetic changes that occurred during tumor development, loss of heterozygosity (LOH) at the *SEP15* locus in breast cancers and cancers of the head and neck among African Americans, but not Caucasians, was demonstrated [36]. Several studies have indicated an association between genetic variants of *SEP15* and the risk of colorectal cancer [37–39] with the risk of colon cancer associated with a specific *SEP15* allele being modified by estrogen status [39] or gender [38]. The targeted downregulation of *SEP15* has been shown to inhibit the growth of human colon cancer cells in culture [40], to inhibit tumorigenicity and metastasis in mouse colon cancer cells [41, 42], and knockout of *Sep15* protected mice against the formation of chemically-induced aberrant crypt foci [43]. For lung cancers, the 811/1125 AA haplotype was associated with a higher risk of cancer in individuals with low selenium status [44]. In contrast, that same haplotype was not associated with the risk of breast cancer [45].

In 2010, Penney et al. reported that there was a statistically significant association between polymorphisms in *SEP15*, plasma selenium levels and importantly, prostate cancer mortality, but not risk [46]. An interaction was revealed between one of the polymorphisms and plasma selenium levels, which impacted the risk of prostate cancer survival. In this study, the polymorphisms were previously shown to be functional, and form a haplotype such that a C at residue 811 was always associated with a G at 1125 and a T at 811 was always associated with an A at 1125. This haplotype exhibited a trend towards association to prostate cancer-specific mortality with a $P = 0.10$. Not reaching statistical significance was perhaps due to the low prevalence of the AA genotype in the particular population examined in this study; it was conducted on self-reported Caucasians obtained from the Physicians Health Study. The allele frequency for the 811 AA genotype was 4.8 % (n = 1195) and 4.6 % (n = 1186) for controls, which is in good agreement with the low frequency we previously reported among Caucasians [36]. Consistent with these results, a more recent study indicated associations between *SEP15* polymorphisms and prostate cancer recurrence following prostatectomy [47].

To date, the best characterized biochemical function of SEP15 has been its involvement with correct protein folding in the endoplasmic reticulum (ER) and the unfolded protein response (UPR) [48, 49]. UPR occurs in response to an accumulation of unfolded proteins in the ER and involves the suppression of protein synthesis,

the increase in the levels of proteins involved with appropriate folding, and increased degradation of unfolded proteins, autophagy and apoptosis, and possibly in tumor growth [50]. Knocking out *SEP15* in Chang liver cells resulted in profound changes in the cytoskeleton and membrane as well as inducing ER stress [51, 52], but it remains undetermined whether these phenotypes are altered by any of the polymorphisms associated with cancer or contribute to cancer development or outcome.

29.5 Glutathione Peroxidase 4 (GPX4)

Another member of the glutathione peroxidase family of proteins, GPX4, is unique among this protein family in that it can reduce phospholipid hydroperoxides [53, 54] and can function in a variety of biological processes (reviewed in [55]). Polymorphisms in *GPX4* have been associated with the risk of cancers of the colon [37, 56] and prostate [57], as well as survival following diagnosis for cancers of the breast [58], prostate [59] and bladder [60].

Several of the studies revealing associations between polymorphisms in *GPX4* and cancer found a variation located in the 3′-untranslated region (UTR) (rs713041) corresponding to position 718 in the GPX4 protein. This polymorphism, which resides in the SECIS element, was shown to be functional, determining the efficiency of GPX4 protein translation for a given availability of selenium [56, 61]. The efficiency of *GPX4* translation is also affected by the gender of the individual who harbors the polymorphism [61]. Different polymorphisms in the *GPX4* gene were associated with bladder cancer recurrence (rs3746162) and prostate cancer survival (rs2074452) that were also located in the 3′-UTR, but functional studies for these variations have not yet been reported [57, 60]. Less clear is the significance of the polymorphism in *GPX4* (rs3746165) associated with prostate cancer survival as it is located 2 kb upstream of the *GPX4* gene [59].

29.6 Thioredoxin Reductases (TXNRD)

TXNRD are Sec-containing proteins that are involved in the reduction of thioredoxin and a wide variety of cellular functions including DNA synthesis, protection from oxidants and the regulation of critical signaling pathways (reviewed in [62, 63]). Two different thioredoxin reductase isoforms, TXNRD1 and TXNRD2, have each been implicated in cancer etiology based on associations between specific polymorphisms and cancer risk or outcome. These cancer types include those of the prostate, colon and rectum [39, 47, 64]. However, while some of the evaluated polymorphisms were in the coding sequence and affected the corresponding amino acid sequence, others were in regions outside of the gene or in sequences encoding introns. Such variations may still impact the regulation of thioredoxin reductase gene in ways that have yet to be determined.

The complexity of studying the impact of polymorphisms in the thioredoxin reductase genes on cancer risk and mortality is likely complicated by impact modifiers. Such interactions for both TXNRD1 and TXNRD2 were reported for aspirin use, estrogen levels and body mass index [39]. The association between polymorphisms in *TXNRD* genes and prostate cancer risk was only observed when patients were stratified by selenium status, and the role of selenium availability on the penetrance of these polymorphisms is discussed below.

29.7 Selenoprotein S (SELS)

SELS is a member of the SELS/SELK family of proteins that functions in ER-associated protein degradation and the UPR [65, 66]. A functional polymorphism in the *SELS* promoter has been identified (rs34713741), which can affect the steady state levels of the SELS protein, have an impact on inflammation [67], and has been associated with increased risk of several diseases linked to deregulated inflammatory responses (reviewed in [68]). Although a number of different polymorphisms in the *SELS* gene have been investigated with regard to their association to cancer, this variation in *SELS* resulting in an A rather than a G has been associated with gastric and colon cancers in Chinese, Japanese, Korean and Czech populations [37, 38, 69, 70].

29.8 Selenoprotein P (SEPP1) and Its Impact on Other Selenoproteins

As a protein family, selenoproteins have evolved intricate and multi-level means by which their activities and levels are regulated by the availability of selenium. These include controls at the levels of transcription, mRNA stability and translation [8, 71, 72] and collectively result in a hierarchy by which the levels of each of the different selenoproteins have distinct responses to the availability of selenium. In turn, selenium levels in tissues are largely controlled by SEPP1, a protein that accounts for approximately 50 % of the selenium in plasma and transports selenium to peripheral issues from the liver [73, 74]. Given the utility of SEPP1 as a biomarker of selenium status [75] and the literature indicating that selenium levels are inversely associated with cancer incidence [76], it is not surprising that lower levels of SEPP1 are also associated with increased cancer risk [77–81]. Polymorphisms in the gene encoding *SEPP1* have been investigated with regard to cancer risk and outcome, including rs3877899 resulting in an alanine/threonine variation at position 234 and rs7579 located in the 3′-UTR. Significant associations between *SEPP1* polymorphisms and cancer have been revealed for cancer of the breast, prostate colon, lung and esophagus [6, 80]. The impact of *SEPP1* variants on cancer could be modified by a host of factors, including selenium status, other selenoproteins and gender [37, 78, 79, 82].

Polymorphisms in *SEPP1* have been also shown to interact with a polymorphism in gene for manganese superoxide dismutase (*MNSOD*, *SOD2*) and together are linked to a significantly increased risk for aggressive prostate cancer [83].

The mechanism by which SEPP1 impacts cancer is not completely understood, but is likely to involve other processes in addition to its role in the delivery of selenium [74]. SEPP1 was shown to affect anti-oxidant defenses, the immune system function and stemness in model systems, all of which are among the possible cancer-modifying mechanisms [84–87].

29.9 Concluding Remarks

Human epidemiological studies have revealed associations for polymorphisms in the genes for several selenoproteins and either cancer risk or outcome. These data, summarized in Fig. 29.1, provide evidence for a contributing role of selenoproteins in these diseases and yield information on how these proteins impact cancer

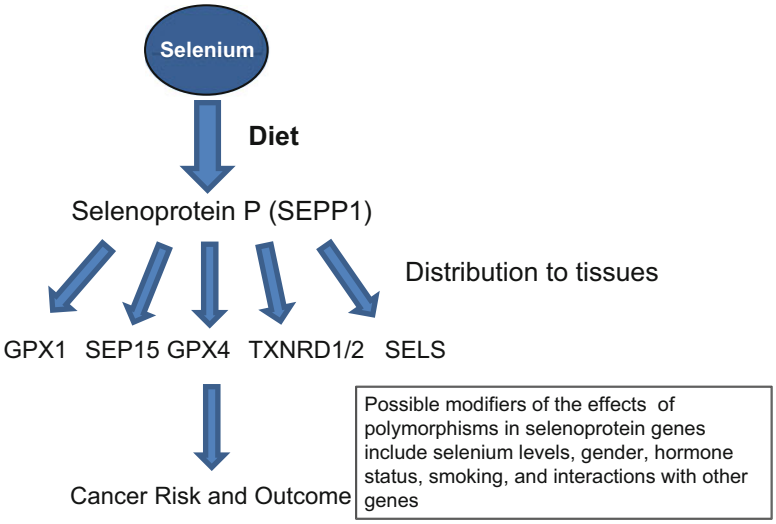

Fig. 29.1 Summary of the impact of polymorphisms in selenoprotein genes on cancer. Selenium consumed in the diet is transported to tissues by SEPP1 and the amount of selenium distributed to those tissues can be influenced by polymorphisms in *SEPP1*. SEP15 was originally identified as a human T cell 15 kDa protein that was labeled with Se^{75} and was expressed at high levels in the prostate. Polymorphisms in the genes of *GPX1*, *SEP15*, *GPX4*, *TXNRD1* and *2* and *SELS* can impact their functions to ultimately have an effect on cancer risk and/or outcome. The expression of the phenotypes associated with these genetic variations could be influenced by a number of variables, including selenium availability, behaviors, and interactions with polymorphisms in other genes

development and progression. This information can be used to identify people who are at risk for certain cancers due to their individual genetic identity, while revealing the possibility of developing novel approaches to reduce that risk.

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Chapter 30
Is Adequate Selenium Important for Healthy Human Pregnancy?

Margaret P. Rayman

Abstract Selenium appears to have a beneficial effect on number of adverse pregnancy health conditions. Higher selenium status has been associated with a lower risk of miscarriage and preterm birth, while there is evidence from randomized controlled trials that selenium supplementation may reduce the risk of pre-eclampsia and post-partum thyroid disease. The ability of selenoproteins to reduce oxidative stress, endoplasmic-reticulum stress and inflammation, and to protect the endothelium, control eicosanoid production, regulate vascular tone and reduce infection, is likely to be important in these apparently protective effects.

Keywords Autoimmune thyroid disease • Insulin resistance • Miscarriage • Pre-eclampsia • Pregnancy • Preterm birth • Selenium

30.1 Introduction

This chapter summarizes what is known about the role of selenium (Se) in adverse health conditions of human pregnancy. However, even in normal pregnancy, oxidative stress rises and there is a systemic inflammatory response, largely triggered by placental events [1, 2]. There is evidence from animal models that Se requirement increases during pregnancy, possibly because of these maternal factors, but also because of the needs of the growing fetus [3]. During normal pregnancy, whole-blood Se concentration falls substantially (e.g., by 12 %) with increasing gestational age [4–6]. Apart from plasma volume expansion, an additional cause of a fall in blood Se concentration throughout pregnancy is likely to be the transfer of Se to the fetus by selenoprotein P (SEPP1) which is expressed in the placenta [7]. In pregnant mice, placental transfer of maternal SEPP1 occurred through the apoE receptor 2 [8]. Even in mice with normal Se status, maternal plasma SEPP1 concentration falls rather dramatically in late pregnancy; hence, pregnancy may be

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putting similar pressure on the Se stores of pregnant women with marginal Se status. The pregnancy conditions for which most evidence for involvement of Se exists are addressed below.

30.2 Pre-eclampsia

Pre-eclampsia, a major complication in 2–8 % of pregnancies [9], is associated with high maternal and perinatal morbidity and mortality; there is currently no cure other than early delivery of the baby [10]. Surviving infants are likely to be small for gestational age and premature, factors that may jeopardize their development and health even into adulthood [9]. Furthermore, pre-eclampsia is linked to an increased risk of maternal coronary heart disease, hypertension and stroke in later life [11].

Deficient placentation in the first half of pregnancy frequently presages the development of pre-eclampsia [1]. Shallow invasion of the trophoblast (embryonic cells that develop into the placenta) and inadequate remodeling of the spiral arteries result in a placenta that is not adequately perfused [1, 12]. Thus, localized areas of ischemia and reperfusion associated with placental oxidative and endoplasmic-reticulum (ER) stress develop [12–15]. This results in increased apoptosis/necrosis of the syncytiotrophoblast layer (outer cells of the placental villi), accompanied by release of anti-angiogenic factors, including soluble vascular-endothelial-growth-factor-receptor-1 (referred to as sFlt-1), that cause endothelial dysfunction, hypertension and proteinuria [1, 16]. Placental microvesicles released into the maternal circulation stimulate a systemic inflammatory response and endothelial activation that characterize pre-eclampsia [1, 17, 18].

30.2.1 Observational Studies of Se and Pre-eclampsia

A positive correlation between Se status and the incidence of pre-eclampsia has been shown in an epidemiological study of 45 countries [19]. A recent meta-analysis of 13 observational studies found significantly lower blood/plasma/serum Se concentrations in women with pre-eclampsia than in control pregnant women [mean difference −8.25 μg/L, 95 % confidence interval (CI) −12.89, −3.61; P=0.0005] ([20] and personal communication, Min Xu, February 2016). A case-control study published since that meta-analysis similarly found that serum Se concentration was significantly lower in pre-eclampsia ($P<0.05$) with a further significant difference between mild and severe cases ($P<0.05$) [21]. Pre-eclampsia has also been shown to be associated with lower plasma glutathione peroxidase (GPx) activity than normal pregnancy [22, 23], while significantly lower levels of GPx and thioredoxin reductase (TrxR) have been found in placentae from pre-eclamptic women than from matched healthy controls [23, 24]. A case-control study found that the concentration of Se in toenails (laid down from 3 to 12 months previously) of women with

pre-eclampsia was significantly lower than that of controls matched for age, gestational age and parity (P=0.001) [25]. Women in the bottom tertile of toenail Se were 4.4-times [95 % CI 1.6, 14.9] more likely to have pre-eclampsia.

30.2.2 Se Supplementation in Pregnancy

Following the results of the case-control study discussed above, a randomized controlled trial (RCT) of Se supplementation in pregnancy was set up in the UK to assess whether a nutritional dose of Se could reduce the risk of pre-eclampsia, as measured by biomarkers of pre-eclamptic risk [26]. In a double-blind, placebo-controlled, pilot trial (SPRINT, Se in PRegnancy INTervention), 230 primiparous pregnant women were randomized to Se (60 μg/day, Se-yeast) or placebo-yeast from 12 to 14 weeks of gestation until delivery. Whole-blood Se was measured at baseline and 35 weeks, plasma SEPP1 at 35 weeks. The primary outcome measure was the anti-angiogenic factor, sFlt-1 (see above). Between 12 and 35 weeks, whole-blood Se concentration increased significantly in the Se-treatment group, but fell significantly in the placebo group. At 35 weeks, plasma SEPP1 concentration was significantly higher in the Se-treated group than in the placebo group. In line with the study hypothesis that Se supplementation would reduce sFlt-1 in women of low Se status, sFlt-1 was significantly lower at 35 weeks in the Se-treated group than in the placebo group in participants in the lowest quartile of Se status at baseline (P=0.039) [6].

Further analysis of data and samples from that trial showed that the risk of pre-eclampsia and/or the related condition, pregnancy-induced hypertension, was significantly reduced in women with higher toenail Se (Odds Ratio 0.38, 95 % CI 0.17, 0.87; P=0.021) [26]. As toenail Se is a likely biomarker of early pregnancy/pre-pregnancy Se status, this result suggests that having an adequate Se intake from very early pregnancy or even pre-pregnancy is important for the health of mother and baby.

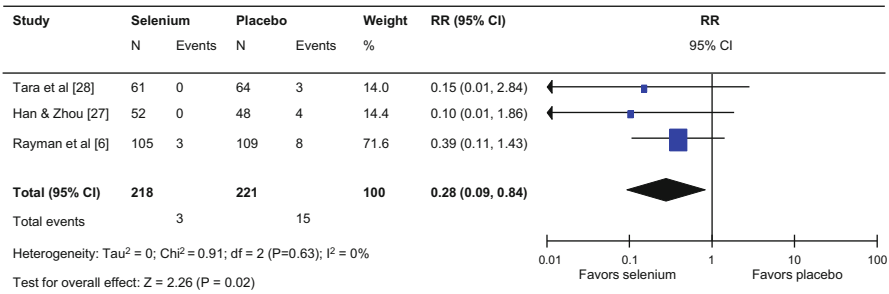

Fig. 30.1 Forest plot of meta-analysis of the effect of Se supplementation on incidence of pre-eclampsia in three RCTs (RR = relative risk) (modified with permission from Xu et al. 2015 [20])

There have been two other small trials of Se supplementation (100 μg/day) in the prevention of pre-eclampsia [27, 28]. The three trials have been entered into a meta-analysis [20]. Using a random-effects model, the relative risk for pre-eclampsia with Se supplementation was 0.28 (95 % CI 0.09, 0.84); P = 0.02 (see Fig. 30.1).

30.2.3 How Might Se Lower the Risk of Pre-eclampsia?

Se/selenoproteins can: i) protect endothelial cells, trophoblasts (and thereby the placenta) from oxidative stress [29–37]; ii) protect the endothelium and regulate vascular tone by controlling eicosanoid production [30, 38, 39]; and iii) help in the production of anti-inflammatory eicosanoid mediators [40] and repress inflammatory gene expression [40–42].

Se/selenoenzymes are also important to the heme-oxygenase (HO) system, which has been linked to antioxidant effects, successful placentation, inhibition of sFlt-1 release, uterine quiescence, placental hemodynamic control, and regulation of the apoptotic and inflammatory cascades in trophoblast cells [43–45]. Se has been shown to upregulate HO-1 by a number of pathways that involve Se or the thioredoxin/TrxR system either directly or indirectly, resulting in reduced expression of pro-inflammatory genes [46–49].

Perhaps the most important role of Se in affecting the risk of pre-eclampsia is its role in selenoprotein S1 (SEPS1), an ER membrane protein involved in the control of inflammation and ER stress [31, 50]. It helps remove stressor-induced misfolded proteins from the ER [31, 50], preventing their accumulation and the subsequent stress response that leads to activation of NF-kB, proinflammatory cytokine gene transcription and the inflammation cascade. Genetic variation in SEPS1 has been shown to influence the inflammatory response [51]. A retrospective study in a large Norwegian case-control cohort compared maternal genotype and allele frequencies of the *SEPS1* g.-105G>A polymorphism in pre-eclamptic and control pregnant women [52]. Women with pre-eclampsia were 1.34 times more likely to have the GA or AA genotype (P = 0.0039; 95 % CI, 1.09, 1.64) suggesting that SEPS1 has a role in protection from pre-eclampsia in that population.

30.3 Miscarriage

First-trimester pregnancy loss affects up to 15 % of clinically recognized pregnancies but 2–4 % of couples will suffer recurrent losses, often with no identifiable cause [53]. Idiopathic miscarriage has been shown to be associated with Se deficiency in a range of human studies. In interpreting the results of such studies, it is important to be aware that excessive inflammation is a probable part of the picture in miscarriage [54] and will lower circulating Se [55, 56].

Significantly lower serum Se was found in 40 UK women who miscarried in the first trimester than in 40 control pregnant women of similar gestational age [57]. In a later study, the same investigators found significantly lower serum Se in 25 women who miscarried in the first trimester (52.1 μg/L) than in 12 (non-pregnant) recurrent aborters (67.1 μg/L) and 25 non-pregnant controls (76.6 μg/L) [58]. An Indonesian study of 46 women with normal pregnancies and 25 women with spontaneous abortion found significantly higher mean serum Se in those with normal pregnancies than in those with spontaneous abortion (76.36 ± 18.22 μg/L, *vs.* 66.71 ± 13.55 μg/L; P = 0.023), though serum GPx activity did not differ between the groups [59]. An Indian study found significantly lower red-cell Se in 20 (non-pregnant) women with ≥ 3 recurrent pregnancy losses than in a similar number of non-pregnant controls with no history of miscarriage [mean (SD) 119.55 (32.94) μg/L *vs.* 150.85 (37.63) μg/L, respectively; P < 0.01] [60]. Although a further UK study found significantly lower mean hair Se in 26 women with a history of recurrent miscarriage than in a control group of 18 women with good reproductive performance (mean (SD) 0.14 (0.09) μg/g *vs.* 0.34 (0.25) μg/g, P < 0.001), there was no difference in serum Se between the groups [61]. However, the method used to prepare the serum samples was inadequate and no certified reference material was used to validate the results.

Not all studies have found associations: whole-blood and plasma concentrations of Se were no different in Polish women who had just miscarried than in women of the same gestational age with viable pregnancies, though their red-cell and plasma GPx activities were significantly lower [62]. A Scottish study found lower plasma Se in non-pregnant women having recurrent miscarriage than in controls, but the difference did not reach significance [63]. However, the control group in that study did not exclude women who had had a miscarriage. A South African study found no difference in hair Se concentration in women with recurrent pregnancy loss and controls (median (range) 0.80 (0.19–4.15) μg/g *vs.* 0.68 (0.43–3.76) μg/g, respectively; P = 0.74) [64]. Hair Se concentration in these South African women, however, was considerably higher than in the earlier UK study that found a difference between groups [61]. Though on balance, there appears to be an association between low Se status and miscarriage, surprisingly, population Se status does not seem to be the discriminator.

Miscarriage resembles pre-eclampsia to some extent, i.e., failure/partial failure of immunoregulatory mechanisms, defective placentation, impaired placental perfusion, excessive placental oxidative stress and inflammation [54, 65]. At the extreme, the outcome is pregnancy loss; if the pregnancy continues, the result may be pre-eclampsia [65]. Thus, many of the mechanisms discussed above in relation to the effect of Se on the risk of pre-eclampsia will also be relevant to its potential effect on miscarriage.

HO-1 is likely to be important in the context of miscarriage: upregulation of HO-1 expression diminished fetal rejection and abortion rates in a murine abortion model [66]. Furthermore, HO-1 upregulation may also augment the levels of regulatory T cells, improving immune suppression [66]. Se/TrxR may upregulate the expression of HO-1 by a number of pathways that would be protective against abortion [46–49].

30.4 Preterm Birth

Preterm birth, i.e., birth before 37 weeks of gestation, occurs in 5–13 % of pregnancies and is the most important cause of perinatal morbidity and mortality [67]. Short- and long-term outcomes include cerebral palsy, respiratory-distress syndrome, neurodevelopmental impairment, difficulties with schooling and behavioral problems [68].

Se concentrations in blood components have been compared between term and preterm mothers. Four studies found no difference between the term and preterm groups [69–72], while two others found significantly lower Se in the preterm group [73, 74]. The discordant results cannot be rationalized on the basis of the Se status of the countries in which the studies took place. Inadequate sample size may well be part of the explanation for the disparity in results.

By far, the largest study to date was carried out in the Netherlands: 1129 pregnant women were followed prospectively from 12-weeks of gestation, of whom 60 (5.3 %) gave birth preterm [75]. The commonest causes of preterm delivery were preterm premature rupture of membranes (PPROM, n = 21) and pre-eclampsia (n = 13), together accounting for 57 % of the preterm births. Those who delivered preterm had significantly lower serum Se at 12 weeks gestation than those who delivered at term [mean (SD): 75.8 (11.1) μg/L and 80.5 (10.3) μg/L, respectively, P = 0.001] [75]. The percentages of women with preterm birth by quartile of serum Se at 12 weeks were significantly different ($P < 0.05$). Even after adjusting for the occurrence of pre-eclampsia, which is associated both with Se status (see above [25, 26]) and with preterm birth, women in the lowest quartile of serum Se at 12-weeks of gestation had twice the risk of preterm birth as the rest (adjusted OR 2.18; 95 % CI 1.25–3.77). Results also suggest that low Se status in early gestation may increase the risk of PPROM, a major cause of preterm birth [69].

The above study does not show that low Se status *caused* preterm birth. Both preterm birth and low plasma Se may have been joint outcomes, for instance, of increased inflammation [77]. Plasma Se concentration decreases in proportion to the magnitude of the inflammatory response [57, 58]. However, the significant reduction also seen in the incidence of PPROM with Se supplementation in a small RCT in Iran suggests that Se status may indeed be relevant [76].

30.4.1 How Might Se Affect the Risk of Preterm Birth?

Se status in the Netherlands is relatively low [77]. Se (probably as selenoproteins) has a number of protective effects that are directly relevant to pathways implicated in preterm birth or its sub-categories, PPROM and pre-eclampsia. These pathways include infection, inflammation, defective placentation, placental ischemia-reperfusion, oxidative stress, the presence of anti-thyroid antibodies, and premature extracellular-matrix-degradation of fetal membranes [67–80].

Se is required for an adequate immune response [81], so low Se status in either the mother or the fetus is a risk factor for infection, a major cause of preterm birth [67]. Inflammation may be an underlying factor linking many of these pathways as suggested by the fact that polymorphisms that affect the magnitude or duration of the inflammatory response were associated with the risk of preterm birth [82]. Se is capable of attenuating the excessive inflammatory response associated with adverse pregnancy outcomes by a number of mechanisms that have already been discussed [30, 40–43, 46–49, 52, 83–85]. Defective placentation and placental ischemia-reperfusion are both counteracted by HO-1, which is upregulated by a number of pathways that involve Se or the thioredoxin/TrxR system, either directly or indirectly [46–49]. Oxidative stress is counteracted by the GPxs [30], by SEPP1 (which scavenges peroxynitrite) [30, 36] and by the antioxidant effects of the products of HO-1 (biliverdin and/or bilirubin) [43, 44, 84]. Higher Se status or supplementation with Se appears to be able to reduce the titer of antithyroid antibodies, in particular, thyroid peroxidase autoantibodies (TPO-Ab) [86] (see next section).

30.5 Autoimmune Thyroid Disease

Autoimmune thyroid disease has a prevalence ranging between 5 and 20 % and is the main cause of hypothyroidism in pregnant women [87]. While the incidence of gestational hypothyroidism is 2.4 %, thyroid autoantibodies are present in 55–80 % of these women [87]. Some 6 % of pregnant women have TPO-Ab [88] though the titer tends to decrease towards term, reflecting the down-regulation of the immune system during gestation [89]. An elevated TPO-Ab titer is associated with poor obstetric outcome including an increased risk of miscarriage [90], perinatal mortality [91], placental abruption [88] and PPROM [92].

Se is important to the thyroid. Not only is it a component of the iodothyronine deiodinase selenoenzymes that convert thyroxine (T_4) to tri-iodothyronine (T_3) and reverse T_3, but it is also a component of GPx3 which protects thyroid cells from the hydrogen peroxide that is generated there [93]. This protective function may be the basis of the beneficial effect of Se supplementation found in patients with autoimmune thyroiditis in a recent meta-analysis of RCTs where a significant decrease in TPO-Ab titer was found at 6 and 12 months [86]. More important in the context of pregnancy is the reduction in thyroid inflammatory activity and the risk of postpartum thyroid disease found in an RCT of Se supplementation in TPO-Ab-positive women in Italy [94]. During pregnancy and the postpartum period, 151 TPO-Ab-positive women were randomized to Se (200 μg/day as selenomethionine) or placebo. TPO-Abs fell significantly during gestation but the reduction was significantly greater in the Se-supplemented group ($P=0.01$) and remained so in the post-partum period ($P=0.01$) (see Fig. 30.2). Importantly, there was a significant reduction in the incidence of post-partum thyroid disease and hypothyroidism in the Se-supplemented group (28.6 % *vs*. 48.6 %, $P<0.01$ and 11.7 % *vs*. 20.3 %, $P<0.01$, respectively) [94].

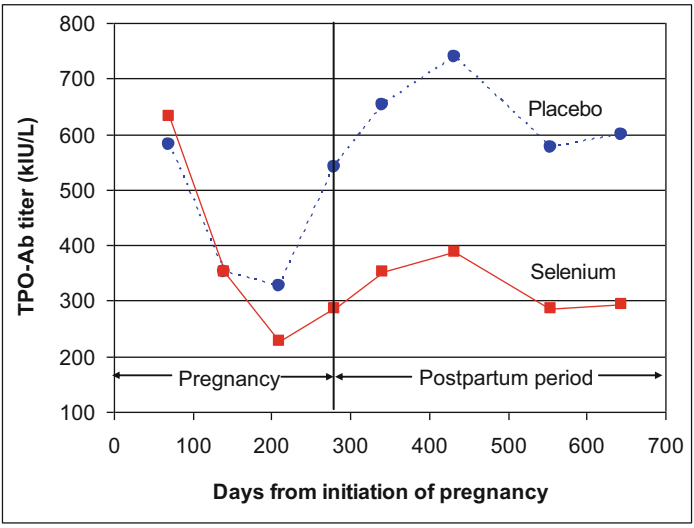

Fig. 30.2 Thyroid peroxidase antibody (TPO-Ab) titers in 151 TPO-Ab-positive Italian women randomized to Se (200 μg/day as selenomethionine) or placebo during pregnancy and the postpartum period. Values significantly different between groups at delivery (280 days) and in the postpartum period (modified from Negro et al. 2007 [99])

The only other RCT that investigated the effect of Se supplementation on autoimmune thyroid disease in pregnancy was a secondary analysis of samples and data from the SPRINT pilot RCT where 230 women at 12 weeks of gestation were randomized to receive 60 μg/day Se or placebo until delivery [95]. No difference was found in the magnitude of decrease between Se and placebo groups (54.2 % *vs.* 65.6 %, P = 0.785). The difference in results from those of the earlier study [94] can probably be explained by three factors: i) there were only 25, as opposed to 151, TPO-Ab-positive women, thus the trial lacked power; ii) the median baseline TPO-Ab concentration in the women was much lower: 110 kIU/L *vs.* 600 kIU/L (higher concentration appears to respond better to treatment [93]); and iii) the Se dose given was much lower, i.e., 60 μg/day *vs.* 200 μg/day. There is clearly a need for another adequately powered RCT in TPO-Ab-positive pregnant women to see if the results of Negro et al. [94] can be replicated.

30.5.1 How May Se Affect the Risk of Autoimmune Thyroid Disease in Pregnancy?

In pregnancy, a woman has to increase her production of T_4 by 50 % to maintain maternal euthyroidism and to transfer thyroid hormone to the fetus in the first trimester before its own thyroid starts to function [96]. The increased synthesis of thyroid hormones triggers a rise in the production of hydrogen peroxide that is used

by TPO in the synthesis of T_4 from iodide and thyroglobulin [93]. As hydrogen peroxide is damaging, any excess must be removed for the protection of the thyroid, largely by GPx3, which is highly expressed in thyrocytes [93]. Thus, the requirement of the thyroid for Se in pregnancy probably increases above the non-pregnant level.

TPO-Abs tend to correlate with lymphocytic inflammation of the thyroid. Hence, apart from the specific role of Se in GPx3, the anti-inflammatory effects of the selenoenzymes might play a role in down-regulating TPO-Abs during gestation as previously outlined [30, 40–43, 46–49, 83–85].

30.6 Insulin Resistance

Concern has been expressed in recent years that Se may increase the risk of type-2 diabetes though there are few studies investigating this potential effect in pregnancy. An adverse effect of Se, or at least of a selenoprotein was suggested by a study in pregnant women without gestational diabetes (GDM), where the activity of GPx1, in erythrocytes increased during gestation and was positively associated with fasting plasma glucose, plasma insulin, C-peptide and the homoeostasis model of assessment for insulin resistance (HOMA-IR) index [97]. By contrast, the literature also shows that pregnant women with impaired glucose tolerance or GDM have much lower serum Se concentrations than women with normal pregnancies [98–101] and that there is an inverse relationship between serum Se and blood-glucose concentration [100, 101]. Furthermore, in a small group of pregnant women, the increase in plasma glucose following an oral glucose tolerance test administered at 12 weeks of gestation was inversely correlated with plasma Se concentration [102]. Recently, an RCT of Se (200 μg/d) in 70 women who had developed GDM found a significant reduction in fasting plasma glucose, serum insulin and HOMA-IR in those on Se [103].

There has been only one RCT of the effect of Se supplementation on the risk of GDM or insulin resistance in normal pregnant women. Stored plasma samples from the SPRINT RCT of Se supplementation in pregnancy [6] were used to test the effect of Se supplementation on a marker of insulin resistance pregnant women in the UK [104]. Plasma adiponectin concentration, a recognized marker of insulin resistance [105] and a strong independent predictor of the risk of GDM and type-2 diabetes [106, 107] that can be used in non-fasted samples was measured at 12 and 35 weeks [104]. There was no significant difference between the two treatment groups in the change in adiponectin from 12 to 35 weeks ($P=0.938$), nor when the analysis was restricted to the bottom or top quartiles of baseline whole-blood Se ($P=0.515$ and 0.858, respectively) [104]. The results of this RCT, though probably underpowered, help to allay fears that a small dose of Se will increase insulin resistance in pregnant women of modest Se status.

30.7 Concluding Remarks

Despite the positive evidence cited above, the role of Se/selenoproteins in the etiology of adverse conditions of pregnancy has still not been clearly established. There are few RCTs, which alone are capable of establishing causation, and those that there are, are largely underpowered, though we do have an excellent study in autoimmune thyroid disease and a meta-analysis with a significant result in the case of pre-eclampsia. In case-control studies of Se in pregnancy, which constitute the majority of the evidence, it is vital to match controls to cases on age, gestational age and parity, all of which can affect Se status. Only one of the cited studies has been matched on those three parameters [25]. Many also lack important information such as the Se status of the groups, and adequate detail of analytical methodology. There are few large prospective studies [75] though in any case they cannot establish causality.

We need more RCTs, properly powered, and these should be run in populations with relatively low Se status, e.g., plasma Se ≤90 μg/L. We already have an excellent RCT in autoimmune thyroid disease that requires replication and a strong rationale for a very early intervention with Se to reduce the risk of pre-eclampsia.

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Chapter 31
The Epidemiology of Selenium and Human Health

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Abstract Few issues involving nutritional and environmental epidemiology have received as much interest as the relation between selenium (Se) intake and human health, as reflected by the large body of evidence from observational and experimental studies with reference to cancer and other clinical endpoints. Se deficiency may play a major role in favoring the onset of a human cardiomyopathy, Keshan disease. Se overexposure has been linked to skin and advanced prostate cancers in recent randomized controlled trials, in contrast with earlier hypotheses of protective effects of Se intake against cancer generally, and prostate cancer in particular. Overexposure has also been linked to higher risk for diabetes and amyotrophic lateral sclerosis. For cardiovascular disease risk, such as ischemic heart disease and stroke, little evidence of any modifying effect of Se exposure has been provided by epidemiologic studies. The results of these studies should be used in public health to set better standards for intake of organic and inorganic Se species, focusing on experimental studies with individual Se compounds more than overall exposure to the element, in order to improve reliability and reduce bias in the studies.

Keywords Cardiovascular disease • Diabetes • Epidemiology • Neoplasms • Neurological disease • Randomized controlled trials • Selenium • Thyroid disease

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31.1 Introduction

There is vast epidemiologic evidence relating selenium (Se) to major human health outcomes (Fig. 31.1). In this chapter we give emphasis to experimental studies, particularly randomized controlled trials (RCTs), as those have greater validity in human biomedical research. For more comprehensive analyses, we refer to some recent reviews on these topics [1–3] and for the outcomes not covered here, such as the relation between Se and infectious disease and AIDS (see [4] and Chap. 28), pre-eclampsia (see [5] and Chap. 30), Kashin-Beck disease osteoarthropathy [6], and critical illness [7, 8]. Also not covered are issues such as the genetic susceptibilities and mutations possibly underlying the relation between Se and human health ([9–11] and see other Chapters in Part III).

31.2 Cancer

Cancer has received more research interest in connection with Se intake than any other human disease [3, 12] with results from laboratory studies showing both carcinogenic and anticarcinogenic effects [13–15]; and more recently, the potential for Se use in cancer therapy, a fascinating issue not further taken into consideration herein [16]. Early epidemiologic research involved ecologic investigations and case-control studies carried out in the 1960s [17] and 1970s [18], and observational prospective studies in the 1980s and 1990s [19]. Most evidence supported an inverse relation between Se exposure and cancer risk, although not all studies were consistent, and in some cases, a positive association was suggested [3]. To resolve the confusing pattern of evidence, RCTs were undertaken, representing a turning point in establishing the role of Se in human cancer (and more generally in human health).

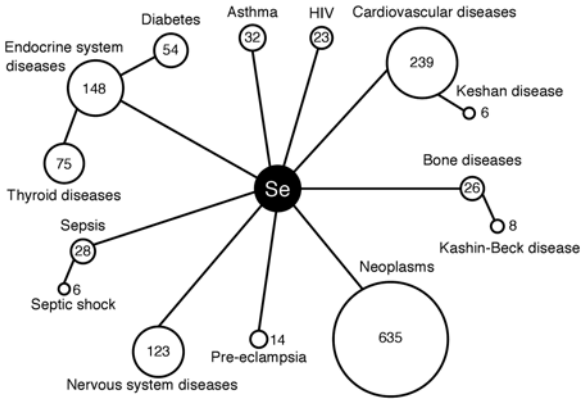

Fig. 31.1 Results of a PubMed search on the epidemiology of Se and human health. Figure shows the number of hits by January 2, 2016 based on a search strategy using the MeSH terms 'selenium' AND 'humans' AND ('epidemiology' OR 'epidemiologic methods'), plus entry terms for the various diseases

If we exclude early Chinese trials due to potential risk of bias [3], the first RCT was the 'Nutritional Prevention of Cancer' (NPC) double-blind trial coordinated by the University of Arizona [20–23]. In this study, 200 μg Se via selenized yeast tablets, or a placebo, were randomly allocated to 1,312 subjects with a previous history of non-melanoma skin cancer (NMSC). Though the trial failed to demonstrate that Se supplementation prevented NMSC incidence (the primary endpoint), an unexpected sharp decrease of secondary endpoints such as other cancer incidence and mortality were found [20]. This led to a premature unblinding of the study, and publications of the blind phase based on 6.4 and 7.4 average years of follow-up [20–23]. The first preliminary report showed a sharp decrease in incidence of lung cancer (hazard ratio (HR) 0.56, 95 % confidence interval (CI) 0.31–1.01), prostate cancer (HR 0.35, 95 % CI 0.18–0.65), colorectal cancer (HR 0.39, 95 % CI 0.17–0.90), and total cancer (HR 0.61, 95 % CI 0.46–0.82) [20]. In the final reports based on the entire period of follow-up, beneficial but weaker effects of the intervention emerged for incidence of all cancers (HR 0.75, 95 % CI 0.58–0.97), prostate cancer (HR 0.48, 95 % CI 0.28–0.80), lung cancer (HR 0.74, 95 % CI 0.44–1.24), and colorectal cancer (HR 0.46, 95 % CI 0.21–1.02) [21]. The protective effect of Se was confined entirely to males (HR 0.67, 95 % CI 0.50–0.89) and was most evident in former smokers [21–23]. However, the authors noted an increased incidence of melanoma, bladder, breast, and head and neck cancer, as well as lymphoma and leukemia, results which 'although non-significant and based on small case numbers, may indicate potential increased risk with Se supplementation' [21]. A strong positive association between baseline plasma Se and the incidence of total cancer in the Se-supplemented subjects also emerged [21], as previously reported for prostate cancer alone [24]. Finally, increased risk for NMSC emerged with a HR 1.17 (95 % CI 1.02–1.34) [23], a finding which was replicated subsequently in a small clinical trial [25].

However, in their 2003 report, the NPC trial investigators acknowledged a serious methodological pitfall in the trial, i.e., that 35 % of men with an abnormal prostate-specific antigen in the placebo group underwent biopsies at some point during the trial, compared with only 14 % in the Se group. This detection bias may have jeopardized the trial's validity [3]. Nevertheless, this bias has been ignored subsequently by many reviewers, and the final 2002–2003 NPC trial reports themselves have received limited attention. Instead, attention has been given to the 1996 report emphasizing beneficial effects of the Se intervention [20], which generated large expectations about a cancer-preventive Se effect and contributed to the implementation of more RCTs to further elucidate the role of Se.

The most famous and widely cited among these is SELECT (Se and Vitamin E Cancer Prevention Trial), a randomized, double-blind, placebo-controlled, 2 × 2 factorial design. More than 35,000 healthy men who received daily supplemental Se as selenomethionine (200 μg/day), vitamin E, Se plus vitamin E or placebo [3, 13, 26–28] were enrolled in this trial. However, SELECT was discontinued before its planned end for three main reasons: i) its inefficacy in risk reduction, ii) concern about increased risk of prostate cancer in vitamin E-treated participants, and iii) concern about increased incidence of type 2 diabetes in the Se-treated participants [13], also taking into consideration the increased diabetes risk in the Se arm reported

in 2007 in a *post hoc* analysis of the NPC trial [29]. Overall, results of SELECT were that Se supplementation did not decrease risk of all cancers (HR 1.01, 99 % CI 0.89–1.15), prostate cancer (HR 1.04, 99 % CI 0.87–1.24), or colorectal cancer (HR 1.05, 99 % CI 0.66–1.67), while lung and bladder cancer were characterized by a HR of 1.12 (99 % CI 0.73–1.72) and 1.13 (99 % CI 0.78–1.63), respectively [26, 30]. More recently, studies on the over 1,700 prostate cancer cases diagnosed in SELECT yielded concerning results. Among Se-supplemented subjects, those with the highest baseline toenail Se showed an increased prevalence of high-grade prostate cancer [31]. A positive association between prostate cancer risk and baseline α-tocopherol plasma levels also emerged in Se-supplemented subjects, as well as an increased risk of prostate cancer (overall and low-grade) among carriers of the NKX3.1 androgen-regulated prostate tumor suppressor protein (the CC genotype at rs11781886) [32, 33]. These results are of interest given the results of a recent cohort study on 4,459 men diagnosed with non-metastatic prostate cancer, which found an increased risk of prostate cancer mortality following self-supplementation of ≥140 μg/day Se [34].

Three other carefully-conducted and low-bias trials were carried out on the role of cancer prevention by Se compounds, mainly focusing on prostate cancer [35–37]. The results show no beneficial effects of Se on primary or secondary cancer endpoints, confirming the results of SELECT, although their smaller size and shorter follow-up provide reduced precision and inability to account for long-term effects. Overall, meta-analyses of all RCTs as well as of low-bias RCTs only clearly show the lack of any beneficial effect on prostate cancer risk (Fig. 31.2) and other cancers.

Therefore, the most recent RCTs, all with a low risk of bias, have demonstrated that Se supplementation in populations with no evidence of deficiency, has no beneficial effect on cancer risk, while supplementation might increase risk of some

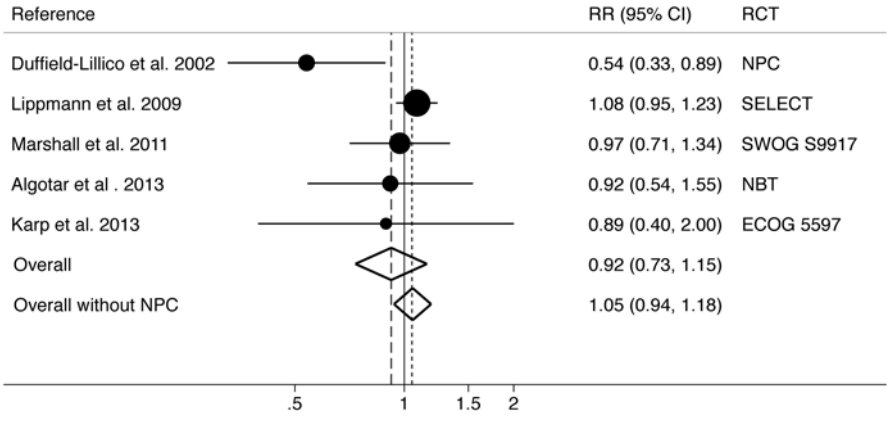

Fig. 31.2 Summary relative risk of prostate cancer following selenium supplementation in RCTs. Figure shows the forest plot of random-effect meta-analysis summary RRs of prostate cancer (95 % CI) from RCTs [21, 26, 35–37]. The vertical dashed lines show the effect of Se on prostate cancer risk for all studies (Overall) and for those with low risk of bias only (Overall without NPC trial)

site-specific cancers. Thus, a question arises about some results of the NPC trial. While detection bias may at least partially explain the results at least for prostate cancer, it has been suggested that two other trial characteristics, i.e., the specific Se species used for supplementation (200 μg/day Se as selenized yeast in NPC trial and selenomethionine in SELECT), and the different baseline Se levels, may explain these discrepancies. However, both hypotheses are unlikely. First, Se is mainly present in selenized yeast as organic Se forms, the major part of which is selenomethionine [12, 38], though we cannot entirely rule out an effect of the other Se forms or metabolites present in yeast despite their smaller amounts. Second, different baseline Se exposure is also unlikely to explain the results [3, 12], as the difference in intakes represented only about 15 μg/day Se [39] with a large overlap between the two populations (Fig. 31.3). In addition, the recent dose-response analyses in SELECT ruled out a beneficial effect of Se even at the lowest levels of exposure [31]. Finally, the specific study population in the NPC trial (subjects with NMSC history) may at least partially explain the different results of that trial [22].

Of interest may also be the results of a 'natural experiment' concerning an Italian municipality, wherein a cohort of 2,065 residents accidentally consumed drinking water with unusually high Se content (~8 μg/L) in its inorganic hexavalent form for several years. Notwithstanding the potential effects of unmeasured lifestyle confounders, such exposure was associated with increased cancer mortality, mainly due to melanoma and colorectal cancer, kidney cancer in males, and lymphoid

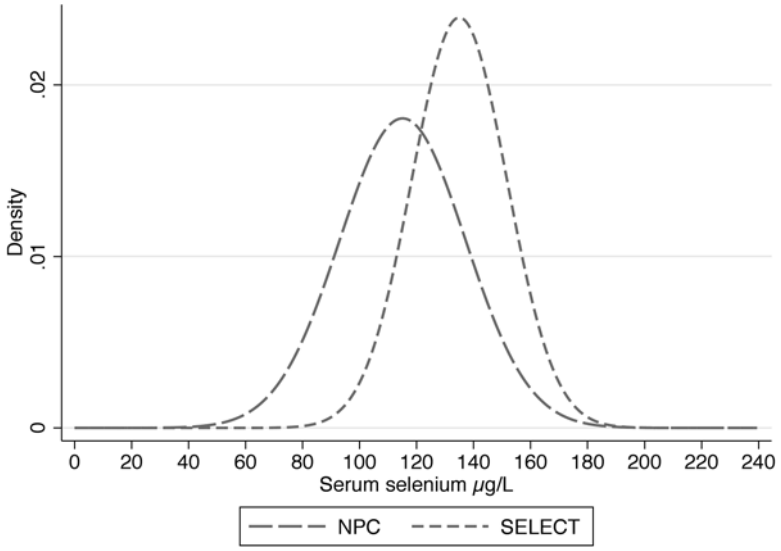

Fig. 31.3 Estimated distribution of plasma/serum selenium in the NPC trial and SELECT. Data extracted for the NPC trial from Duffield-Lillico et al. 2003 [22] (mean (SD) plasma Se 115.1 μg/L (22.05)), and for SELECT from Lippmann et al. 2009 [26] (Table 2, mean (SD) serum Se computed as 136.3 (18.66) μg/L from median and interquartile values using the equation found in http://handbook.cochrane.org/chapter_7/7_7_3_5_mediansand_interquartile_ranges.htm [101])

malignancies in females [40]. These results are of interest being the only data so far available for inorganic hexavalent Se exposure, and also for some similarities with increased risks noted in the NPC trial [21]. The excess melanoma risk, validated by an incidence study [41], finds support from an observational cohort study [42], a case-control study [43], and from time trends of the excess melanoma incidence detected in a French trial after administration of Se and other substances [44].

31.3 Cardiovascular Disease

While some support for a beneficial effect on cardiovascular health from a higher Se status originally came from observational cohort studies, the RCTs have demonstrated that no effect is induced by Se supplementation independently of baseline Se status [1, 45]. In SELECT, the relative risk (RR) for any cardiovascular events (including death) was 1.02 (99% CI 0.92–1.13) based on 1,080 cases in the Se group and 1050 in the placebo group [26], while corresponding estimates in the intermediate NPC trial report were 0.96 (95% CI 0.64–1.44) on the basis of 47 and 46 events in the Se and placebo groups, respectively [20]. Conversely, no other trial, nor the final NPC report itself, has investigated this issue. The above mentioned natural experiment reported in an Italian community was also unable to show any beneficial effect on cardiovascular mortality since standardized mortality ratio among residents exposed versus unexposed was 1.06 (95% CI 0.81–1.38) in males and 1.04 (95% CI 0.80–1.34) in females [40].

Furthermore, two recent RCTs have investigated the possible effect of Se administration as selenized yeast on a putative cardiovascular risk factor, i.e., serum cholesterol levels. In the first of these trials, administration of 100 μg/day Se for 6 months in 501 elderly UK subjects decreased total cholesterol levels compared with placebo. However, no dose-response relationship was found with higher doses, leading to the authors' statement that 'Se supplementation seemed to have modestly beneficial effects on plasma lipid levels in this sample of persons with relatively low Se status' [46]. More recently, a RCT carried out in 491 Danish elderly individuals with a considerably longer period of follow-up, also using Se doses of 100, 200 and 300 μg/day, was unable to find relevant and dose-response related changes in total cholesterol in the Se-supplemented group compared with placebo [47].

In addition to overall cardiovascular disease, Keshan disease, an endemic and severe childhood cardiomyopathy occurring in some Chinese areas, has been frequently related to Se status. Two main lines of epidemiologic evidence suggest that low Se intake may represent a risk factor for Keshan Disease: its increased prevalence in low-Se areas of China, and the ability of Se administration as sodium selenite to lower its incidence [48–51]. However, Chinese investigators noted some peculiar characteristics of this disease, such as its seasonal trend in incidence, which cannot be explained by nutritional deficiency [48, 49], suggesting an infectious etiology like a Coxsackie virus [52]. Furthermore, background levels of intake as low as 16 μg/day appear to be enough to prevent this disease [52]. In addition, the effectiveness of Se in reducing disease incidence does not *per se* imply that Se deficiency was a cause, due to the absence of reports of Keshan disease in other areas of the world with very low Se status. The possibility

that Se had a beneficial effect in the Chinese trial due to its pharmacological effects, considering its high doses (500–1000 μg/week sodium selenite), should also be considered. Selenite has the ability to inhibit Coxsackie virus replication even at low concentrations [53, 54] as well as other microorganisms [12]. Thus, the etiology of Keshan disease, including the possible role of Se, is still a matter of debate [51, 55].

31.4 Diabetes

A relationship between Se (specifically its overexposure) and risk of type 2 diabetes represents a new issue in Se research. The link was established unexpectedly following a secondary analysis of the NPC trial results [29], and then adding this outcome to the safety endpoints of the subsequent RCTs [56]. The possibility that Se may modify diabetes risk had long been suspected [57], but it was the NPC trial report which raised the possibility of a strong diabetogenic effect of Se. After SELECT, the RCTs have systematically confirmed an increased diabetes risk in Se-supplemented subjects [58], although mostly statistically imprecise due to the low number of observed cases in these smaller intervention studies [26, 27, 36, 37]. A diabetogenic effects of Se finds support from most, but not all, observational studies and from several laboratory studies [57, 59]. Currently, an excess risk of diabetes represents one of the most concerning adverse effects which might be attributed to Se overexposure [12], calling for further research to carefully assess the association (see also Chap. 49).

31.5 Thyroid Disease

Se is an essential component of the iodothyronine deiodinases and therefore is required for normal thyroid function [60, 61]. However, even low dose administration has been unexpectedly suggested or demonstrated to alter thyroid function in case reports, ecological and clinical intervention studies [62], including RCTs [63–65]. On the other hand, recent RCTs have shown a potential efficacy of Se compounds in the treatment of Hashimoto thyroiditis and other autoimmune thyroid diseases, and Graves' disease, which is clearly worth further investigation, though the exact pharmacological activity and safety of Se in these diseases still need to be defined [2, 66, 67].

31.6 Neurological Disease

Several recent studies suggest that Se and selenoproteins have key roles in brain and, more generally, in nervous system functions [68–70]. This is generating speculation for a beneficial role of this element in both prevention and treatment of central nervous system (CNS) diseases [69, 71]. Conversely, epidemiologic studies have suggested a broad spectrum of neurotoxic effects of environmental Se [72].

The original epidemiologic evidence for Se neurotoxicity came from studies among occupationally-exposed subjects, subjects consuming misformulated Se-supplements and populations from seleniferous areas of China [72]. However, most of these studies cannot be properly considered as epidemiologic studies, since they were frequently case reports of acute Se intoxication with neurological symptoms in workers exposed to high levels of Se compounds or attempting to use Se for suicide [72]. In addition, most investigations were flawed methodologically, e.g., inadequate Se exposure assessment, or lack of adequate control groups. Nevertheless, these studies provided evidence linking Se overexposure with neurological endpoints such as confusion, memory loss, depression, tremors and ataxia, lethargy, dizziness, sleep disturbances and paresthesias, weakness and fatigue [72]. In the seleniferous areas of China, selenium exposure has been associated with acroparesthesia and dysesthesia, hyperreflexia, convulsions, motor weakness, paralysis and even hemiplegia [72–74]. However, the exact clinical nature of this broad spectrum of neurological alterations, the specific signs and symptoms due to Se overexposure, the exposure levels, and the possible role of modifying factors, would require a comprehensive, in-depth epidemiologic investigation [72].

The most specific CNS disease associated with Se exposure is amyotrophic lateral sclerosis (ALS), first associated with Se overexposure among farmers in a seleniferous South Dakota area in 1977 [75]. More recently, investigation of the small Italian cohort exposed to hexavalent inorganic Se showed an excess ALS incidence [76, 77]. Case-control studies have also supported this association, despite their inherent methodological limitations, including a recent study analyzing Se species in a key CNS biomarker, cerebrospinal fluid [78]. In that study, the various Se species showed profound and inconsistent differences between cases and controls: higher levels of selenite and lower levels of organic Se forms in ALS patients [78]. This suggests an involvement of the most neurotoxic Se compounds in disease etiology, and also the potential for exposure misclassification when Se speciation is not used [79]. An association of Se overexposure with ALS risk is also strongly supported by animal [80] and in vitro studies [81, 82], showing a selective toxicity of Se species on neural cells, particularly motor neurons.

The Italian cohort study of subjects overexposed to inorganic Se through drinking water has also shown an excess mortality from Parkinson's disease, but epidemiologic studies on this issue are lacking, with the exception of three case-control studies, all of which found higher blood Se levels in patients compared to controls [72]. Few studies exploring potential etiologic or therapeutic roles of Se in Alzheimer's disease show little evidence of association [72]. The influence of Se on cognitive performance has also been investigated in a cross-sectional and two prospective studies, finding in two cases a positive association [83, 84] and no relation in the remaining study [85]. However, well-known limitations in nutritional epidemiology, mainly inadequate long-term exposure assessments and potential for unmeasured confounding variables, limits the reliability of these findings. Depression has been investigated in relation to Se status in six prospective studies with observational [86, 87] and experimental design [88–91]. A small Australian cohort-nested case-control study based on 18 major depression events showed a

triplicated risk in the lowest Se intake category at baseline (Odds Ratio (OR) 2.95, 95 % CI 1.00–8.72) compared with the highest category [86]. Conversely, a large prospective study, based on 25 years of follow-up and a total of 407 depression cases, found a positive dose-response association between baseline toenail Se content and disease risk [87]. Multivariate OR of depression was 4.25 (95 % CI 1.79–10.14) in the highest category of toenail Se (1.61–1.98 μg/g) versus the lowest one (0.51–0.88 μg/g), and a doubling of Se levels was associated with 56 % higher odds of having depressive symptoms at an exam. Concerning RCTs, their findings have been inconsistent in that the two small case-crossover studies yielded conflicting results and only weak evidence supporting a beneficial Se effect on mood [88, 89]. Furthermore, Se administration had no effect on mood and quality of life in a trial with 501 UK participants aged 60–74 randomly allocated to 100, 200 or 300 μg/day Se [90]. A slightly lower score of a postpartum depression was associated with 100 μg/day Se administration in a small Iranian trial on 85 women [91], though the study suffered from a high risk of bias for several reasons, including high attrition rate [92]. Finally, no beneficial effect on depression and anxiety scores was induced by 200 μg/day Se administration in patients with initially untreated thyrotoxicosis [67]. Overall, a link between Se and depression risk remains thus far unclear, though it warrants further investigation.

Finally, three recent studies raised the possibility that Se may affect neurological functions in children. A case-control investigation in Inuit children found a positive association between blood Se and longer visually evoked potentials latencies, and thus with possible optic nerve demyelination, even after adjusting for methylmercury toxicity in multivariate analysis [93]. A cohort study in Chinese neonates showed a U-shaped relation between Se umbilical cord and the Neonatal Behavioral Neurological Assessment score [94], that apparently supported a very narrow safe range of Se intake in neonatal life. A second cohort study in Sweden showed little evidence of any association between Se (and manganese) levels in umbilical cord serum and diagnosis of Attention-Deficit/Hyperactivity Disorder (ADHD) in childhood, with the exception of a high OR (2.6, 95 % CI 1.2–5.5) for Se levels above the 90th percentile compared with the 10th–90th category, suggesting the need to further assess a possible association between Se and ADHD [95].

31.7 Concluding Remarks

Why have results of observational studies been so inconsistent with each other, and with those of RCTs? Indicators commonly used in observational studies to assess Se exposure include biomarkers such as blood (serum, plasma and erythrocyte), toenail, hair and urine content, as well as dietary intake assessments (24 h recalls, food frequency questionnaires). Each method has its own characteristics, strengths and limitations for epidemiologic research, as reviewed elsewhere [13, 96]. Correlation between dietary Se intake and Se biomarkers has been found to be weak in some studies [13], due for instance to a high variability in actual food Se content

compared to average values reported in food composition databases. However, caution should be used when favoring biomarkers of Se intake over dietary assessment methods. The various Se species are distributed and excreted to different extents [79], and organic species have higher retentions as well as lower toxicological activity, compared with inorganic forms [79]. Moreover, the various Se species (both organic and inorganic) have markedly different and sometimes even opposite biological effects [79, 97], thus emphasizing the need to consider individual Se species levels when assessing Se exposure. Unfortunately, food composition data on Se species, and elemental speciation generally, are rarely available [98]. Finally, a major limitation of epidemiologic studies is the use of circulating biomarkers of Se exposure and not Se levels found in target tissues, an important issue when assessing health outcomes such as neurological disease [78].

Discrepancies between observational and experimental studies, and among observational studies themselves, may also be due to unmeasured confounding variables, thus confirming the key role of RCTs to understand Se health effects. However, due to the null results or unforeseen risks arising in some RCTs, replication of Se trials appears unfeasible for ethical reasons. Thus, we must rely on secondary analyses for additional outcomes from the most recent and powerful low-bias trials, which have greatly clarified the major controversial relationships between Se and cancer. In addition, the exact nature of the relation between Se and Keshan disease needs to be further investigated through well-conducted epidemiologic studies. These studies, along with a newly emerging body of research, will better define the still-debated safe range of intake of this metalloid [12, 99, 100].

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Chapter 32
Sex-Specific Differences in Biological Effects and Metabolism of Selenium

Lutz Schomburg

Abstract Women and men differ in several aspects of development, metabolism and endocrine regulations, among many other issues. In most clinical studies women are underrepresented, have a higher risk for certain diseases, e.g., autoimmune or allergic diseases, have a different perception of pain, and suffer from a higher frequency of adverse drug reactions. Moreover, pharmacodynamics and pharmacokinetics differ considerably between the sexes. This notion is increasingly recognized in basic and clinical science. Selenium (Se) is a very instructive example for a micronutrient that displays some sexual dimorphic aspects in relation to its metabolism, effects and roles in health and disease. The available experimental and clinical data indicate that in general, males are more responsive to acute changes in the Se supply, their Se status responds with faster kinetics and stronger amplitude to inflammatory stimuli, and likewise they seem more sensitive to adverse health effects upon surplus Se intake. These differences complicate Se research and interpretation of clinical studies on the importance of Se for maintaining health or reducing disease risk in men and women, and especially for the (adjuvant) treatment of certain diseases by Se-containing supplements. It is therefore inappropriate to extrapolate Se-related findings from studies conducted with one sex to the other sex, or to design, conduct or interpret future clinical studies without considering the sex of the participants.

Keywords Cancer • Gender • Infectious diseases • Selenium metabolism • Sexual dimorphism

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32.1 Selenium Metabolism in Female and Male Animals

During the initial phases of Se research, Schwarz and Foltz studied a rat model of vitamin E deficiency-induced necrotic liver degeneration, and the emphasis was placed on the effects of selenocompounds and their relative characteristics, bioavailability and protective role [1]. In these pioneering studies, female and male rats were used without discrimination, and the results were generalized since the selenocompounds afforded a comparable degree of protection in both sexes. But soon thereafter, males and females were analyzed separately and sex-specific differences were observed, e.g., when growth of second generation Se-deficient animals was analyzed [2], when plasma and cell concentrations of Se were compared [3], or when the retention of isotopes was determined in tissues of male and female rats (Table 32.1, data from [4]).

Of all the 25 human genes encoding selenoproteins [5], none is located on the Y- or X-chromosome. This notion excludes that the copy number of selenoprotein genes is a major reason for sexual dimorphic Se metabolism. Selenium is essential for reproduction, especially in males by affecting testes development and spermiogenesis [6]. Testes are among the preferentially supplied organs residing high in the hierarchy of Se supply among mammalian tissues [7, 8]. The kinetic profile of injected ^{75}Se in the form of SeO_3^{2-} highlights that Se is taken up by the testes with a certain delay and then is transferred to the epididymis while the ovaries show a fast accumulation and an almost linear loss of the tracer [4]. The molecular mechanisms behind this male-specific metabolism of Se in the reproductive tract have been intensively studied [9]. The selenoproteins, glutathione peroxidase 4 (GPx4) and selenoprotein P (SePP), take center stage in Se metabolism in the testes; GPx4 contributes to the sperm architecture [10], while SePP transports the essential trace element to testes, and becomes secreted during ejaculation likely in order to protect the spermatozoa during their dangerous journey [11]. Accordingly, under limiting Se supply, a direct competition between brain and testes can be demonstrated in transgenic mouse models [12].

Circulating SePP mainly derives from liver, where dietary Se is taken up, used for selenoprotein biosynthesis, and channeled back into the circulation in form of

Table 32.1 Time course of 75-Se in reproductive organs[a]

Time	Ovaries	Testes	Epididymis
3 h	0.3	1.9	0.8
1 day	0.3	9.1	0.3
1 week	0.3	24.5	0.6
2 weeks	0.3	29.5	3.3
3 weeks	0.3	31.5	10.3
4 weeks	0.2	25.4	14.1
6 weeks	0.2	23.9	8.0

[a]Data were taken from [4]

SePP for supplying other organs. Uptake by target cells is mediated by receptors of the lipoprotein receptor-related protein (Lrp) family, especially Lrp2 (megalin) in kidney [13, 14], and Lrp8 (ApoER2) in brain [15], bone [16] and testes [17]. SePP supply to testes is essentially needed for supporting the generation of vital and motile sperm [18]. Sertoli cells bind and internalize SePP [17], causing a strong Se enrichment specifically in late spermatids which apparently use SePP as Se source for GPx4 biosynthesis [19]. During spermiogenesis, GPx4 undergoes a functional metamorphosis from an active enzyme into a structural component needed for stability and motility of spermatids [20].

Accordingly, SePP and ApoER2 are abundantly expressed in male testes, but their expression is marginal or absent in female ovary or uterus. This pronounced sex-specific difference may contribute to the differential Se retention in males and females. But it is unlikely that sperm and seminal fluid are major factors controlling male-specific Se metabolism and Se flux. In humans, the total amount of this element secreted with one ejaculation averages at 100–250 ng Se (mean volume: 2–5 ml, mean Se concentration in seminal plasma: 50 μg/l) [21]. In comparison, blood loss during menstruation is around 35 ml corresponding to an average loss of 1750–3500 ng Se per month, i.e., in a similar range as the Se loss via sperm in males. In vivo, there must thus be other and more important pathways causing the sexual dimorphic kinetics of Se uptake, retention and selenoprotein expression patterns, which are not yet fully understood. But the growing number of sexual dimorphic effects observed in clinical studies argue that these differences are important for human health.

32.2 Sex-Specific Regulation of Selenoprotein Expression

Protein biosynthesis is regulated at multiple levels. The dependence on the limiting trace element Se increases the relative importance of translation for controlling selenoprotein expression [22]. The role of gender-specific circuits and sex steroid hormones affecting transcription of selenoproteins in different tissues is a complex and dynamic issue. In experimental animals, castration alters the expression of a number of selenoproteins in a sex-specific manner [23]. The effects are not only exerted at the transcription level but involve posttranscriptional mechanisms giving rise to tissue-specific expression in males and females which vary with age [24], and Se status (Fig. 32.1, data taken from [25]).

Interestingly, it is not only selenoproteins, but also Se-binding proteins, that are controlled in a Se- and sex-specific manner. The ubiquitously expressed Se-binding protein 2 (SP56) is among the most differentially expressed proteins in livers of male and female mice [26]. Recent research has highlighted its potential relevance for tumorigenesis [27]. Its sex-specific expression opens another pathway potentially contributing to the observed sex-specific differences in hepatic Se metabolism and chemopreventive effects of supplementation.

This chapter illustrates the underlying complexity of trying to elucidate the mechanisms controlling selenoprotein expression in males and females and high-

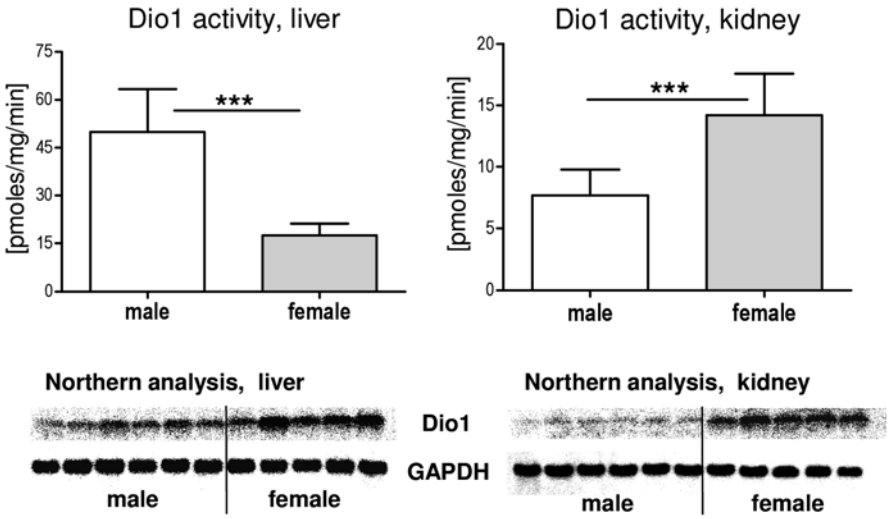

Fig. 32.1 Sexual dimorphic selenoprotein expression (*top*, activity; *bottom*, mRNA) in liver and kidney of adult mice (taken from C. Riese et al. [25]). Enzymatic activity of type 1 iodothyronine deiodinase (Dio1) is higher in male compared to female liver, whereas the relation is opposite in kidneys. This sexually dimorphic expression pattern is paralleled by respective differences in Dio1 mRNA in kidney, but not in liver where translational efficiency is obviously higher in male compared to female hepatocytes

lights that different levels of regulation are involved which converge under physiological conditions ensuring a time-, tissue- and cell-specific expression pattern of a given selenoprotein. The aforementioned multifunctional selenoenzyme, GPx4, can serve as a very instructive example, since it is ubiquitously expressed and of functional importance for diverse processes, including brain development, arachidonic acid metabolism and fertility.

According to its sexual dimorphic importance in reproduction, GPx4 expression in testes depends on gonadotropin stimulation and increases after puberty in rat testes [28]. Surprisingly, testosterone or gonadotropins do not directly affect *GPx4* transcription. The increased biosynthesis of GPx4 rather correlates to the maturation stage of spermatids, i.e., to a differentiation process that in turn is controlled by local testosterone from Leydig cells [29]. In addition, the primary transcript is subject to dynamic alternative splicing, and depending on environmental parameters, the cells synthesize different patterns of cytosolic, mitochondrial or nuclear GPx4 isozymes [30, 31]. Besides this inherent complexity, posttranscriptional mechanisms involving sequence-specific RNA-binding proteins recognizing the 5′-untranslated region of *GPx4* mRNA control translation efficiency, e.g., during brain development [32]. Finally, single nucleotide polymorphisms (SNPs) have been identified in human *GPX4*, affecting Se-dependent expression and turnover in a sex-specific way [33]. This additional gender difference may be of importance for sex-specific effects of Se in clinical trials [8, 34].

Collectively, there are several sex-specific mechanisms affecting transcription, alternative splicing, translation and posttranslational activity of GPx4 in vivo, ensuring the appropriate cell-, age- and Se-status-dependent expression pattern in the different tissues.

32.3 Sexual Dimorphic Findings in Clinical Studies

32.3.1 Cancer

The trace element Se belongs to the nutritional supplements that are used in clinical studies and are regularly taken as over-the-counter drugs. The initial enthusiasm for supplemental Se intake was based on its alleged function as an antioxidative compound potentially slowing down degenerative processes and protecting genome integrity. Early analyses indicated an inverse association of Se intake and cancer prevalence [35], with some sex-specific differences (Table 32.2, data taken from [36]).

A large intervention study, the Nutritional Prevention of Cancer (NPC) trial, supported Se's potential role in cancer prevention [37]. The NPC data indicated that a daily supplementation with 200 μg selenized yeast reduces the incidence of lung, colorectal, and prostate cancers, especially in those participants with a relatively low baseline Se status [38]. However, this conclusion is derived from the analysis of a men-centric clinical trial. Females were underrepresented in this important prospective cancer prevention trial and constituted only 25 % of the enrolled participants. Even worse, the follow-up trial was designed as the largest-ever chemoprevention trial testing Se supplementation specifically for preventing prostate cancer [39].

Table 32.2 Age-specific deaths by sex per 100,000 population[a]

	Females			Males		
Age group	Low	Medium	High	Low	Medium	High
All cancer						
45–54	196	253	221	177	166	152
55–64	354	301	312	530	478	442
65–74	588	513	481	1077	910	892
Digestive organs						
45–54	36	27	28	47	36	35
55–64	89	72	74	144	117	112
65–74	253	156	150	320	238	238
Breast						
45–54	60	48	43	0.5	0.3	0.2
55–64	82	70	72	0.4	1.1	0.4
65–74	101	87	79	1.8	1.2	2.0

[a]Data are subdivided into geographically different Se regions, (low vs. medium vs. high) and are taken from [36]

This was a mistake and a major injustice, as the costs for initiating the Selenium and Vitamin E Cancer Prevention Trial (SELECT), and for the continuous observational analyses of the participants and in the future are covered by the taxpayers' money, i.e., from males and females alike.

That a generalization across both sexes is not justified is indicated by several clinical studies, e.g., the data from the European SU.VI.MAX trial (SUpplementation en VItamines et Mineraux AntioXydants). This randomized double-blind intervention trial indicated a significantly reduced total cancer incidence in men, but not in women 7.5 years after initiating low-dose antioxidant supplementations, including 100 μg Se/day [40]. A more detailed analysis of this surprising finding indicated that the baseline antioxidant status was already sexually dimorphic. However, this finding was insufficient to explain the differences observed in the supplementation effect between the sexes.

The trend of a male-specific antioxidant effect is in agreement with two earlier reports from European epidemiological studies; increased cancer risk was associated with lower serum Se levels in men but not in women in a Dutch [41], and independently, in a Finish case-control study, in which the strongest associations were observed for stomach and lung cancer [42]. Conversely, a recent meta-analysis indicated that bladder cancer risk is inversely associated with Se in women, but not in men [43]. The sex-specific trend is in agreement with a case-control study in the USA associating low toenail Se with higher bladder cancer risk in women, but not in men [44].

Similarly, a recent nested case-control analysis in Europe indicated an inverse association between colorectal cancer risk and higher Se status, especially in women [45], whereas a previous study in the USA indicated an inverse association, especially in men [46]. These data indicate that there are sex-specific differences in Se supplementation effects on cancer risk in both observational and intervention studies, and that extrapolations from one sex to the other for a particular cancer risk and across different populations of varying Se status are not appropriate.

32.3.2 Infectious Diseases and Sepsis

Serum Se and SePP are negative acute phase reactants and decline in response to inflammatory signals [47, 48]. Plasma Se concentrations are significantly lower in patients on the intensive care units than in controls [49]. More importantly, low plasma Se is associated with reduced survival odds of intensive care patients [50]. This association is valid for both females and males, and mortality risk can be predicted from the minimal Se levels observed in plasma [51]. Accordingly, clinical trials have been conducted trying to correct this trace element deficiency by supplementation. The results are inconsistent at present, and different selenocompounds, chosen dosages and application regimen have been discussed as potential reasons underlying this heterogeneity [52, 53]. A large placebo-controlled multicenter study, SIC (Selenium in Intensive Care), has yielded positive supplementation effects reducing the 28-day

mortality rate in patients with severe sepsis [54]. Unfortunately, female participants were underrepresented, and the positive effect may be confined to males [55]. This hypothesis was supported by an experimental study where an LPS-induced immune response was reduced by selenite supplementation in male, but not in female mice [56]. A large retrospective analysis of Se supplementation in critically ill patients observed no positive benefit, neither in males or females [57]. It remains to be determined what underlying factors in sepsis are modulating the Se effects, and whether indeed supplementation is more successful in males than females.

HIV infection is another inflammatory burden causing weight loss and certain mineral and vitamin deficiencies. Serum Se concentrations decline during HIV disease, and low Se also correlates with poor survival [58]. A sex-specific difference was noted before and after introduction of a highly-active antiretroviral therapy (HAART), and males displayed particularly low Se concentrations before HAART [59]. Interestingly, the Se status normalized during HAART along with improved weight stabilization, and the sex-specific difference disappeared. These findings indicate that disease severity impaired Se metabolism more severely in male as compared to female HIV patients.

32.3.3 Autoimmune Thyroid Disease

Among the autoimmune diseases, Hashimoto's thyroiditis (HT) is a destructive disorder of the thyroid gland characterized by hypothyroidism, transient goiter and loss of active thyroid tissue. It is a highly prevalent disease with a skewed sex ratio being about 10-times more frequent in adult females than males [60]. There is no established curative therapy preventing the thyroid destruction process. The accompanying hypothyroidism is corrected by an adequate daily supplementation with L-thyroxine in order to establish euthyroidism and subjective well-being. As the destructive process involves activated lymphocytes and reactive oxygen species (ROS), a number of Se supplementation trials have been conducted in HT patients [61]. In line with the disease prevalence, the studies were conducted mostly with women, and there is only one particular trial reporting the enrollment of nine male patients [62]. A global statement on the effects of Se on HT disease can thus not be given for the full population, but for women only. It might well be that the Se effects will differ between male and female patients. This hypothesis is in line with a genetic analysis, demonstrating a strongly increased risk for HT in male carriers of a certain genotype in selenoprotein S [63].

The analysis of the baseline status in participants of the European SU.VI.MAX trial indicated that serum Se concentrations inversely correlate to thyroid volume, risk of goiter and hypoechogenicity in women [64]. None of these interactions were found in male participants. Similarly, the association of thyroid volume and nodule formation was determined in a cohort of Danish adults, and a significant association was found for females only [65]. A recent intervention trial has tested the effects of Se supplementation

on the other prevalent thyroid autoimmune disease, Morbus Basedow (Graves' disease), with patients suffering from mild Graves' opthalmopathy, i.e., protruding eyeballs. Se supplementation efficiently reduced signs of inflammation, proptosis and improved quality of life [66]. Unfortunately, the study was not sufficiently powered to elucidate sex-specific differences, and no larger follow-up study has been conducted for verifying these important treatment effects.

32.3.4 Cardiovascular System

The cardiovascular system is a prime target exposed to oxidative stress with the metabolically highly active myocardium and the widespread network of arteries, veins and capillaries transporting a colorful cocktail of partly reactive and potentially damaging molecules throughout the body. Key events for development of arthrosclerosis comprise blood lipids, and the activity of ROS in oxidation of LDL triggering the development of proatherogenic foam cells in the vasculature. Selenoproteins of the GPx family are catalyzing the degradation of peroxides thereby reducing ROS concentrations. A genetic analysis indicated a sex-specific association of a functional SNP in *GPx1* encoding P198L with blood pressure specifically in men [67]. GPx1 activity in red blood cells of patients with suspected coronary artery disease (CAD) turned out as a strong univariate predictor of risk for cardiovascular events with sex as an important modifier of the effects [68]. In a Belgian cross-sectional and longitudinal analysis, a significant inverse correlation between blood pressure and Se concentrations was found in men, but not in women [69, 70]. In extrapolating these data, it appears that increasing the average daily Se intake to improve blood Se concentrations by a margin of 20 μg Se/l might already suffice to lower CAD and myocardial infarction (MI) rates in European men by an impressive 7 % and 10 %, respectively. This male-specific trend was verified in a Finnish study, but was not replicated in a similar French analysis [71].

CAD is the end result of a degenerative process affecting the coronary arteries finally impairing oxygen and nutrient supply to the heart and eventually causing myocardial infarction. CAD is the leading cause of death in developed countries.

A large observational study analyzed cardiovascular patients of moderate Se status, and reported that survival rates in angina pectoris were unrelated, while survival of acute coronary syndrome patients was positively associated with Se status [72]. In this study, the influence of sex was of borderline significance. In a European cross-sectional study, metabolic syndrome as a risk constellation for cardiovascular disease was associated with higher Se concentrations in women, but not in men [73]. The data from a cross-sectional study in the USA indicate a positive association of serum Se with blood lipids, and again highlight that the interaction may not be linear but U-shaped [74]. This notion is supported by a Chinese study reporting on an inverse association of Se with blood lipids in subjects with relatively low Se status [75]. The importance of sex on the different associations in well- versus poorly Se supplied subjects is not yet fully elucidated.

32.4 Sexual Dimorphic Risk/Benefit Ratio of Se Supplementation

In general, a low Se status which is insufficient for full expression of selenoproteins seems to confer an increased risk for developing a number of diseases. It is thus widely accepted that correcting a Se deficit is a meaningful measure for reducing health risk, especially in poorly supplied individuals. This applies to both sexes. In comparison to correcting a Se deficit, the risk of over-supplementation is poorly defined and the data are inconclusive. Two independent reports from 2007 highlighted a potentially increased risk of developing type 2 diabetes mellitus (T2DM) upon high Se intake, i.e., the follow-up analysis of the NPC intervention trial, where the participants took 200 μg Se/day [76] and an epidemiological cross sectional analysis as part of the Third National Health and Nutrition Examination Survey (NHANES III) [77]. Notably, both studies were conducted mainly in the USA and analyzed a population of high baseline Se concentrations. But most importantly, the reported increase in T2DM risk was confined to males, both in the NPC trial, where n = 8 in the placebo and n = 9 in the Se arm developed T2DM [76], and in NHANES III, where there was no interaction in pre- and postmenopausal women [77]. Animal studies have clearly established that there are molecular pathways supporting the U-shaped interaction of Se and T2DM risk [78] with both too little and too high Se intake conferring an increased risk. However, the importance of age on the interaction, and whether the threshold is similar in males and females, remains to be determined in human subjects. At present, avoiding a Se deficit is recommended for both men and women, and an intake of 1–2 μg Se/day/kg body weight appears adequate for this purpose, as it indirectly takes sex into consideration in a personalized manner. While Se intake differs between men and women, the intake per kg body weight levels out [79]. An insufficient intake is associated with an increased risk of obesity in both sexes (Table 32.3, data taken from [79]). Linear regression analyses indicated that Se intake [μg/day] is inversely associated with total and body fat in men but not in women, and all indices of obesity become significantly associated with Se intake in both sexes when corrected for body weight.

32.5 Concluding Remarks

Even though there is a clear lack of mechanistic insights into the underlying molecular pathways, both animal experiments and clinical data highlight that the health effects of Se, the associations of Se intake and Se status with certain disease risks and the potential side-effects from too high a daily Se supply differ between the sexes. In general, males seem to be more dependent and more responsive to acute changes in the Se status, their Se status responds with faster kinetics and stronger amplitude to inflammatory stimuli, and likewise they seem more likely to develop adverse health effects upon surplus Se intake. The current sexual dimorphic data

Table 32.3 Inverse association of Se intake with obesity in males and females[a]

	Women (n = 2232)			Men (n = 822)		
Indices of obesity	R^2	β	p-Value	R^2	β	p-Value
Dietary Se intake [μg/day]						
Weight	0.051	0.032	0.348	0.044	0.095	0.139
BMI	0.085	0.026	0.447	0.090	0.049	0.430
WC	0.039	–0.052	0.117	0.199	0.010	0.862
WHR	0.039	–0.049	0.157	0.100	0.063	0.310
TF [%]	0.176	–0.013	0.692	0.260	–0.128	0.023
BF [%]	0.175	0.001	0.985	0.221	–0.121	0.036
Dietary Se intake [μg/kg(bw)/day]						
Weight	0.315	–0.781	<0.001	0.266	–0.821	<0.001
BMI	0.301	–0.708	<0.001	0.283	–0.761	<0.001
WC	0.341	–0.692	<0.001	0.374	–0.734	<0.001
WHR	0.074	–0.289	<0.001	0.144	–0.368	<0.001
TF [%]	0.315	–0.569	<0.001	0.405	–0.670	<0.001
BF [%]	0.315	–0.571	<0.001	0.373	–0.682	<0.001

[a]Data were taken from Y Wang et al. [79]
BF body fat, *BMI* body mass index, *TF* trunk fat, *WC* waist circumference, *WHR* waist-hip ratio

clearly advocate more balanced study designs in future trials. Wherever possible, we should not conduct studies with one sex only. But more importantly, we should refrain from generalizations of the findings when a single sex has only been analyzed. Males and females differ with respect to Se metabolism, selenoprotein expression and medical Se effects, and some sex-specific differences are conserved across the species and may thus be meaningful for health and disease.

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Chapter 33
Selenium and Endocrine Tissues

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Abstract Selenium (Se) status and individual selenoproteins are important regulators of hormonal homeostasis during development and adulthood. Because endocrine cells and organs are highly perfused and continuously involved in hormone secretion and feedback sensor function, adequate control of their redox state is required to sustain the hormone secretory machinery. Several selenoproteins contribute to this essential function, which might explain the high relative Se content in endocrine tissues. Specifically, hypothalamus-pituitary-feedback regulation is responsive to Se, and the thyroid, islets of Langerhans in the pancreas, adrenal cortex, and gonads are strongly affected by Se status. Deiodinase selenoproteins contribute to the hypothalamic control of satiety, food intake, and energy expenditure, as well as to the development and proper function of several endocrine tissues. Inadequate Se status is linked to autoimmune diseases of the thyroid, impaired insulin secretion and resistance, delayed chondrocyte differentiation, defective bone formation, and reduced gonadal function. Single-nucleotide polymorphisms of several selenoprotein genes affect nutritional Se status and several hormonal axes. An inactivating mutation of thioredoxin reductase 2 causes familial glucocorticoid deficiency. Thus, adequate Se supply is crucial for the appropriate function of the endocrine system and hormone action.

Keywords Adrenal • Antioxidant defense • Autoimmune disease • Bone • Cancer • Deiodinase • Diabetes • Gonad • Kashin-Beck disease • Pancreas • Redox regulation • ROS • Selenoprotein • Testes • Thyroid

33.1 Introduction

Endocrine tissues in rodents stand out by their high relative selenium (Se) tissue content per weight and thyroid, kidney, and pituitary show highest concentrations [1–3]. Experiments involving dietary Se depletion, ^{75}Se labeling, and Se repletion indicate a remarkable hierarchy of Se distribution, retention, and repletion for endocrine organs and the brain, compared to large parenchymal tissues and organs, such

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as the liver, muscle, and adipose tissue [1]. Analysis of the expression or function of various selenoproteins in endocrine tissues during these nutritive manipulations revealed a further superimposed 'hierarchy' among individual selenoproteins within these tissues. Endocrine tissues are highly vascularized and perfused to allow rapid delivery of their hormones via circulation to target tissues. Thus, they are also strongly exposed to heavy metals and other adverse agents that circulate in the blood and affect tissues. The accumulation and precipitation of insoluble heavy metal selenides in endocrine tissues and kidneys may account for a fraction of the observed high Se content. However, even a correction for Hg, Cd, and Pb tissue content [4–6] leaves endocrine tissues and kidneys among those that rank the highest with respect to their relative Se content and bioavailability.

In the meantime, several other factors contributing to the observations of high Se content in endocrine tissues have been identified, such as i) distinct selenocysteine insertion sequence structures of selenoprotein transcripts, ii) expression of apolipoprotein E receptor 2 (ApoER2) in the testes, kidney, and brain, and iii) the local expression of abundant selenoproteins, including glutathione peroxidase 1 (GPX1), that retain Se at their active sites [7]. ApoER2, which is the membrane transport protein that is involved in the cellular uptake of the Se transport and storage protein selenoprotein P (SEPP1) [8], is expressed at low levels in the thyroid. The thyroid has the highest Se content in mice and humans and expresses most known selenoproteins [9]. It may be speculated that the functional role of endocrine tissues to actively secrete hormones throughout life requires a highly efficient Se-related redox system that regulates both the regeneration of the cell machinery involved in hormone secretion and the function of the quality control system for secreted peptides, proteins, and glycoproteins [10] acting as hormones on target tissues and cells. Several selenoproteins reside in the endoplasmic reticulum (ER) and are involved in protein biosynthesis [10]. Many of these functions can be adequately covered by various selenoproteins in endocrine tissues (for review see [3]). In addition, thyroid as well as steroid hormone-producing glands, such as gonads and adrenals, experience high and life-long exposure to H_2O_2 and reactive oxygen species (ROS), which are required for hormone synthesis. Several selenoprotein families are perfectly suited to provide adequate antioxidative function, along with the cellular redox control that is required to quench any excessively produced H_2O_2-derived ROS and to protect endocrine cells for their integrative function in development, growth, and metabolism. At the same time, most endocrine tissues have very low proliferation rates and may thus accumulate and deposit insoluble heavy metal selenides.

33.2 Hypothalamus–Pituitary-Feedback Axis, Selenium, and the Hormonal Regulation of Energy Metabolism

Various groups of hypothalamic neurons, called nuclei, are involved in the central coordination of hormone secretion by integrating sensory input from the central nervous system and metabolic signals from peripheral tissues. These nuclei translate such

information into the production of various 'releasing hormones', which instruct anterior pituitary cells to secrete their glandotropic hormones. Various selenoproteins and high Se content have been found in both endocrine structures. Of special interest is the role of the thioredoxin (TXN)-like ER-resident, selenoprotein M, whose genetic inactivation in mice alters body composition and increases white adipose tissue mass [11]. This likely occurs by decreasing the leptin sensitivity of hypothalamic centers regulating satiety. Leptin, which is produced in white adipose tissue, is a main regulator of energy expenditure, satiety, and food intake. It is unclear whether this observation is related to the anorexic effect of diphenyl diselenide [$(PhSe)_2$] and p-chloro-diphenyl diselenide [$(p\text{-}ClPhSe)_2$]. These agents decrease satiety, food intake, fat mass, and body weight after pharmacological or dietary administration [12, 13].

The TXN-like selenoprotein T (SELT) is another selenoprotein that is expressed in the anterior pituitary, as well as in several other endocrine tissues (e.g., thyroid, pancreas, and testicular Leydig cells). SELT is also involved in the hormonal regulation of energy metabolism [14]. The pituitary adenylate cyclase-activating polypeptide (PACAP) represents a main activator of SELT expression, especially in the β and δ cells of the pancreatic islets of Langerhans. In these Langerhans islets, PACAP mobilizes intracellular Ca^{2+} secretion and significantly contributes to the enhanced secretion of insulin and somatostatin in β cells and δ cells, respectively [15, 16]. The genetic inactivation of *Selt* in β cells impairs glucose tolerance, and the number and size of these Langerhans islets are decreased compared to those in wild type mice [16].

The paraventricular nucleus of the hypothalamus is a key structure in the regulation of energy metabolism and contains thyrotropin-releasing hormone (TRH)-producing neurons. TRH stimulates the production of anterior pituitary thyrotropin (TSH), which is the main stimulator of thyroid hormone (TH) formation [17]. Both TRH and TSH feedback regulation are negatively controlled by the active TH 3,3′,5-triiodo-L-thyronine (T_3), which binds nuclear T_3 receptors. Local production of T_3 from the prohormone L-thyroxine (T_4) occurs by 5′-deiodinases (DIO1, DIO2), which are selenoproteins ([3] and Chap. 41). In the hypothalamus, T_4 and T_3 may also be inactivated to TH metabolites, which do not modulate the function of cellular T_3 receptors. This reaction is catalyzed by the selenoenzyme deiodinase 3 (DIO3). DIO2 is mainly expressed in tanycytes, which are specialized ependymal cells that are located in the third ventricle and use their extensions to bridge the distance between the ventricular floor and the mediobasal hypothalamus. These tanycytes convey signals, such as locally produced T_3, to hypothalamic TRH neurons [17]. Neurons exhibit low DIO2 activity but express T_3-inactivating DIO3. A complex pattern of TH transporters expressed in neurons, astrocytes, and other brain cells contributes to local TH bioavailability [18]. In contrast to other tissues, hypothalamic DIO2 is less sensitive to T_4-induced, ubiquitination-dependent inactivation, which may explain the high sensitivity of the hypothalamus to T_4 feedback inhibition [19]. Whether nutritional variations in Se status can significantly alter hypothalamic and pituitary DIO activity remains unclear. However, severe Se depletion has been shown to affect DIO-regulated TRH and TSH feedback [2, 20]. Interestingly, the strong inhibitory action of proinflammatory cytokines on both DIO2 and DIO3 function results in an inappropriate TH-dependent feedback regulation of the TH axis in severe non-thyroidal illness and starvation [21].

33.3 Selenium in the Thyroid Gland and Thyroid Hormone Synthesis, Metabolism, and Action

The effects of Se status on TH homeostasis have been demonstrated. The pathogenesis of cretinism is associated with Se and iodine deficiency [3, 22], and several investigator teams have concomitantly discovered DIO as the second family of selenoenzymes [23–25]. Adequate Se status is required for proper TH synthesis, which depends on the life-long production of H_2O_2 by dual oxidase inside the functional angiofollicular units of the thyroid gland [26]. The thyrocytes express several selenoproteins that contribute to continuous cellular redox control and antioxidative defense [9] to protect the ROS-exposed gland. In females, the thyroid gland is highly affected by the development of autoimmune thyroiditis [27–29] and thyroid cancer [30, 31], which is the most frequent endocrine tissue-related malignancy. Both diseases might be linked to the continuous exposure of the gland to H_2O_2 and ROS [32–34]. In terms of TH metabolism and action, it is important to know that all three DIOs are selenoproteins, and that severe nutritional Se deficiency in experimental animal models impairs their function, mainly resulting in the decreased enzymatic formation of the active TH T_3 [3, 29]. However, even under severe systemic Se deficiency induced by genetic inactivation of the *Sepp1* gene in mice, tissue DIO function is maintained close-to-normal levels [35]. Furthermore, serum TH concentrations, including TSH feedback regulation, remain at normal status. A remarkably mild phenotype with respect to the TH axis and metabolic function is observed in mice exhibiting liver-specific or complete knockout of *Dio1* or *Dio2* [36]. Similar phenotypes are observed in mice exhibiting knockdown of all three *Dio* isoenzymes. A major phenotype is only observed with the inactivation of *Dio3*, an imprinted gene. This inactivation alters the setpoint of the hypothalamus-pituitary-thyroid (HPT) axis, thus leading to impaired growth, development, metabolic function, and transient hypothyroidism [37].

Surprisingly, the thyroid-specific genetic inactivation of $tRNA^{[Ser]Sec}$, which depletes all functional selenoproteins in thyrocytes, had only minor effects on thyroid morphology and function in mice [38]. This was the case even after experimental challenge by iodine deficiency, which did not destroy this 'unprotected gland'. In fact, the gland continued to produce close-to-normal levels of serum TH, albeit being exposed to increased oxidative stress. These results suggest that the expression of selenoproteins is not essential for thyroid function and/or other components of cellular redox control. Antioxidative defense might adequately handle follicular exposure to H_2O_2 and ROS, even under stimulatory conditions of iodine deficiency. This finding in mice contrasts with current clinical observations showing that Se supplementation and adequate Se status, especially under conditions of combined iodine deficiency, might i) protect against benign and malignant thyroid disease, ii) improve thyroid function, iii) reduce thyroid volume and goiter size, and iv) decrease the titer of circulating anti-thyroid peroxidase (TPO) antibodies in autoimmune thyroiditis (see recent reviews [28, 29, 38, 39]). However, with few exceptions [27, 40–42], such intermediate conclusions were mainly drawn from small and controversial studies,

which provided only low-grade evidence for clinical guidelines and the code of daily practice [43–47]. Currently, several prospective and adequately powered Se interventional studies are being conducted to clarify these open questions [48–50].

Proposed links between low Se status and thyroid cancer incidence have not been confirmed [45, 46, 51, 52]. At this point, it is not clear whether low Se status, which is observed under various conditions of inadequate thyroid function and autoimmune disease, is a cause or effect of this condition. An altered thyroid status has been shown to affect the homeostasis of trace elements, such as Se, copper, zinc, and iron [46, 53–56]. Currently, there are divergent opinions concerning the chemical form, duration, and dose of Se to be administered in the prevention and treatment of thyroid-related (autoimmune) diseases. Effects were mainly observed if doses higher than 100 μg/day and longer than 3 months were used, whereas selenite, selenomethionine, and other organic forms appeared to be effective in patients with inadequate Se status. Some studies have suggested a gender-specific action of supplementation with Se compounds [28, 29, 43].

There is evidence that the rate-limiting step in iodide uptake by thyrocytes is sensitive to Se status, as Se increases the expression and function of the sodium-iodide symporter (NIS) that is localized in the basolateral membrane of angiofollicular units. Se-dependent TXN/thioredoxin reductase and an altered cellular redox state have also been shown to enhance the binding of the redox-sensitive transcription factor Pax8, which is essential for TSH-dependent NIS transcription [57].

Although Se compounds might enhance tissue-specific DIO activity [58], they may also interfere with or inhibit TH metabolism [59, 60]. DIO expression is affected by proinflammatory cytokines, both at the transcriptional and posttranscriptional levels. Typically, *Dio1* and *Dio2* are decreased, and *Dio3* may be increased under such circumstances in cell cultures in vitro or in appropriate animal models ex vivo [61–63]. However, Se supplementation does not fully rescue cytokine-impaired DIO activity in various cell lines in vitro [64].

Recently, a study in Portugal reported a twofold elevation in the risk for carriers of the 105 GA and AA SNPs in *SEPS1* and autoimmune thyroiditis [65]. Carriers of the GG phenotype might be more efficient in antioxidant selenoprotein synthesis, as indicated by their higher GPX activity [66], and their SEPS expression might be less impaired under the influence of proinflammatory cytokines [67]. The effects of Se on various components of the humoral and cellular immune system have been described [68]. Selenomethionine treatment (80 or 160 μg/day) for 6 or 12 months had no effect on TPO autoantibody titers and thyroid morphology in patients with autoimmune thyroiditis. However, decreases in serum interferon-γ-dependent chemokines were observed, which suggests that these chemokines might serve as Se-responsive biomarkers for autoimmune thyroiditis [69]. In an experimental autoimmune thyroiditis female rat model, high Se intake (2 μg Se/kg body weight) improved thyroid morphology, decreased circulating thyroid-related autoantibody titers, increased the TH_1/TH_2 cytokine ratio, and mitigated inflammatory response [70].

SNPs of selenoprotein genes and several components of the HPT axis have an impact on TH and Se status, as indicated for several SNPs of the *DIO* genes (see also Chap. 13). There is evidence that such SNPs alter Se bioavailability and incorporation

into selenoproteins, as indicated by Se biomarkers [71–73]. SNPs in the promotors and (non-) coding regions of *DIO* genes impair their expression, correct posttranslational handling in the ER and Golgi apparatus (e.g., Thr92Ala-DIO), and affect the efficiency of TH deiodination [74–76]. Such SNPs have been linked to a susceptibility to type 2 diabetes mellitus (T2DM), age-related disorders, neurocognitive function, and neurodegenerative diseases. Most of the currently available data are limited to animal experiments or underpowered human studies and thus require confirmation in replicate cohorts by independent groups to strengthen the claims of cause-effect relationship levels. None of the SNPs in the *DIO* genes directly affect the Se-dependent reaction mechanism, and no major gene defects in human *DIO* genes linked to severe disease have been reported [56, 74]. The Ala variant of Thr92Ala-DIO2 appears to have a longer half-life and might accumulate in the Golgi apparatus and disturb proper Golgi function, as observed in transfected human embryonic kidney cells [76].

Several factors are known to modify both TH status and Se-related parameters and biomarkers, but these might again reflect associations rather than cause-effect relationships. In particular, chronic kidney disease and hemodialysis interfere with Se homeostasis, and impaired renal function and advanced chronic kidney disease are associated with increases and decreases in serum SEPP1 and T_4 levels, respectively [77]. Seasonal variations in serum TH concentrations have also been reported to be related to alterations in nutritional Se intake under extreme climatic living conditions [78]. In a small observational study, nutritional Se intake was analyzed in relation to anthropomorphic parameters, and high serum Se levels were positively correlated with body mass index, waist circumference, and the T_3/T_4 ratio [79]. Several other studies, however, did not observe such direct 'simple' associations [3, 30, 73].

Se supplementation alone or in combination with other trace elements has been studied in euthyroid and (subclinically) hypo- or hyperthyroid patients at various doses and with different Se compounds resulting in rather variable outcomes on the serum parameters of thyroid function [39, 50, 80]. These studies have not provided any conclusions for interventions for the purpose of normalizing thyroid function. Recently, a systematic, dose-related (100–300 μg Se-enriched yeast), randomized, controlled, double-blinded trial that was designated the Danish PREvention of Cancer by Intervention with Selenium pilot study (DK-PRECISE) was performed [50]. Of 491 subjects aged 60–74 years old, 361 subjects completed the 5-year intervention period. No changes in free T_3 levels or T_3/T_4 ratios were found, but Se dose-relatedly reduced serum TSH and free T_4 concentrations. Until further studies can show a therapeutic benefit of Se compounds in thyroid disorders, iodide supplementation should be used for prevention of goiter and T_4 alone or in combination with iodide should continue to be prescribed to treat hypothyroidism. Administration of antithyroid drugs is the first choice in therapy of hyperthyroidism. Se compounds in supplementation doses (100–200 mg/day in adults) appear to have adjuvant benefit in these disorders without adverse effects.

Compared to healthy pregnant controls, lower serum TSH and Se concentrations and higher TH levels have been observed in pregnant women with hyperthyroidism [81, 82]. However, no cause-effect relationships between pregnancy and Se or TH are known [47]. Whether altered maternal Se status during pregnancy impacts on

fetal and postnatal child development (either directly or via Se-dependent TH status of the maternal-fetal unit) requires further long-term and follow-up studies. An alteration in Se status has been observed during pregnancy and might affect placental DIO activity and fetal supply with maternal TH [3]. The limited number of studies in experimental animals and human (term) placenta do not support a direct effect of nutritional Se status on altered placental DIO activity, especially DIO3, even under conditions of preeclampsia [83, 84].

Combined nutritional Se and iodine deficiency has been proposed as a mechanism underlying the development of cretinism caused by inadequate TH availability and action during fetal and postnatal (brain) development [22]. While "neurological cretinism" might rise from maternal and fetal Se and TH deficiency during pregnancy, myxedematous cretinism appears to develop postnatally by combined Se and iodine deficiency [3, 22, 29, 85]. Elevated TSH-stimulated H_2O_2 production under the latter condition leads to fibrotic changes irreversibly destroying functional thyroid tissue. Considering that more than 700 publications on human and animal experimental studies addressing the relationship between Se status and thyroid function in context of iodine intake have been published during the last three decades, it is surprising that the exact molecular mechanisms causing the devastating but preventable disease cretinism, which affects more than five million individuals and may be associated with Kashin-Beck disease [85], are still unknown.

33.4 Selenium, Bone, and Calcium-Regulating Hormones

Se is an organic component of bone but not a constituent of the mineral hydroxyapatite deposit. Severe Se deficiency strongly impairs chondrocyte differentiation, bone development, and calcification, as indicated by the classical Kashin-Beck disease phenotype [85]. However, no clear effects of Se status on hormones regulating Ca^{2+} and phosphate homeostasis have been reported, albeit several mouse selenoprotein knockout models exhibit marked bone phenotypes [86]. Bone is clearly a target for SEPP1-mediated Se delivery via ApoER2, as indicated by the decreased Se content in bones in *Sepp1* knockout mice and the restoration of normal Se status after transgenic expression of human *SEPP1* in this model [87]. Hormones regulate several selenoproteins in bone. For example, estradiol stimulates *Gpx1* expression in osteoclasts, and 1α,25-dihydroxyvitamin D_3 rapidly induces *Txnrd1* in osteoblasts along with their differentiation [88, 89]. Similarly, local expression of the selenoprotein Dio2 generates T_3 which binds to the T_3 receptor, thus initiating steps that are essential for proper bone development [90]. Adequate Se supply is also essential for the development and function of bone marrow stromal cells during their differentiation into bone cells [91].

Interestingly, supplementation of adult Saudi T2DM patients with vitamin D_3 tablets (2000 IU/day) for 6 months decreased serum parathyroid hormone concentrations but also increased serum Se and magnesium concentrations; this

intervention was accompanied by several gender-specific changes in metabolic serum parameters [92]. The mechanisms underlying these observations for positive links between vitamin D and Se status remain unclear. In a 6-year, prospective, European Osteoporosis and Ultrasound Study (OPUS) evaluating fracture-related factors in 2374 postmenopausal euthyroid women associations between parameters of Se status and bone were observed [93]. A positive association between SEPP1 concentration and hip and lumbar spine bone mineral density (BMD) was observed and accompanied by decreased serum markers of bone formation (osteocalcin) and bone resorption (C- and N-telopeptide). Investigators have concluded that adequate Se status positively and inversely correlates with parameters of BMD and bone turnover, respectively, in elderly, euthyroid women. However, hormonal links among Se, vitamin D, Ca^{2+}, and metabolic parameters need to be studied in more detail. Furthermore, it has not been elucidated whether Se status affects fibroblast growth factor 23 (FGF23). This is the main phosphatonine hormone secreted by osteocytes which regulates phosphate homeostasis and its renal excretion [94].

33.5 The Effect of Se on Renal and Adrenal Hormones, Hypertension, and Steroid Biosynthesis

Recently, a homozygous inactivating mutation of thioredoxin reductase 2 (TXNRD2) was shown to result in familial glucocorticoid deficiency and impaired adrenal cell redox-homeostasis, thus linking another selenoprotein gene defect to the endocrine system [95]. The authors proposed that an impaired mitochondrial antioxidant defense has a marked impact on adrenal steroid hormonal biosynthesis. Lack of functional TXNRD2 fails to reduce mitochondrial TXN2, which inactivates H_2O_2 together with glutaredoxin 2. The regeneration of glutaredoxin 2 by glutathione reductase and GSH may initiate a compensatory pathway involving TXN2. Furthermore, accumulation of H_2O_2 might harm adrenal function and cause impaired angiofollicular TH biosynthesis.

No convincing data have associated Se status with hormones that regulate blood pressure and salt homeostasis, such as renal (renin-angiotensin-aldosterone system), adrenal (catecholamines, mineralocorticoids), cardiac (natriuretic peptides), and pituitary (antidiuretic hormone/vasopressin) hormones. In addition, a close relationship between Se status and hypertension has not yet been demonstrated, even in epidemiological studies with sufficient sample sizes [96]. However, clinical conditions related to obesity, diabetes, metabolic syndrome, hyperlipidemia, PCOS, and other cardiovascular diseases might indirectly affect Se status in the context of their proinflammatory effects and thus also change hormonal parameters and actions [97]. In particular, low Se status during pregnancy might represent a risk factor with adverse impacts on hypertension, preeclampsia, and cardiovascular complications [98]. Whether Se status modifies endothelin production and endothelin receptor function is unclear, but Se might protect the endothelium against exposure to heavy metals.

33.6 Selenium, Glucoregulatory Hormones, and Diabetes

Several selenoproteins are expressed in the islets of Langerhans in the pancreas [99], and Se status has been linked to insulin secretion [3]. Controversially, high Se intake has also been suggested to increase the prevalence of T2DM [100–102]. H_2O_2 and ROS-related oxidative stress, as well as the hyperglycemia-induced damage to β cells, might contribute to β cell failure. These cells exhibit low expression of the antioxidative enzymes (catalase and GPX) but show high expression of SEPP1, which may protect β cells and α cells under exposure to high glucose [103]. Apart from GPX isoforms, TXNRD family members are expressed in pancreatic islets, and SEPW1 strongly responds to Se exposure in parallel to selenocysteyl-$tRNA^{[Ser]Sec}$ synthase (SEPSECS) but not selenophosphate synthetase 1 (SEPHS1) in the bird pancreas [104]. In another study investigating the response of the selenoprotein transcripts to Se status in the bird pancreas, one group of genes (*Txrnd1*, *SelS*, *SelU*, *SepX1*, and *Sps2*) was highly expressed under Se-deficient conditions. Another gene set was strongly decreased under these same conditions (*Txnrd2*, *Gpx1*, *Gpx3*, *SelI*, *Dio1*, *Sepp1*, *SepW1*, *SelO*, *SelT*, *SelM*, *SepX1*, *Sps2*); this was partly associated with nitric oxide (NO) synthase activity and NO content [99].

A major role in the regulation of insulin secretion has been attributed to the PACAP-regulated selenoprotein, SEPS1, which is induced by high glucose [16]. In mice, the conditional inactivation of SEPS1 in β cells impairs glucose tolerance and glucose-induced insulin secretion. These mice have smaller and fewer islets. The role of SEPS1 for PACAP-stimulated somatostatin secretion by δ cells has not yet been clarified.

Similar to other tissues, the development of the pancreas and β cells requires the timely expression of DIO3 to protect against inappropriate T_3 exposure. Knockout of *Dio3*, which is maternally inherited in mouse islets, results in smaller islets with fewer β cells, decreased glucose-induced insulin secretion, and glucose intolerance [105, 106].

The expression and secretion of the hepatokine Sepp1 is stimulated by high glucose and inhibits pancreatic insulin secretion [101]. This mutual dependency might represent part of an endocrine feedback loop, but this might have been overinterpreted that high serum Se would favor T2DM. In contrast, elevated Sepp1 appears to be the result and not the cause of high glucose concentrations [107]. Epidemiological studies have also shown associations between SEPP1 SNPs and insulin resistance in a Spanish cohort [108], as well as associations between Se status and (gestational) T2DM or other aspects of metabolic diseases, such as lipid status [109]. Several other studies suggest that adequate Se status improves glucose regulation and might be beneficial or without effect [110]. Observations on impaired glucose regulation in a Gpx1-overexpressing mouse model clearly indicate that the regulation of H_2O_2 and ROS signaling and redox control by selenoproteins in β cells is essential for normal glucose-regulated insulin secretion [100, 101, 111].

Various groups have investigated whether high Se supply might improve β-cell function while decreasing insulin sensitivity in target tissues, such as the liver, muscle, and adipose tissues. These studies used several animal models (e.g., mouse, rat, chicken) under conditions of compromised energy or lipid metabolism

and implemented different Se intervention protocols [101, 111]. Although high Se might improve β-cell function and insulin secretion [102, 112], it might also induce steatosis via the increased exposure of liver to fatty acids released by adipose tissue and higher hepatic oxidative stress resulting therefrom [102, 111, 113].

The first randomized controlled trial involving moderate doses of Se (60 μg/d) during pregnancy revealed no adverse effects on glucose metabolism or insulin resistance, as assessed by adiponectin [114]. Furthermore, a larger epidemiological study in the elderly population revealed no association between Se status and T2DM [110].

In patients with diabetes and hyperglycemia, a decreased activity of GPX4 and higher concentrations of oxidative stress markers were observed in heart tissue [115]. These observations match cardiometabolic impairments in mice haploinsufficient in *Gpx4* and exposed to high fat and high sucrose diet, which results in elevated lipid peroxides. This situation differs from lower expression of *Gpx1* in mice, where impaired insulin secretion is found albeit they are protected from insulin resistance and steatosis [115].

33.7 Concluding Remarks

Phenotypes with disturbed thyroid or endocrine functions are only observed in a small number of knockout or transgenic mouse models for various selenoproteins. This might convey the impression that the endocrine system is capable of adaptation to alterations that are caused by the systemic or tissue-specific loss of selenoprotein gene function. Alternatively, such phenotypes might have been overlooked during the initial characterization of these mouse models due to the lack of attention to hormonal changes. Furthermore, investigators may not have considered the prominent role of several selenoproteins, such as Dio3, in the development of endocrine tissue function and hormonal regulation.

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Chapter 34
Selenium Antagonism with Mercury and Arsenic: From Chemistry to Population Health and Demography

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Abstract Selenium (Se) has been shown to act as a functional antagonist to mercury (Hg) and arsenic (As). Se may influence Hg and As toxicity by modulating redox homeostasis and inflammation. At the same time, the clinical significance of such interactions is questionable. Despite extensive experimental data, human studies on the interaction between these trace elements, as well as on the influence of such interaction on human health are limited. Current data are reviewed on how Hg and Se interplay impacts on cardiovascular diseases, neurotoxicity, neurodegeneration, diabetes and obesity. Studies also demonstrate that the interaction between

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Se and As significantly affects the development of certain cardiovascular diseases and cancer. This notion is further supported by the results of our analysis of 63,118 adults and 13,734 children from different regions of Russia indicating that the hair Se/Hg ratio is characterized by a tighter association with demographical indices (birth rate, mortality, life span, total morbidity) and morbidity than Hg or Se individually. It is proposed that modulation of the Se/As and Se/Hg ratios in humans may help to improve population health and demography.

Keywords Arsenic • Cancer • Demography • Interaction • Mercury • Morbidity • Mortality • Neurotoxicity • Toxicity

34.1 Introduction

Selenium (Se) is an essential trace element involved in regulation and function of metabolic systems in humans through its role as a component of selenoproteins [1]. Consequently, its deficiency is associated with numerous pathologic states. However, excessive intake of Se may also have adverse health effects [2]. Hg and As are key inorganic pollutants having a significant impact on human health [3, 4]. Various studies suggested antagonism between Hg, As, and Se, as well as clinical implications of such interactions [5, 6]. Despite extensive experimental data on this topic, results from human studies are less clear. Herein, we briefly discuss the main crossroads between biological effects of As, Hg, and Se and review the influence of this interaction on human health.

34.2 Oxidative Crossroad Between Se, As and Hg

Se may be viewed as both an antioxidant and pro-oxidant nutrient that produces its effects through action of Se compounds (supranutritional levels) [7] and its role in selenoenzymes such as glutathione peroxidases (GPx), thioredoxin reductases (TrxR) [8], methionine sulfoxide reductase and other selenoproteins [9, 10]. Se deficiency is associated with oxidative stress in organisms due to deficiency of selenoproteins, although this effect is partially alleviated by induction of detoxification programs, most notably the nuclear factor (erythroid-derived 2)-like 2 (NFE2L2 or Nrf2) response. It was shown that a Se-poor diet resulted in induction of phase II detoxification and antioxidant enzymes [11]. At the same time, the levels of Se above nutritional requirements may lead to a pro-oxidant effect of this element, also activating the Nrf2 response. For example, certain Se compounds like selenite (SeO_3^{2-}), selenocysteine, and diphenylselenide take part in generation of superoxide [12]. Thus, in the case of Se-induced oxidative stress, the activity of antioxidant selenoproteins is increased as a compensatory mechanism to alleviate Se toxicity [13].

In contrast to complex chemical biology of Se, Hg is characterized by a significant pro-oxidant effect due to its high affinity for –SH groups of low molecular weight thiols and proteins, and presumably this element also targets selenocysteine residues in selenoproteins, including GPx and TrxR [14]. In turn, As-induced oxidative stress is mediated through the direct pro-oxidant effect of arsenite (AsO_3^{3-}) [4] and induction of NADPH oxidase [15]. However, our previous data indicate that arsenate (Na_3AsO_4) treatment in pregnant mice dams simultaneously affected Se status and oxidative stress parameters in the liver of the offspring [16].

Numerous studies indicate that Se shows a protective effect against Hg- [17] and As-induced [18] oxidative stress. Taking into account the pro-oxidant effect of certain Se compounds [12], a possibility of potentiation of Hg and As pro-oxidant action by Se should be kept in mind [19].

34.3 Inflammatory Crossroads Between Se, As and Hg

Hg is a potent activator of nuclear factor kB (NF-kB) [20] and mitogen activated protein kinase (MAPK) [21] signaling pathways involved in regulation of an inflammatory response [22]. Experimental studies demonstrated a significant Hg-induced stimulation of tumor necrosis factor α (TNFα), TNF receptor-1, interleukin (IL)-1β, IL-2, IL-17, IL-4, IL-6, IL-8, interferon-γ (IFN-γ) expression or production [23]. However, depending on the dose and form of Hg, it may also exert immunotoxic effects resulting in immunosuppression and autoimmunity [24].

Various As compounds have also been characterized as activators of certain pathways like NF-kB, activator protein 1 (AP-1) and MAPK [25]. Clinical studies indicated a significant association between As exposure and proinflammatory cytokine levels in lymphocytes [26] and maternal and cord blood [27]. However, some studies indicate a significant anti-inflammatory activity of As trioxide (As_2O_3) in allergen-induced inflammation [28] through inhibition of NF-kB-mediated gene transcription [29].

In contrast, Se when present in physiological concentrations acts as a potent anti-inflammatory nutrient mediating its action via down-regulation of NF-kB and lipoxygenase/cyclooxygenase pathways [30]. Conversely, high Se may activate NF-kB in immune cells leading to increased production of IL-2 and IFN-γ [31].

Modulation of Hg- or As-induced inflammatory response by Se may also serve as a potential mechanism of antagonism between these trace elements. Interesting results were obtained by Jin and coauthors who demonstrated that Se pre-treatment in various doses may differentially modulate an inflammatory response to methylmercury (MeHg) administration [32]. Se administration also has been shown to modulate immunotoxic effects of Hg in mice [33]. Previous studies indicated that Se in combination with other biologically active compounds may prevent As-mediated alteration of NF-kB and TNF-α expression [34, 35]. Selenomethionine pretreatment in sufficient and excessive levels (0.2 ppm and 2 ppm, respectively) also provided protective properties against As-induced immunotoxicity by increasing secretion of IL-4, IL-12 and IFN-γ [36].

34.4 Clinical Outcome of Hg–Se Interaction: Cardiovascular Diseases

The influence of interaction between Se and Hg on the incidence of cardiovascular diseases (CVD) has not been extensively characterized [37]. A recent study indicated a significant decrease in the Se/Hg ratio in scalp hair, serum, and urine in myocardial infarction [38] and hypertensive patients [39]. Our investigation also demonstrated a significant positive association between Hg/Se and body mass index, triglycerides, and serum atherogenic index values all being risk factors of CVD. The observed association was stronger for Hg/Se than for hair Hg or Se concentrations [40]. In turn, blood concentrations of Hg and Se were differentially associated with paraoxonase 1 activity in Inuit adults from Nunavik [41]. It is also notable that human subjects of this ethnicity were characterized by a significant statistical interaction between Hg/Se ratio and F_2-isoprostanes and isofurans [42] being used as cardiovascular risk markers [43]. In addition, the inverse association between dietary polyunsaturated fatty acids intake and the incidence of hypertension was more expressed at low Hg and high Se toenail levels [44].

An investigation of the role of Hg in hypertension in Inuit adults demonstrated that, in both systolic and diastolic blood pressure models, low Se decreased the regression coefficient [45]. However, examination of two prospective cohorts of US men and women failed to reveal any significant influence of the Hg-Se interaction on the incidence of hypertension [46]. Park and colleagues also demonstrated that serum Se did not efficiently modify the association between Hg markers and hypertension [47]. The absence of significant modification of the relationship between Hg and coronary heart disease by toenail Se levels was also demonstrated [48]. Despite the fact that the trend was insignificant, the highest Hg level was associated with a higher risk of coronary heart disease than those with the lowest one in the highest toenail Se category [48]. Finally, a significant association between the increased Hg/Se ratio and 15-year mortality in men was demonstrated [49]. However, a recent study indicated that the association between daily ambient concentrations of Hg and Se is not characterized by a significant relationship with daily cardiovascular mortality [50].

34.5 Clinical Outcome of the Hg–Se Interaction: Neurotoxicity and Neurodegeneration

It has been proposed that the main health effect of the Se-Hg interaction is its influence on the central nervous system [51]. However, human data to support this claim are insufficient and in part contradictory. In particular, umbilical cord blood Se did not significantly affect an association between prenatal exposure to MeHg and neurobehavioral deficits in memory function of Faroese children of school age [52, 53]. An investigation of the influence of seafood contaminants from the maternal diet during pregnancy on neonatal neurologic function indicated only a slightly improved

association of Hg/Se ratio with a variable outcome [54]. Therefore, the role of maternal Se intake in prevention of developmental MeHg neurotoxicity in humans is questionable [55].

An investigation involving adult lamp factory workers exposed to Hg indicated a significant protective effect of Se and/or vitamin E supplementation resulting in reduced cases of anxiety-insomnia, improved short-term memory, perceptual ability, short-term visual memory and trail-making [56]. It has been also noted that subjects suffering from depression are characterized by increased blood Hg/Se ratios [57].

Recent studies have demonstrated the association between Hg exposure and the incidence of autism [58]. However, the possible protective potential of Se in autism patients due to its antagonism with Hg is unclear. It has been demonstrated that children with autism are characterized by a significantly increased hair and nail Hg content in parallel with decreased Se levels. Moreover, the observed changes were especially marked in children with low functioning autism [59]. In contrast, we observed a significant increase in hair Se associated with lower Hg content in autistic and mentally retarded children. However, no such relationship was observed in serum and urine [60].

Recent studies indicated a significant role of Hg in the development of neurodegenerative diseases [61]. Despite the protective role of certain selenoproteins in neurodegeneration, data on the influence of Se are inconsistent [62]. Neurodegenerative diseases are characterized by differential patterns of Se and Hg. In particular, Alzheimer's disease (AD), but not multiple sclerosis patients, were characterized by a significant decrease of blood Se with a nearly threefold decrease in the Se/Hg ratio in comparison to control groups [63].

Similarly, the value of plasma Hg/Se ratio in amyotrophic lateral sclerosis patients from Hokkaido exceeded the control values by a factor of more than 4 [64]. This observation was partially confirmed by Roos et al. [65], who demonstrated a decrease in plasma Se/Hg ratio accompanied by the elevation of this index in cerebrospinal fluid of ALS patients. Thus, AD was associated with an increased plasma Hg/Se ratio, whereas CSF values were not related to the disease state [66]. Examination of motor neuron disease patients did not reveal a significant difference in blood Hg/Se in comparison to the respective control values [67]. In contrast, a recent study on the population of Brazilian Amazon residents exposed to Hg through fish consumption revealed that Se biomarkers were significantly associated with better motor function, especially when controlling for blood Hg levels [68].

The changes in brain trace elements levels in patients suffering from neurodegenerative diseases were rather distinct. In particular, no significant alteration in the Hg/Se ratio in the pituitary gland was detected in AD patients [69]. Analysis of different brain regions revealed a significant, almost threefold decrease of the Hg/Se ratio in hippocampus of multiple sclerosis patients, whereas no significant differences were observed in subjects with AD [70]. In contrast, a significant increase in Hg/Se values in various brain subcellular fractions was also revealed in AD [71].

34.6 Clinical Outcome of the Hg–Se Interaction: Diabetes and Obesity

A significant association between Hg and Se and type 2 diabetes mellitus has been detected [72–74]. In particular, diabetic patients were characterized by a significant increase in serum Hg levels and Hg/Se ratio in comparison to the healthy controls [72]. However, an earlier study involving diabetic patients and subjects with impaired glucose tolerance or impaired fasting glucose did not detect any significant changes in plasma Hg and Se when compared to control values [73]. An 18-year follow-up study of 3875 Americans revealed a significant positive association between toenail Hg and diabetes that was improved after adjustment for toenail Se [74]. At the same time, a later prospective cohort study did not confirm this observation [75].

34.7 Clinical Outcome of the As–Se Interaction: Cancer

Potential mechanisms of the As and Se interaction regarding carcinogenesis may involve modification of angiogenesis [76], modulation of DNA repair processes [77], and DNA methylation [78]. Despite the presence of multiple indications of the carcinogenic effect of As [79] and the role of Se in cancer [80], the interactive effect of As and Se on the incidence of cancer is insufficiently studied [81]. A recent study indicated that hair and blood samples from As-exposed females with skin cancer are characterized by significantly elevated As and lower Se levels [82]. Moreover, a significantly higher risk of As-related premalignant skin lesions in persons with low blood Se levels was demonstrated [83]. However, data obtained from a Health Effects of Arsenic Longitudinal Study indicated that Se intake was not significantly associated with As-induced hyperkeratosis [84]. A possible mechanism of the association between low Se status and As-associated skin lesions may involve impaired As methylation that increases metalloid toxicity [85]. Antagonistic relationships between Se and As were also confirmed by the intervention trial in Inner Mongolia, where Se supplementation significantly improved As-induced skin lesions being characteristic for arsenism [86]. The results of this study are in agreement with the reported efficiency of Se treatment of endemic arsenism [87, 88]. A later study in general confirmed this observation [89]. However, the effect of Se treatment on skin lesion status was not significant. It has been also shown that long-term Se supplementation may change the patterns of gene expression in subjects with premalignant As-induced skin lesions [90].

Finally, our earlier investigation [91] of 184 control and cancer patients from an As-polluted area in Plast city (South Ural region, Russia) characterized by the highest oncology-related morbidity in Russia demonstrated a nearly two and threefold increase in the hair As/Se ratio in lung and skin cancer patients as compared to the respective control values. However, no significant changes in the As/Se ratio were observed in gastrointestinal, breast and ovary cancer patients.

34.8 Clinical Outcome of the As–Se Interaction: Cardiovascular Diseases

Previous studies made clear that As exposure is associated with multiple CVD forms, including coronary heart disease, stroke and peripheral artery disease [92]. However, the role of the interaction between As and Se was widely demonstrated only in the latter. Lin and Yang provided the first indication of increased As/Se ratio in biological samples from Blackfoot disease patients [93]. Later investigations of Blackfoot disease patients have also demonstrated a significant increase in As concentration associated with low Se levels in hair [94], blood [95], urine [96, 97] and bone [98]. Moreover, these changes became more marked at later stages of the disease [94, 96]. Interestingly, As content in arterial tissue from Blackfoot disease patients was sixfold higher than in the control volunteers, whereas Se was not altered significantly [99].

34.9 Se-Hg Interaction and Population Health and Demography in Russia

Generally, previous research provides sufficient evidence that the interaction between Se and As, and especially between Se and Hg, may have a significant impact on public health. At the same time, the interactions between these elements may also impact the quality of life and demographic indices.

During a cross-sectional observation involving 63,118 adults and 13,734 children from different regions of Russia [100], scalp hair concentrations of Hg and Se were assessed using inductively-coupled plasma mass spectrometry. The obtained values of hair Hg, Se and the Se/Hg ratio and the statistical data on demographical indices and morbidity from the leading causes for every region were used for correlation analyses using Spearman's rank correlation coefficient.

The data indicated that, in the adult population, hair Hg content inversely correlates with birth rate, whereas Se levels are significantly positively associated with life span and negatively correlate with mortality (Table 34.1). At the same time, the hair Se/Hg ratio is interrelated with all the above mentioned parameters (birth rate, mortality, life span and total morbidity) and characterized by higher correlation coefficients. It is also notable that total morbidity directly correlated with the hair Se/Hg ratio, but not with Se or Hg alone.

Further analysis also indicated a significant association between Hg and Se, and especially their interaction (Se/Hg ratio) with the most incident diseases of the adult population in Russia. The hair Se/Hg ratio was found to be significantly inversely associated with the incidence of tumors, type 2 diabetes, acute myocardial infarction, cerebrovascular diseases, stomach and duodenal ulcers, skin and subcutaneous tissue diseases, musculoskeletal disorders and arthrosis. The correlation coefficients were higher than those for individual hair Se and Hg levels.

Table 34.1 Correlation between hair Hg, Se content, Se/Hg ratio and parameters of morbidity and demography in adult population from 83 regions of Russia

	Hg		Se		Se/Hg	
Parameter	r	p	r	p	r	p
Birth rate	–0.260	0.018*	0.059	0.602	0.382	<0.001*
Mortality	0.147	0.188	–0.253	0.022*	–0.415	<0.001*
Life span	–0.170	0.128	0.447	<0.001*	0.501	<0.001*
Total morbidity	0.125	0.263	–0.113	0.312	–0.258	0.019*
Tumors	0.247	0.025*	–0.032	0.775	–0.333	0.002*
Type 1 diabetes	–0.030	0.790	0.131	0.240	0.038	0.734
Type 2 diabetes	0.278	0.011*	–0.066	0.556	–0.330	0.002*
Obesity	–0.082	0.466	0.013	0.910	0.011	0.923
Hypertension	–0.050	0.653	–0.141	0.205	–0.133	0.232
Coronary heart disease	0.088	0.431	0.056	0.595	–0.094	0.404
Acute myocardial infarction	0.322	0.003*	0.008	0.941	–0.340	0.002*
Recurrent myocardial infarction	0.275	0.012*	0.078	0.485	–0.216	0.052
Cerebrovascular diseases	0.227	0.041*	–0.124	0.267	–0.312	0.004*
Stomach and duodenal ulcers	0.105	0.350	–0.131	0.241	–0.236	0.033*
Liver diseases	–0.012	0.915	0.146	0.190	0.022	0.845
Skin diseases	0.200	0.072	–0.234	0.035*	–0.380	<0.001*
Musculoskeletal disorders	0.237	0.032*	–0.270	0.014*	–0.431	<0.001*
Congenital abnormalities	–0.048	0.668	–0.126	0.260	–0.091	0.416

r—correlation coefficients; p—individual p value for a certain interaction; *—correlation is significant at $p<0.05$

Generally, the same trends were observed in children (Table 34.2). In particular, hair Se/Hg was inversely associated with total morbidity, the incidence of tumors, type 1 diabetes, obesity, skin and subcutaneous tissue diseases, atopic dermatitis, and musculoskeletal disorders. The significance of such association was higher than that for hair Se or Hg alone. Similar associations were found in follow-up investigations of certain Russian territories such as Russian North that is characterized by challenging life conditions, increased morbidity and mortality, and short lifespan [101].

Our data indicate that the interaction between Hg and Se may not only affect biological systems in an organism but also may influence public health and even demographic indices. Further studies aimed to assess the association between As and Se and demography on the territory of Russia are currently being carried out.

Table 34.2 Correlation between hair Hg, Se content, Se/Hg ratio and morbidity in a children's population from 59 regions of Russia

	Hg		Se		Se/Hg	
Parameter	r	p	r	p	r	p
Total morbidity	0.231	0.079	–0.389	0.002*	–0.638	<0.001*
Infectious and parasitic diseases	0.400	0.002*	–0.012	0.930	–0.354	0.006*
Tumors	0.064	0.632	–0.156	0.237	–0.336	0.009*
Type 1 diabetes	–0.030	0.823	–0.342	0.008*	–0.402	0.002*
Type 2 diabetes	0.213	0.105	0.007	0.957	–0.080	0.549
Obesity	0.130	0.326	–0.313	0.016*	–0.487	<0.001*
Stomach and duodenal ulcers	0.018	0.894	–0.040	0.765	–0.121	0.362
Gallbladder and bile ducts diseases	0.193	0.144	–0.057	0.669	–0.192	0.146
Skin diseases	0.211	0.108	–0.293	0.024*	–0.506	<0.001*
Atopic dermatitis	0.145	0.273	–0.248	0.058	–0.414	0.001*
Musculoskeletal diseases	–0.091	0.491	–0.311	0.016*	–0.358	0.005*
Congenital abnormalities	0.229	0.080	0.036	0.785	–0.210	0.111

r—correlation coefficients; p—individual p value for a certain interaction; *—correlation is significant at $p<0.05$

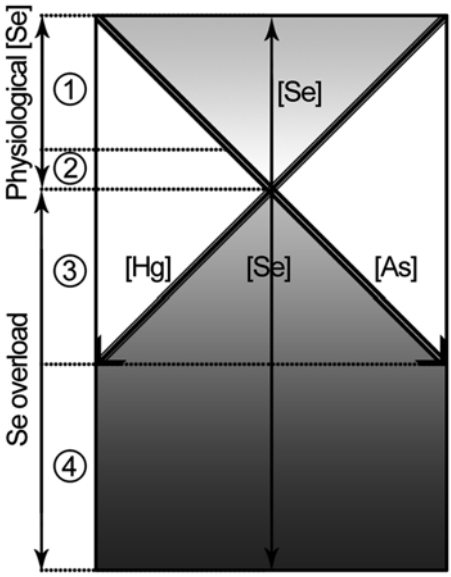

Fig. 34.1 Possible effects of interaction between Se, As, and Hg in humans. Density of *grey color* is proportional to Se burden. (*1*) At physiological concentrations Se is capable of preventing Hg- and As-induced toxicity (especially at the molar ratio of 1:1); (*2*) Se deficiency along with increasing Hg and As concentrations results in clinical manifestations of Hg and As toxicity; (*3*) An increase in Se concentrations in biological fluids over a physiological maximum (Se overload) in the presence of Hg and As hypothetically results in synergistic toxic effects; and (*4*) In the presence or absence of heavy metals, excessive Se levels in the organism may exert toxic effects

34.10 Perspectives

Additional studies are required to assess the influence of the interaction between Se and As or Hg on human public health both in clinical and epidemiological studies. Taking into account a possibility of both antagonistic and synergistic toxic effects of these trace elements (Fig. 34.1), future studies should estimate their levels and ratios in humans that are associated with healthy conditions and adverse health status. Simultaneous speciation analysis for investigation of Se, Hg and As metabolism in health and disease is of particular interest. Such information may be used in biomonitoring, risk assessment and/or treatment of chronic As and Hg poisoning. In particular, it will help estimate whether Se supplementation may assist in alleviating or aggravating heavy metal toxicity in each individual case. Finally, modulation of the ratio between Se, As and Hg in the human population may help to improve population health and demography.

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Chapter 35
Biological Selenium Species and Selenium Speciation in Biological Samples

Katarzyna Bierla, Joanna Szpunar, and Ryszard Lobinski

Abstract The chapter summarizes the state-of-the-art of the analytical methodology for the speciation of selenium (Se) in biological samples relevant to human health (body fluids, cell cultures, tissues, food supplements). Selenoproteins with genetically encoded selenocysteine, Se-containing proteins with Met substituted by selenomethionine, and Se-containing metabolites are discussed. Whereas gel electrophoresis followed by radiography of ^{75}Se is the benchmark for selenoprotein and Se-containing protein detection, the recent advances in laser ablation-ICP MS allow the scanning of the gels for stable Se isotopes, increasing the number of biological systems to be investigated and enhancing the depth of the studies. The democratization of proteomics approaches opens the way to the high throughput identification of selenoproteins, although several bottlenecks, such as loss of Sec during sample preparation, identification on the basis of the part of the proteins without Se, and insufficient sensitivity, are still demanding considerable improvements of analytical methodology. In the field of metabolomics, the combined use of multidimensional HPLC with the combined Se-specific ICP MS detection and Orbitrap MS^n detection, seems to be an ultimate tool for the comprehensive quantitative and qualitative Se speciation analysis.

Keywords Electrospray MS orbitrap • Food supplements • Foodstuffs • HPLC-ICP MS • Human health • Selenium speciation • Selenoproteins

35.1 Introduction: Interest in Selenium (Se) Speciation Analysis in Biology

Knowledge of speciation: the identity and the concentration of the exact chemical form(s) in which Se is present in a sample is fundamental to describe its molecular mechanisms of the biological activity of this element and to delineate the specific

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metabolic pathways in cells and tissues. The processes concern medical, physiological and nutritional research: the role of Se in health and disease, the metabolism and activity of Se-containing active therapeutic molecules and nanoparticles, the biotechnology of Se-enriched food and feed supplements [1–3], and Se bioavailability and metabolism in food [1, 2].

35.2 Se Species of Interest

There are numerous foods and food supplements. The most important Se species of interest are described below.

35.2.1 Selenoamino acids: Selenomethionine (SeMet) and Selenocysteine (Sec)

The SeMet concentration is an important parameter to evaluate the degree of conversion of inorganic Se into organic Se. It is produced by the replacement of sulfur with Se in the biosynthesis of Met. The SeMet concentration is used for the characterization of the efficiency of the Se-rich yeast biotechnology and of that of the conversion of soil inorganic Se by plants. SeMet represents >60 % in yeast food supplements [4] and ca. 80 % of the total Se in staple food [5, 6]. SeMet is the primary form of Se in storage proteins produced during the production of Se-rich food of animal origin (meat, milk, eggs, etc.). In humans, the concentration of SeMet in plasma (and the SeMet/Sec ratio) increases with an increase of total Se [7].

Sec, referred to as the 21st proteinogenic amino acid [8], concentration is a measure of selenoprotein content, as demonstrated in the microanalysis of gel bands and spots [9, 10]. Note that Sec can also be introduced into proteins in a similar way as SeMet, i.e., by a simple substitution of sulfur by Se, without a gene coding for a selenoprotein biosynthesis pathway being present. Substantial concentrations of Sec can be found in some yeasts [9], plants [5, 6] and bacteria [11]. Therefore a caution should be made when the speciation between SeMet and Sec is used as a measure of the “true” intracellular selenoprotein content.

35.2.2 Selenoproteins and Se-Containing Proteins

Selenoproteins (proteins containing genetically inserted Sec) are believed to be of paramount importance in health and disease [12] and to be responsible for many of the beneficial effects of Se [13, 14]. The most important ones are known for their catalytic activity, e.g., glutathione peroxidase (GPX) and thioredoxin reductase

(TXNRD), or are used by mammals as storage protein, e.g., selenoprotein P (SEPP1). The maximum GPX3 activity in plasma was used as the basis for the current Recommended Dietary Allowance (RDA) of 55 μg Se/day of Se [15]. Fundamental work by Gladyshev's group [16] allowed the prediction of selenoproteomes in a number of mammals, including man, and in other organisms. Studies using both cell culture and animal models have shown that expression of selenoproteins is differentially regulated by Se availability. Whereas the expression of a subclass of selenoproteins called stress-related selenoproteins, e.g., GPX1, MSRB1, SEPW, and SELH, is significantly decreased under Se-deficient conditions, while the expression of the other subgroup, housekeeping selenoproteins, e.g., TXNRD1 and TXNRD2, is less regulated by dietary Se [17]. Thus, these observations demonstrate the dependence of stress-related selenoprotein expression on dietary Se.

In contrast to true genetically encoded selenoproteins, the mechanism of incorporation of Se into Se-containing proteins is chemically mediated and consists in the replacement of sulfur in Met and/or in Cys by Se. Examples of such proteins include, selenoalbumin in mammals [18], and the storage proteins, glutenins and gliadins, in plants [5].

35.2.3 Selenometabolites

This generic category of molecules includes low molecular weight (<1000 Da) Se compounds (Fig. 35.1). The largest characterized pool of metabolites is known to accompany the production of SeMet in yeast and can be divided in several subcategories, including selenols (R_1–CH_2–Se-H), diselenides and Se-sulfides, di- and tripeptides containing SeMet and Sec, selenoethers (R_1-CH_2-Se-CH_2-R_2) and selenoxides (R_1-CH_2-Se(O)-CH_2-R_2) [4]. The beneficial health effects of a number of natural Se-rich plants, grown on seleniferous soils or cultivated Se-rich garlic, are assigned to a number of non-proteinaceous, low molecular weight Se species [19]. Selenometabolites, e.g., selenoneine, account for a minor fraction of Se in blood and serum [20, 21]. Low molecular weight species including tetramethylselenonium (TMSe) and selenosugars (Se-methylseleno-N-acetyl-galactosamine, Se-methylseleno-N-acetyl-glucosamine and Se-methylseleno-galactosamine) can be found in urine which is a major excretory route for Se [22, 23].

35.2.4 Nanoparticles and Selenodrugs

An exhaustive list of Se compounds studied for their cytotoxic effects is given in a recent review [3]. Se-containing nanoparticles have recently attracted attention as potential cancer therapeutic payloads, due to their biological activity and low toxicity [3]. The metabolic pathways between different Se compounds differ significantly and can produce various Se metabolites [3].

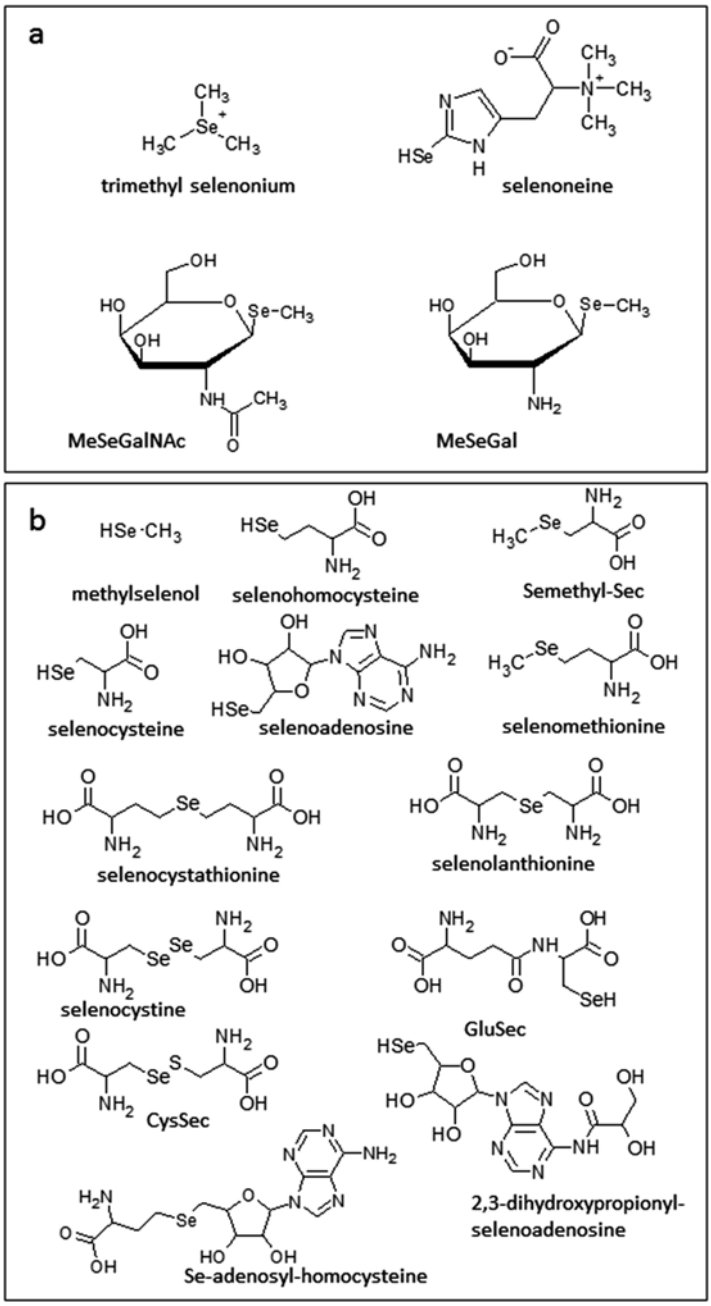

Fig. 35.1 Summary of the most popular selenometabolites found in (**a**) body fluids (**b**) plants and yeast. Compound structures and names are shown in the figure

35.3 Speciation of Selenoaminoacids: Determination of SeMet and Sec

35.3.1 SeMet

Chemical protocols, typically used for amino acid determination often fail as the conditions are too harsh and do not always preserve the integrity of SeMet leading to biased results. The most popular have become protocols based on the enzymatic digestion including multiple incubation of a sample with different types of proteases [4]. The procedures do preserve the integrity of SeMet but may not be sufficiently aggressive to release all the SeMet present. SeMet is subsequently determined by HPLC-ICP-MS or, after derivatization, by GC-MS. A standard reference yeast material with a certified concentration of SeMet is available (SELM-1, NRCC, Canada), but caution is necessary to extrapolate the validity of a method developed for SELM-1 to other biological materials, including other types of yeast. The mass balance (the sum of the concentrations of the species in comparison with the total Se content) of all the Se forms present is the *sine qua non* condition for validation of the method.

35.3.2 Sec

Accurate determination of Sec is still subject to controversy because of its by far lower stability resulting in its oxidative conversion to dehydroalanine or reactivity with derivatizing reagents. Derivatization is, however, necessary in order to stabilize Sec. It should be carried out on the purified protein fraction, otherwise a number of artefacts resulting from side-reactions with selenometabolites interfere with the analysis. The simultaneous derivatization of SeMet may occur leading to the splitting of its content between the native and the derivatized form; and degradation can be minimized by optimizing the procedure for a given matrix and in a given analyte concentration range. The risk of the potential loss of Sec, especially at the low and sub-ppm levels (e.g., in meat, milk, eggs) has to be controlled by the Se mass balance [24–28]. On the basis of the identification of ten proteins richest in Se, it was demonstrated that Se was incorporated by the *Lactobacillus reuteri* strain exclusively as Sec; its exact location within the primary sequences was determined [11].

35.4 Speciation of Protein-Bound Se and Selenoproteomics

The three types of analytical approaches to identifying Se-containing proteins include the targeted analysis of known selenoproteins, Se-filtered exploratory analysis of selenoproteins, and generic (e.g., SWATH) proteomics.

35.4.1 Targeted Analysis of Known Selenoproteins

The classical approach for analysis of known proteins is Western blot analysis, which, although sensitive does not guarantee the presence of Se in the detected species. For example, two potential isoforms of SEPP1 have been identified by Western blot analyses (51 and 61 kDa); the biological relevance of the smaller isoform has been called into question by several studies confirming the presence of at least one Sec in SEPP1 structure [29–31], and thus, suggesting that the shorter 51 kDa form might be an artifact of protease activity during the purification process. This ambiguity demonstrates limitations of Western blot analyses providing no information regarding the Se content of the potential isoforms [32]. Also, the feasibility of the analysis for a particular selenoprotein depends on the availability of an antibody.

Indicative levels of the most abundant selenoproteins, GPX, SEPP1 and selenoalbumin, in a commercially available human serum (BCR-637 CRM), with a certified level of total Se, were calculated using the results obtained by 13 different analytical methods on the basis of affinity high-performance liquid chromatography coupled to ICP-MS [33]. The methodology was further developed to allow the simultaneous speciation of GPX, SEPP, selenoalbumin and selenometabolites (eluting as a single peak) [21].

35.4.2 Se-Targeting Proteomic Techniques

35.4.2.1 Bottom-Up Approach

A preliminary comprehensive screen for the presence of Se-containing proteins can best be carried out by gel electrophoresis (GE), either one (isoelectric focusing, IEF, or SDS PAGE) or two dimensional (2D). Classical procedures, especially for model studies, used radioactive ^{75}Se-labeling [34, 35]. They can be replaced by the Se-specific ICP MS detection [36] via a coupling with laser ablation (LA), thus avoiding problems with handling of radioactive isotopes. Quantitative analysis is possible upon calibration of the in-gel detection by commercial or in-house prepared protein standards [37, 38]. The use of ICP MS also has the advantage of the isotopic specificity allowing the use of stable Se isotopes for tracer studies and isotope dilution quantification.

As LA-ICP MS is destructive and the ablated parts of the gel cannot be further analyzed, the proteomics analysis should be carried out using spots in a gel prepared in parallel or for a non-ablated part of bands in 1D gels [5, 6, 11, 39]. The workflow is shown in Fig. 35.2.

Canonical HPLC–MS/MS proteomics protocols can be used for the protein identification [40], but it should be noted that they may easily miss Sec because of H_2Se-elimination leading to dehydroalanine, especially for proteins with a terminal Sec. Also, the protein identification is often based on the part of the protein sequence which does not contain Se; hence, the interest in the parallel ICP MS and ESI MS detection (Fig. 35.2).

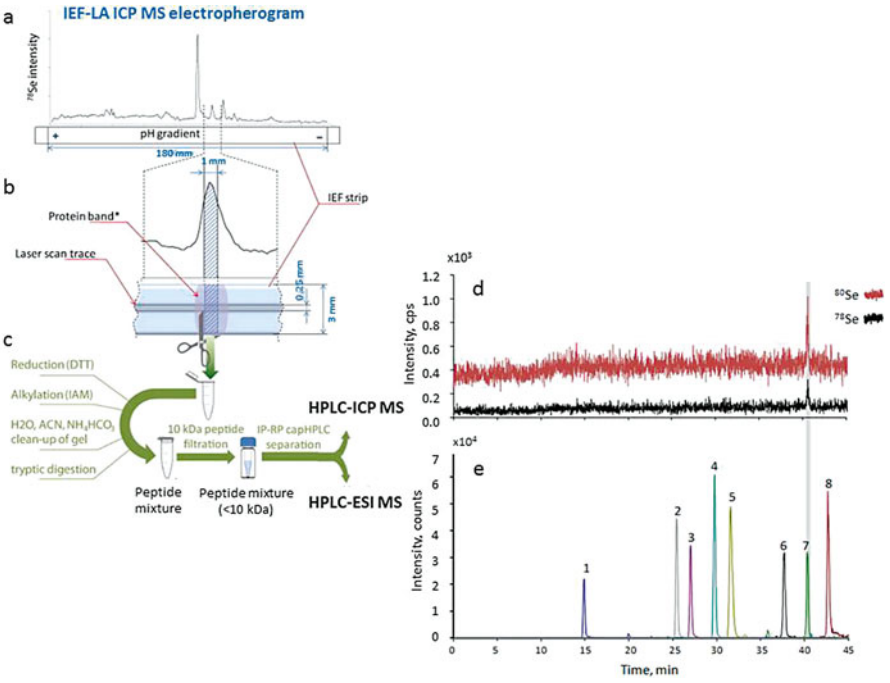

Fig. 35.2 Analytical workflow for bottom up selenoproteomics using (**a**, **b**) 1D IF protein separation and LA-ICP MS detection of Se-containing bands followed by (**c**) in-gel tryptic digestion and HPLC separation of tryptic peptides by HPLC with parallel (**d**) ICP and (**e**) ESI MS detection; the example shown is the identification of GPx with the following peptides: multi-ion XIC chromatogram for all the ions corresponding to the target protein (*1*—DYTQM(OX)NELQR, *2*—NEEILNSLK,*3*—NDVAWNFEK,*4*—LITWSPVCR,*5*—FLVGPDGVPLR,*6*—GLVVLGFPCNQFGHQENAK, *7*—VLLIENVASLUGTTVR, *8*—YVRPGGGFEPNFMLFEK) [58]

35.4.2.2 Shotgun Approach

The parallel HPLC-ICP MS and HPLC ESI MS can be applied to a tryptic digest of the whole proteome which has the advantage of combining the elemental and molecular information on Se-containing peptides [6, 41]. Because of the complexity of the samples, initial fractionation of the samples based on their solubility [6] or molecular weight [41] was used for real samples.

35.4.2.3 Top-Down Approaches and Structural Studies

The state-of-the-art protocols still do not address some challenges such as the identification of truncated isoforms, post-transitional modifications, possible formation of dimers [42], and the formation of bonds with selenite. The elucidation of reaction mechanisms [42] or structural studies of isoforms of SEPP1, the only human selenoprotein containing multiple Sec residues [32] remains a challenge.

35.4.3 High Throughput Non-targeting Approaches

A future trend in analytical proteomics is non-focused data acquisition of the whole proteomes after their digestion with trypsin and identification of all the produced peptides. The data obtained in this way can be reanalyzed later for the presence of useful information. Se-containing species that were found included, e.g., (1) TXNRD1 and SEP15 identified among 450 proteins in bovine colostrum after high-speed centrifugation and 2D-HPLC fractionation [43], and (2) SEPP1 among 311 proteins identified in the study of biomarker candidates of alcohol abuse [44]. Because of the instability of Sec in the protocols not optimized for this purpose and the consequent lack of the link between Se and the protein other than prediction, no difference between Sec, Cys, and serine proteins in the active site (crucial from the point of view of insight into the role of Se) could be detected. Mutant enzymes with Cys instead of Sec had reduced hydroperoxidase activity, thus Se is required for the catalytic activities of TXNRD explaining the essential role of this trace element for the cell growth [45].

High throughput approaches, when optimized, should allow estimating Met/SeMet and Cys/Sec amounts, and thus, the identification of the privileged or random replacement of sulfur.

35.5 Speciation Analysis of Selenometabolites

The information on Se speciation is essential in animal and plant physiology [19]. It can also be useful for the control of the technological enrichment processes and the determination of the origin of the commercial products [46]. Special interest concerns studies on Se detoxification and/or extraction. Moreover, in food science, low molecular weight of a species is considered as favorable for its bioaccessibility.

A critical step in the analysis for selenometabolites is the sample preparation procedure. For solid samples, it is expected that extraction with water removes metabolites, but dedicated extractions with less polar solvents may be essential to recover more hydrophobic species. Generally, immediate analysis to avoid possible changes in speciation due to oxidation is recommended. Risk of volatilization is significant for some samples, in particular for garlic and allium plants [47]. Deproteinization and desalting are necessary for body fluids.

The principle of a typical analytical approach consists of the parallel elemental and molecular MS detection in HPLC, allowing one to take advantage of both techniques, i.e., the quantitative character and matrix-independent response of ICP MS and the structural information provided by ESI MS^n (Fig. 35.3). The optimization of the HPLC separation in the context of subsequent MS detection is important for assuring the compatibility of eluent with the requirements of both detectors (note that plasma and ESI ionization conditions are changing during the gradient elution). Identification by molecular MS is based on the accurate mass and the characteristic isotopic pattern (*cf* inset in Fig. 35.3). The high accuracy and preci-

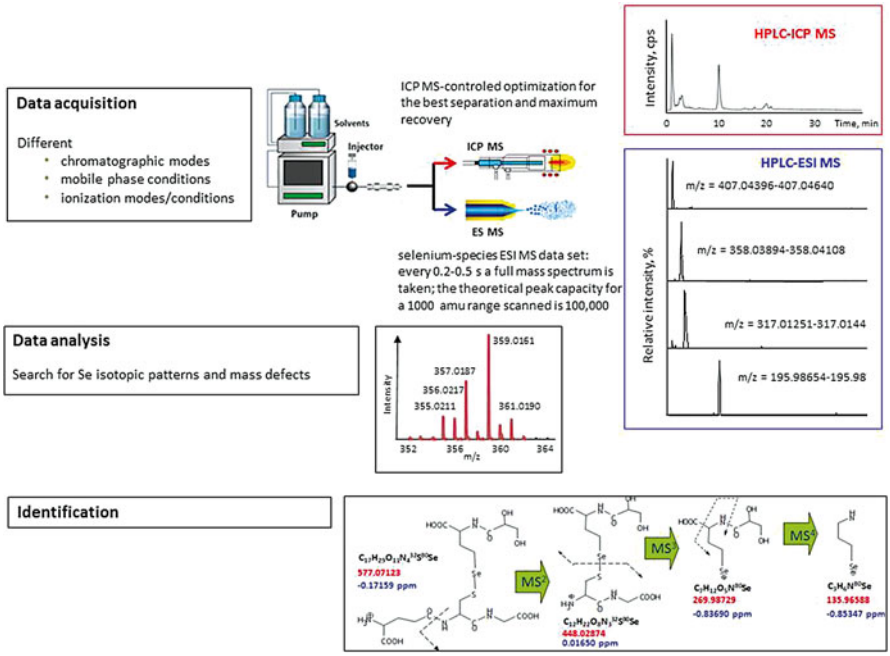

Fig. 35.3 Analytical workflow for selenometabolomics. The cation-exchange HPLC chromatograms shown in the *insets* were obtained for ammonium acetate extracts of maize (the species eluted: methylseleno-Se-pentose-hexose, deamino Sec-Se-hexose, SeMet)

sion together with the multistage fragmentation capability of state-of-the-art molecular spectrometers are necessary for de novo species identification [48]. In contrast to selenoproteomics studies, where predictions can be made on the basis of the genome, structures of selenometabolites are difficult to predict and their full elucidation is a complex task often beyond the capacity of mass spectrometry.

The major compounds found in the plant samples enriched with Se(IV) were MeSec, SeMet and γ-Glu-MeSec, and accumulation of these organic forms, particularly MeSeCys and γ-Glu-MeSec, may also be increased by genetic modification [49]. Selenohomolanthionine was identified in the water extract of pungent Japanese radish [50]. The comprehensive list of selenometabolites identified in Se-enriched yeast includes more than 60 compounds [39, 51].

An assessment study of the analytical methods used for identification of urine Se metabolites revealed assignment of incorrect structures to several compounds; and especially, the long-held view that trimethylselenonium ion is a major human urinary metabolite was judged unjustified [23], wherein the major forms are selenosugars [22], e.g., Se-methyl-N-acetylselenohexosamine Se speciation in blood serum is dominated by selenoproteomics, and the selenometabolite fraction (as a whole) was quantified [21] in a global approach focused on selenoproteins. However, the low molecular weight species detected together as a single chromatographic

peak may have resulted from, e.g., degradation of selenoproteins. The key metabolite seems to be selenoneine [52, 53] (Fig. 35.1a) and its methylated derivative [20] which were identified in tuna [52] and human blood [20, 53].

35.6 Solid-State Speciation of Se (X-Ray Absorption Spectrometry, XAS)

Although the coupled techniques offer excellent sensitivity and the ability to identify unequivocally individual compounds at trace and ultratrace levels, they require extensive sample preparation of biological tissues. The digestion and acidification of samples can alter the speciation of Se, particularly with regard to its oxidation state. Note that, except for body or plant fluids, a significant fraction of the Se often remains insoluble. It can be accessed by soft protocols that degrade the original species in a controlled way in order to dissolve it, while preserving speciation information (e.g., digestion of selenoproteins to Sec or SeMet). However, there remain a number of questions that can only be answered by the direct (solid-state) techniques, such as X-ray absorption spectrometry (XAS), including Extended X-Ray Absorption Fine Structure (EXAFS), and X-ray Absorption Near Edge Structure (XANES).

X-ray absorption spectrometry also offers high spatial resolution that can be used to acquire information on the distribution of Se within organs which is lost during the homogenization of the samples [54]. In comparison with hyphenated techniques, XAS lacks sensitivity at low concentrations and is unable to detect trace components. Also, it is limited to the determination of the coordination and oxidation environment of Se, rather than giving the exact identity of the Se compounds. The speciation of Se in Se-enriched yeast was investigated using both XAS and HPLC-ICP-MS. The results were in agreement, although the restricted model compound library and absence of an EXAFS spectrum limited the information that could otherwise be derived from XAS [55]. XANES was applied to the study of Se speciation in tissues from rainbow trout exposed to SeMet [56]. SeMet- and MeSec-treated cancer cells showed XAS spectra distinct from selenite-treated cells [57]. Although sample preparation is minimal compared to the preparation required for HPLC-ICP-MS, it may still interfere with the sample by selectively mobilizing, redistributing or washing out sample components [57] (Fig. 35.4).

35.7 Concluding Remarks

Considerable progress has been made during the last decade in the analytical methodology allowing qualitative and quantitative Se speciation in biological samples. Valid quantitative methods for the determination of SeMet and Sec exist in a variety of samples. The coupling of HPLC with the parallel ICP MS and electrospray MS^n detection is a generic method allowing the global speciation of Se on the metabolome levels.

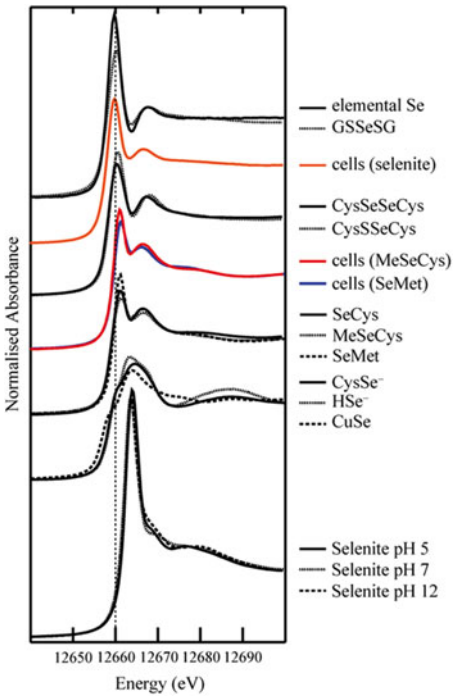

Fig. 35.4 Library of Se K-edge X-ray absorption spectra of model Se compounds used in the linear combination fitting of experimental spectra. XANES spectra of cancer cells treated with 100 μM SeMet (*blue*), 100 μM MeSec (*red*) or 5 μM selenite (*orange*) are also shown for comparison [57]

The use of canonical high throughput protocols for proteomics such as shotgun or data independent analysis protocols gives the illusion of producing meaningful data for selenoproteins as well. However, the prerequisite of the validity of the selenoproteomics method is the detection of the selenoproteins via the Se-containing peptide(s) and a rigorous control of the Se mass balance during the sample preparation aiming at the preservation of the Sec-containing entity. Solid-state speciation techniques, such as XAS, offer the spatial resolution at the micrometer level, but their applicability is limited to fairly high Se concentrations and simple systems such as Se(IV), Se(VI), SeMet, and Sec.

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Part IV
Selenoproteins in Human Health

Selenoproteins have important roles in many aspects of human health. Congenital disorders of selenoprotein expression can lead to disorders of muscle, the endocrine system and the brain. The chapters in this section focus on the roles that selenoproteins have in cancer, the nervous system and numerous other facets of human health.

Chapter 36
Selenoproteins in Nervous System Development, Function and Degeneration

Ulrich Schweizer

Abstract It is now firmly established that the mammalian brain depends on adequate expression of selenoproteins. Genetic studies in the mouse have paved the way and suggested that neurodevelopmental and neurodegenerative disorders can be caused by impaired selenoprotein expression within the brain. With technical advances in human genetics, it became clear that inborn errors of selenium (Se) metabolism or selenoprotein expression do occur and can lead to specific developmental and degenerative disoders. The discovery of spontaneous neurological phenotypes in selenoprotein P-deficient (*Sepp1*$^{-/-}$) mice clearly marked a turning point in our appreciation of Se and selenoproteins within the nervous system. Since then, several selenoproteins have been identified that are essential for normal brain function. Feeding animals low Se-containing diets was not sufficient to impair brain function unless the Sepp1-receptor ApoER2 was inactivated. Sepp1 and its receptors are thus pivotal in preferential Se transport into the brain. The two selenoproteins essential for mouse neurons are glutathione peroxidase 4 (Gpx4) and selenoprotein T (Selt). This chapter will focus on neurological diseases and phenotypes observed in mouse models or patients with impaired selenoprotein expression in the brain. Common observations and divergent findings will be discussed.

Keywords Atrophy • Cerebellum • Cerebrum • Epilepsy • Interneuron • Seizure

36.1 Reduced Brain Selenium Availability Impairs Brain Function

Only one report demonstrated spontaneous neurological symptoms in Se-deficient mammals, which is “leg-crossing”, in Balb/c mice maintained on a Se-deficient diet [1]. Brain Se levels remained rather stable in experimental animals during periods

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of severe dietary Se restriction [2, 3]. A role for Se in brain function was therefore not apparent, in particular, since brain Se levels are relatively low [4, 5]. However, there have been case reports that suggested a link between intractable childhood epilepsy and low Se levels [6, 7].

Mice genetically deficient in selenoprotein P (*Sepp1*$^{-/-}$) represented the key tool to establish a role of Se in the brain [8, 9]. As detailed in Chap. 22, the protein is involved in the molecular mechanism that preferentially supplies Se to the brain. Disruption of this mechanism renders the brain dependent on dietary Se intake and lowers brain Se levels during times of Se restriction. Accordingly, selenoprotein expression in brain is reduced and complex neurological phenotypes develop [8–10]. These mice exhibited reduced plasma Se levels and reduced Se content in many organs and tissues including the brain [8, 9]. These mice show a neurological phenotype which depends on dietary Se intake [11, 12]. When the mothers were fed a low Se diet during lactation, *Sepp1*$^{-/-}$ mice develop a severe runting phenotype and die. When dietary Se restriction starts from weaning, the offspring develop a severe neurodegenerative phenotype leading to death within 2 weeks. Brainstem axonal damage and gliosis were reported in Se-deficient *Sepp1*$^{-/-}$ mice [13]. Even when fed an RDA diet (recommended dietary allowance, which is 0.15 mg Se/kg diet), *Sepp1*$^{-/-}$ mice developed a neurological phenotype including a movement disorder with a wide waddling gait [11, 12, 14]. Seizures are frequent from 5 weeks of age, but can be controlled by increasing dietary Se intake [11]. Synaptic transmission is altered in *Sepp1*$^{-/-}$ mice, even if fed a high Se diet, but the mechanism remains unclear [15]. The Se-rich C-terminus is likely important for efficient Se transport, since mice expressing a truncated Sepp1 protein containing only the single N-terminal selenocysteine residue (*Sepp1*$^{\Delta C/\Delta C}$), also exhibit diminished Se content in brain and brain pathology [13, 16]. When Se transport to the testes is abrogated by prepubertal castration, male *Sepp1*-deficient mice divert more Se to their brains, thus delaying the development of the neurological phenotype [17]. This is an interesting finding, because preferential Se transport to brain and testis is thought to depend on Sepp1, which is not present in these mice. This means that the redistribution of Se in castrated mice is achieved by a Sepp1-independent mechanism, possibly as selenite which was present in the mouse diet and is known to enter the brain independent of Sepp1 [11].

Expression of a hepatocyte-specific human *SEPP1* transgene in *Sepp1*$^{-/-}$ mice fully rescued the *Sepp1*$^{-/-}$ neurological phenotype when mice were fed a Se-sufficient diet, although their brain selenoenzyme levels were not fully restored to the levels found in control litter mates [18]. When *Sepp1*$^{-/-}$ mice were fed a Se-resticted diet, brain Se levels and cerebral selenoprotein expression decreased, and ultimately the mice succumbed to Se-deficiency. This means that lowering dietary Se intake failed to sustain hepatic Sepp1 expression, and hence, Se supply to the brain diminished. In the absence of cerebral Sepp1 expression, *Sepp1*$^{-/-;\ SEPP1}$ mice were not able to maintain brain Se levels, unlike Se-deficient wild type mice [18]. SEPP1 expression is not only found in mouse brain [8, 19], but also in human brain [20], where SEPP1 was easily detectable in cerebrospinal fluid. In support of a dual role of *Sepp1* expression in brain and liver, we have observed that hepatic inactivation of *Sepp1*

expression lowered plasma Se and kidney Se contents, but not brain Se content and selenoprotein expression [14]. These findings were later corroborated with a different conditional mouse model [21].

A major advance in the field was the identification of ApoER2 (also known as *Lrp8*) as a specific Sepp1 receptor in testis and brain [22, 23]. ApoER2 belongs to the lipoprotein receptor-related protein family of endocytic receptors (see Chap. 22). *Apoer2*$^{-/-}$ mice fed a Se-deficient diet recapitulated the *Sepp1*$^{-/-}$ phenotype [24]. We found that *Apoer2*$^{-/-}$ mice fed a Se-sufficient (RDA) diet do not exhibit the characteristic movement phenotype known from *Sepp1*-deficient mice fed an identical diet for the same time [25]. An explanation is that Sepp1 expression in the brain helps trap Se in the brain [18, 26]. There are two other possibilities that may explain this conundrum: Se in the diet may be directly bioavailable to the brain, e.g., as selenite, and/or other Sepp1 receptors participate in uptake of Sepp1 into the brain. Megalin/*Lrp2* was identified as another Sepp1-receptor [27, 28]. Brain Se content and selenoprotein expression are reduced in *Lrp2*$^{-/-}$ mice and the effect is enhanced when the animals were fed a low Se diet [28, 29]. After several weeks of feeding low Se diet to adult *Lrp2*$^{-/-}$ mice, movement coordination deteriorated, reminiscent of *Sepp1*$^{-/-}$ mice [28]. Since megalin is expressed along the blood-brain-barrier, but not on neurons or astrocytes, these data support a role of megalin in cerebral Se uptake. ApoER2 is specifically expressed in a subtype of interneurons [10], which we have shown to be most dependent on selenoproteins [30] (see below).

36.2 Neurologic Phenotypes of Selenoprotein-Transgenic and –Deficient Mouse Models

The brain expresses almost all selenoproteins [31], and within the brain neurons are the primary site of selenoprotein expression [30, 31]. Thus, it appears likely that different aspects of cerebral selenoprotein deficiency are mediated by the lack of different selenoproteins in affected structures.

36.2.1 Transgenic Mouse Models with Global Selenoprotein Deficiency

36.2.1.1 Transfer RNA$^{[Ser]Sec}$

The tRNA$^{[Ser]Sec}$ gene, symbol *Trsp*, is absolutely essential for functional selenoprotein expression (see Chap. 46). We have conditionally inactivated *Trsp* in neurons [30]. This experiment represented a *proof-of-principle* whether selenoproteins play any important role in brain function, because the alternative hypothesis, that the complex phenotypes of *Sepp1*$^{-/-}$ or *Apoer2*$^{-/-}$ mice led indirectly to neurological deficits, could initially not be refuted. In neuron-specific *Trsp*-deficient mice, cerebral selenoprotein

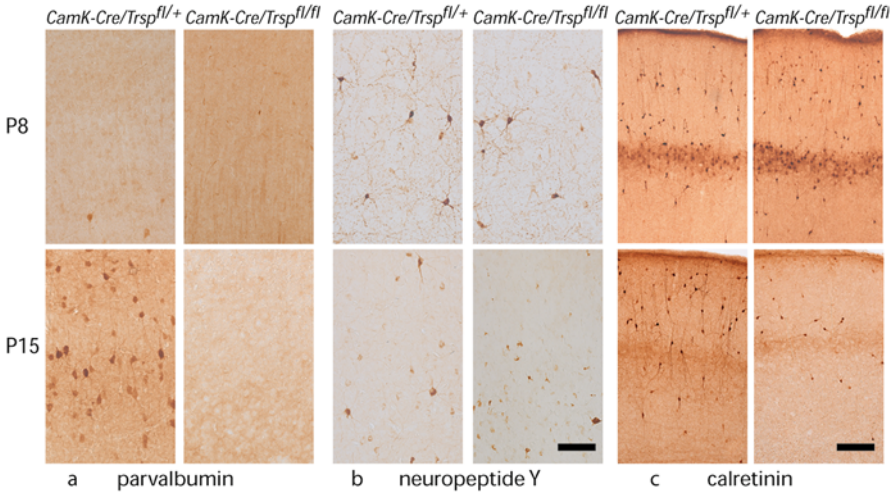

Fig. 36.1 Numbers of GABAergic interneurons in cerebral cortex of mice with genetic inactivation of $tRNA^{[Ser]Sec}$ (*Trsp*) in neurons are selectively reduced. Interneurons expressing a set of non-overlapping markers are labeled by immunohistochemistry. (**a**) Parvalbumin-expressing cells. (**b**) Neuropeptide Y-expressing cells. (**c**) Calretinin-expressing cells. P8, P15 postnatal days 8 and 15, respectively. Scale bar: 100 μm

expression was significantly reduced. Mutant mice showed growth retardation, loss of balance, and increased excitability. Hippocampal slices from mutants exhibited spontaneous epileptiform activity in vitro. Massive neurodegeneration followed, shortly thereafter, with signs of apoptosis, enlarged ventricles, and microcephaly [30].

A key finding was the specific lack of parvalbumin-expressing (PV+) cortical and hippocampal GABAergic interneurons in *Trsp*-mutant animals (Fig. 36.1). PV+ interneurons represent the largest fraction of cortical interneurons. Interestingly, neuropeptide Y-expressing interneurons and the third major subtype, calretinin (CR)-expressing interneurons, developed normally [30]. We showed that PV+ cells are similarly affected by inactivation of *Gpx4* [30, 32]. Hence, it seems as if PV+ cells are particularly sensitive towards *Gpx4* inactivation or lipid peroxides. In fact, when dissociated cortical neurons are cultured in vitro using a chemically defined medium, combined deprivation of Se and vitamin E leads to a reduction in the number of PV+ GABAergic neurons [33].

When a Cre-transgenic mouse line was used which directed *Trsp* deletion to the cerebellum, massive Purkinje cell death and cerebellar hypoplasia were observed [34]. Cerebellar hypoplasia was associatd with decreased proliferation of granule cell precursors and increased cell death (Fig. 36.2). Purkinje cell loss was also observed upon *Gpx4*-inactivation [34]. Cerebellar interneurons, in particular, stellate cell numbers were reduced as well, but to define the roles of selenoproteins during development in the various cerebellar cell types will require more study.

Mice expressing hypomorphic *Trsp* alleles manifest neurological phenotypes similar to $Sepp1^{-/-}$ mice fed an RDA-diet from weaning [35, 36]. These mice carry

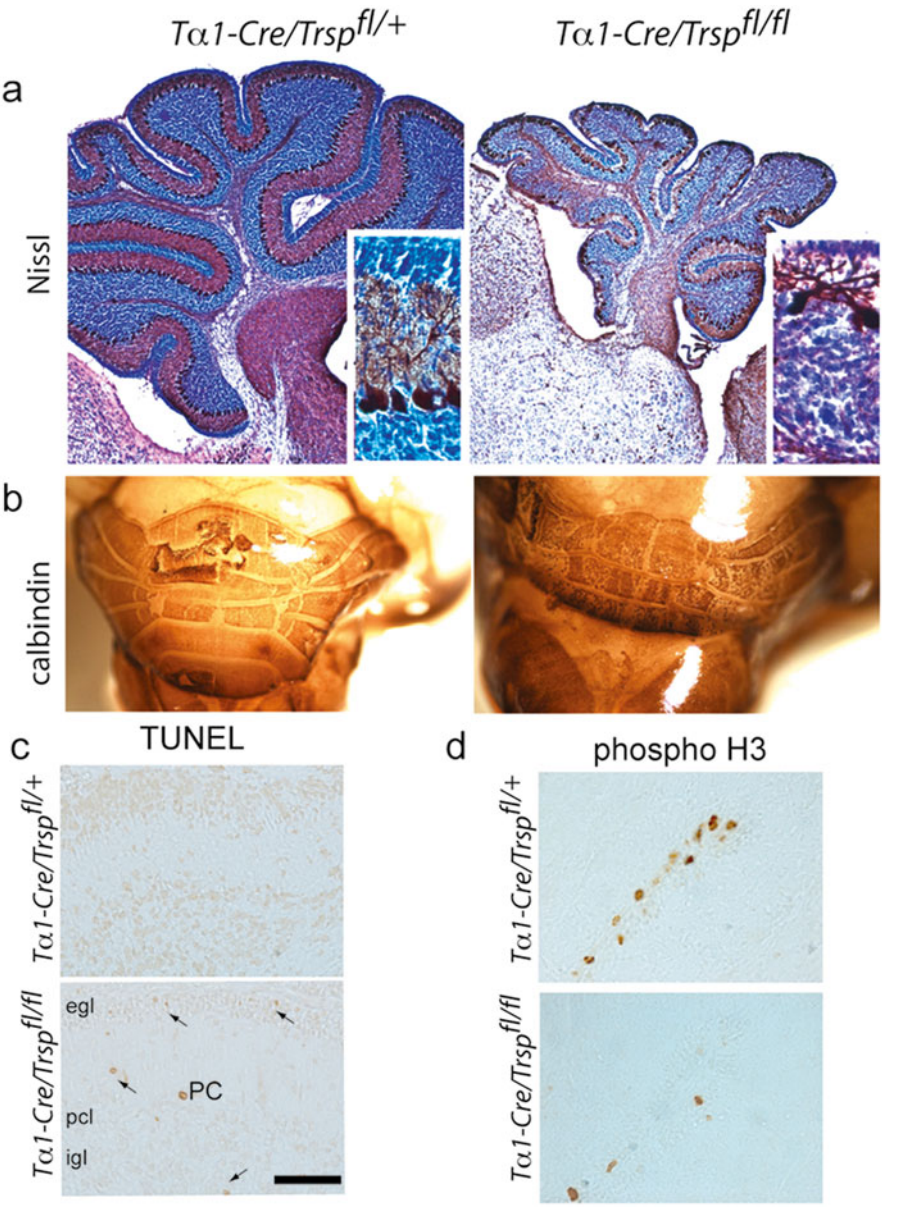

Fig. 36.2 Cerebellar hypoplasia and atrophy in mice with genetic inactivation of tRNA$^{[Ser]Sec}$ (*Trsp*) in neurons. (**a**) Nissl stain of sagittal sections through the cerebellar vermis on postnatal day (P)12. Calbindin is stained brown by immunohistochemistry. (**b**) Whole mount immunohistochemistry for the Purkinje cell marker calbindin showing the loss of these cells. (**c**) TUNEL staining in the developing cerebellar cortex shows increased apoptotic cell death on P8. Scale bar: 50 μm. (**d**) Reduced cell proliferation in the developing cerebellar cortex shown by staining for phosphorylated histone H3

Trsp-transgenes, either lacking the *activation element* (Δ*AE*) in the promoter or carrying the A37G mutation (see Chap. 46). Brain selenoprotein expression is significantly reduced and the mice show signs of progressive neuronal loss, including a significant reduction of cortical PV-interneurons.

36.2.1.2 Secis-Binding Protein 2

Secis-binding protein 2 (*Secisbp2*) inactivation in mice supported its role in selenoprotein expression in hepatocytes [37]. Selenoprotein levels were reduced in the livers of *Alb-Cre*; *Secisbp2*$^{fl/fl}$ mice. However, part of the effect may be explained by significant reduction in the abundance of selenoprotein mRNA levels. It even appeared as if some selenoproteins allowed a certain low level of Sec-incorporation in the absence of *Secisbp2*. Neuron-specific inactivation of *Secisbp2* in *CamK-Cre*; *Secisbp2*$^{fl/fl}$ mice also reduced selenoprotein expression in the brain, but less completely than in hepatocytes [38]. In short, these mice represent a model of reduced selenoprotein expression in the brain. There is clearly more selenoprotein expression than in tRNA$^{[Ser]Sec}$-knockout mice, but less than in *Trsp*$^{\Delta AE}$ mice. Accordingly, mutant mice were euthanized around weaning. Neuron-specific *Secisbp2*-deficient mice also appear hyperexcitable and have reduced numbers of PV-internenurons in cortex and hippocampus. In situ hybridization against the GABAergic neuron marker *Gad67* supported the absence of interneurons, and in addition to their failure to express the subtype marker PV. Interestingly, these mice displayed a movement phenotype which was not related to the cerebellum, because the *CamK-Cre* mouse spares the cerebellum. Rather, the numbers of PV+ interneurons, as well as the number of cholinergic interneurons, were reduced in the striatum of these mice [38]. Thus, striatal interneurons also depend on selenoproteins and their reduction leads to a movement phenotype characteristic of *Sepp1*$^{-/-}$ mice (Fig. 36.3).

36.2.1.3 Selencysteine Lyase

Selenocysteine lyase (*Scly*) inactivation does not disrupt selenoprotein expression in the brain [39], but combination with *Sepp1*$^{-/-}$ exacerbates the neurological phenotype [17].

36.2.2 Transgenic Mouse Models with Specific Selenoprotein Deficiencies

36.2.2.1 Glutatione Peroxidase 4

The *Gpx4* gene has been inactivated in several experimental models across various laboratories [32, 40, 41]. Inactivation of *Gpx4* is embryonic lethal and leads to cell death in several cell types, including embryonic fibroblasts [32]. We have studied

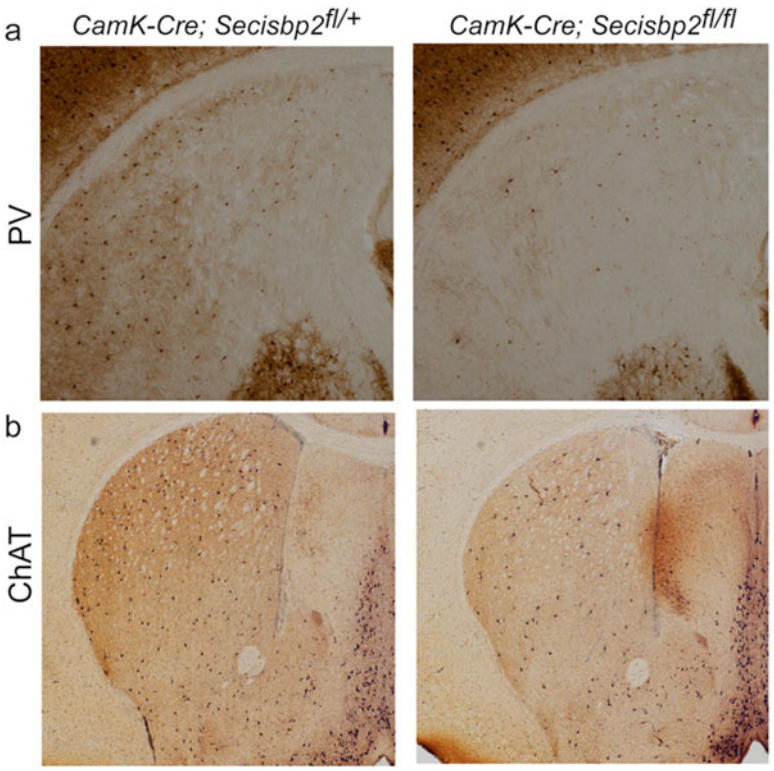

Fig. 36.3 Striatal interneuron loss in mice lacking neuronal Secisbp2 (*CamK-Cre*; *Secisbp2*$^{fl/fl}$). (**a**) Immunohistochemical staining for parvalbumin (PV). (**b**) Immunohistochemical staining for choline acetyltransferase (ChAT)

CamK-Cre; *Gpx4*$^{fl/fl}$ mice and found massive neurodegeneration before weaning [32]. The mice displayed a somewhat milder phenotype than *CamK-Cre*; *Trsp*$^{fl/fl}$ mice, suggesting early on that Gpx4 is one, but not the only, essential selenoprotein for neurons [30]. *CamK-Cre*; *Gpx4*$^{fl/fl}$ are hyperexcitable, similar to *Trsp*-knockouts, but appear to better keep their balance [32]. In culture, *Gpx4*-mutant neurons progressively degenerate, unless they are rescued with α-tocopherol [32]. An important neuropathological finding is the massive reduction of PV+ interneurons in cortex and hippocampus of *CamK-Cre*; *Gpx4*$^{fl/fl}$ mice, while CR-expressing cells remained normal [30]. Thus, selective loss of PV+ interneurons is a common phenotype in mice with a global reduction of cortical selenoprotein expression (*Sepp1*$^{-/-}$, *Trsp*$^{\Delta AE}$, neuronspecific *Trsp*- or *Secisbp2*-knockout) and neuronspecific *Gpx4*-knockout mice. Inducible systemic inactivation of *Gpx4* was reported to lead to death of the mice with astrogliosis and loss of neurons in the hippocampus [42]. However, fatality in mice may have been caused by massive ferroptosis in kidney as described by another group [43]. The former group subsequently demonstrated progressive paralysis in another model of *Gpx4* inactivation in adult mice [44], which suffered

from spinal motoneuron degeneration. Interestingly, these neurons were cholinergic, which was reminiscent of the degeneration of striatal cholinergic interneurons in *Secisbp2*-knockout mice [38]. Why these authors did not see neuronal loss in the cortex or hippocampus would be interesting to discuss. Unfortunately, these authors did not comment on the discrepancy or acknowledge this earlier work [30, 32]. More details on *Gpx4* are found in Chaps. 18 and 43.

36.2.2.2 Thioredoxin Reductase

Txnrd1 and *2* genetic inactivation is embryonic lethal and perinatal lethal, respectively [45, 46]. Neural-specific inactivation of *Txnrd2* using *nestin-Cre* did not lead to any apparent neurological phenotype [47]. In contrast, neural-specific inactivation of *Txnrd1* using the same *nestin-Cre* caused cerebellar hypoplasia associated with a movement phenotype in mice [47]. The layering of the cerebellar cortex was disrupted, in particular in the more anterior lobules of the vermis, and Purkinje cells appeared scattered within granule cells [47]. Interestingly, when we inactivated *Txnrd1* using the *tubulinα1-Cre* and *CamK-Cre* mice (as used previously to inactivate *Trsp* and *Gpx4*), no neurological or neuroanatomical phenotype was noted, including cerebellar cortex layering and cerebral interneuron expression [47]. However, we observed a compensatory upregulation of cellular Gpx activity in *Txnrd1*-deficient brains [25]. Moreover, in older animals, movement coordination was decreased in the *Txnrd1*-mutants. Finally, loss of rotarod performance was accelerated by feeding a low Se-diet. Thus, Txnrd1 and Txnrd2 are not essential for neurons, albeit Txnrd1 plays some role in radial glia biology during development and may be important for long-term maintenance of neurons. More data on the thioredoxin system can be found in Chap. 16.

36.2.2.3 Deiodinases

Deiodinases, gene symbols *Dio1-3*, catalyze the elimination of iodide from iodothyronines, i.e., thyroid hormones and their metabolites (see Chap. 41). *Dio1*$^{-/-}$ mice do not exhibit any neurological phenotype [48]. Although *Dio2*$^{-/-}$ mice exhibit pituitary resistance to thyroid hormone, cerebellar development is reportedly normal [49]. While it is known that cortical PV+ neuron maturation depends on thyroid hormones [50], it is apparently not changed in *Dio2*-deficient mice. The most striking developmental phenotype of *Dio2*$^{-/-}$ mice is failure of cochlear development and subsequent sensorineural hearing loss [51]. *Dio3*$^{-/-}$ mice also suffer from failed cochlear development [52]. Moreover, retinal photoreceptor development is deranged in *Dio3*$^{-/-}$ mice [53]. Taken together, the auditory and visual systems are obviously affected by *Dio* deficiency, while much less data are available on other neuronal systems.

36.2.2.4 Selenoprotein T

Selenoprotein T (*Selt*) is an essential selenoprotein for neurons [54]. The protein resides in the ER and exhibits a thioredoxin-like structure. Its activity is upregulated in dopaminergic neurons after challenge with the neurotoxin, MPTP [55]. Selt is involved in Ca^{2+}-regulation and induced in developing, regenerating, and endocrine tissues (see also Chap. 23). Interestingly, *Selt*-deficient mice are hyperactive [54].

36.3 Syndromes of Impaired Selenoprotein Expression in Human Patients

Only two early case reports specifically hinted towards a possible link between Se metabolism and intractable childhood epilepsy [6, 7]. The molecular cause was never identified, but the phenotypic similarity between patients [7] and *Sepp1*$^{-/-}$ mice became apparent. Se-responsive epilepsy during childhood or adolescence, brain atrophy, movement disorder with spasticity, low circulating Se levels, low plasma GPX activity, and elevated liver enzymes suggested a common mechanism, but may as well be consistent with a syndrome discovered much later, Progressive Cerebello-Cerebral Atrophy (PCCA).

36.3.1 Progressive Cerebello-Cerebral Atrophy (SEPSECS-Mutations)

Progressive Cerebello-Cerebral Atrophy (PCCA) is a novel syndrome discovered in several non-consanguineous Jewish Sephardic families of Moroccan or Iraqi ancestry [56, 57]. Affected individuals are homozygous or compound heterozygous for missense mutations in the selenocysteine synthase gene (*SEPSECS*) [57]. SEPSECS carrying the Moroccan (p.Ala239Thr) or the Iraqi (p.Tyr334Cys) mutations were incapable of complementing the deletion of bacterial *SelA* in *E. coli* and sustain selenoprotein biosynthesis [57]. Mutations in *SEPSECS* have been recently described in Finnish patients [58]. Two mutations were found in these patients, p.Thr325Ser and p.Tyr429*, and it was shown that the expression of selenoproteins GPX1, GPX4, TXNRD1, and TXNRD2 were reduced in a patient's brain indicative of residual SEPSECS activity [58]. The p.Tyr429* variant had no detectable activity. It is noteworthy that the English mutation (p.Tyr334His) affected the same amino acid as the Iraqi mutation [59]. This amino acid is located near the pyridoxalphosphate binding site ([60] and Chap. 9). Very recently, *SEPSECS* mutations have been reported in patients of Japanese origin, carrying p.Asn119Ser in two patients combined with p.Arg26Profs*42 or p.Arg156Gln mutations [61]. The phenotypes of these patients point to a milder course of the disease than in previously known cases.

The SEPSECS syndrome involves profound mental retardation, progressive microcephaly, and hypotonia that develops towards severe spasticity. Myoclonic or generalized tonic-clonic seizures are often observed and cerebral and cerebellar atrophy involving grey and white matter. Axonopathy, astrogliosis, neuropathy, and pain have also been reported. The English patient was diagnosed with ponto-cerebellar hypoplasia type 2D and optic nerve atrophy, which was not mentioned in the Iraqi patients [59]. The milder PCCA cases recently described by a Japanese group are characterized by late disease onset, absence of epilepsy, and the ability to walk, albeit with a wide gait [61]. Yet both patients developed spasticity. In one patient, cerebellar atrophy was discovered at the age of 9 years, in the other after graduation from a special support school. This report shows that a wide phenotypic continuum exists in PCCA and it will be interesting to see whether the phenotype correlates with the residual activity of SEPSECS.

In the Finnish patients, mitochondrial disease was suspected because of elevated lactate [58]. The English patient also showed elevated lactate [59]. The reason for mitochondrial disease in these patients is unclear, but it is known that TXNRD2 and GPX4 are present in mitochondria.

36.3.2 Spondylometaphyseal Chondrodysplasia of the Sedaghatian Type (GPX4-Mutations)

In 1980, Sedaghatian desribed a new autosomal recessive disorder which was characterized by skeletal abnormalities and perinatal death due to cardio-respiratory insufficiency [62]. Later reports confirmed the new syndrome and death within days of birth, but also pointed to cerebellar hypoplasia, reduced size of the corpus callosum, simplyfied gyral pattern, and enlarged lateral ventricles [63]. Recently, the genetic cause was uncovered as mutations in the *GPX4* gene [64]. Myoclonic seizures developed in patients, who became older because they were artificially ventilated. While Sedaghatian initially observed biochemical evidence of decreased Ca^{2+} and increased phosphate in plasma, later reports did not mention markers of kidney function. Recently, it was shown that induction of *Gpx4* inactivation in adult mice led to kidney failure [43].

36.3.3 Deficiencies in Thioredoxin Reductases

Given the embryonic failure of mice deficient in *Txnrd2* [45], it was a surprising finding that humans carrying the homozygous nonsense mutation, p.Tyr447*, also have familial glucocorticoid deficiency [65]. Six patients ranging in age from 2 to 33 years were reported to have low plasma cortisol and accordingly elevated ACTH levels. Some, but not all, of the homozygous carriers showed hyperpigmentation,

because melanocyte-stimulating hormones are the product of the same pituitary hormonal precursor as ACTH. It is remarkable that interference with the mitochondrial *TXNRD2* gene produces a surprisingly mild phenotype in humans, while a homozygous stop mutation, p.Trp24*, in *TXN2* leads to infantile-onset neurodegeneration and cerebellar atrophy [66].

Inactivating mutations in *Txnrd1* are embryonic lethal in mice [46]. Nervous system-specific inactivation of *Txnrd1* leads to cerebellar hypoplasia, while neuron-specific *Txnrd1* deficiency resulted in increased Gpx activity in the brain, but did not disrupt cerebellar development [47]. We have found mutations in *TXNRD1* in patients with epilepsy (unpublished).

36.3.4 SECISBP2-Syndrome

Mutations in *SECISBP2* were initially identified in patients with a rare form of resistance to thyroid hormone ([67] and Chap. 44). Patients showed delayed growth and retarded bone age likely associated with reduced expression of deiodinases. Accordingly, patients exhibit high T4 levels, low/normal T3, slightly elevated TSH and clearly elevated rT3 (consistent with a lack of DIO1 activity). Accordingly, DIO2 actvity was low in patient-derived fibroblasts. Plasma GPX activity and SEPP1 were low indicative of a general defect of selenoprotein biosynthesis. These patients carry the homozygous p.Arg540Gln missense mutation [67]. More patients have been subsequently identified, wherein some also manifest neurological and muscular impairments [68–71]. One girl was described with mental retardation, sensorineural hearing loss, and waddling gait [69]. These phenotypes are consistent with cochlear DIO2-deficiency, selenoprotein-deficiency in the brain, and muscular dystrophy caused by lack of SEPN1 (Chap. 40). Two other patients with even lower SECISBP2 bioactivity also exhibited bilateral sensorineural hearing loss and undetectable plasma SEPP1 [68], as well as additional phenotypes (see Chap. 44). Overt neurodegeneration/brain atrophy, epilepsy, or spasticity were not reported in *SECISBP2*-syndrome suggesting that residual selenoprotein expression is higher than in PCCA.

36.3.5 tRNA$^{[Ser]Sec}$ (TRNAU) Mutation in Humans

Very recently, a patient was reported who clinically resembled patients with *SECISBP2* mutations, but carried a single mutation in *TRNAU* [72]. Plasma T4 was high, T3 normal, and rT3 elevated, plasma selenoproteins GPX3 and SEPP1 were reduced. Cells from the patient showed unaltered expression of selenoproteins GPX4 and TXNRD1, while so-called stress-related selenoproteins, SEPW and GPX1, were reduced. The homozygous C65G mutation in the tRNA$^{[Ser]Sec}$ apparently reduces its 2′-O methylation at ribose 34, a modification needed for efficient expression of stress-related selenoproteins, while housekeeping selenoproteins like

GPX4 and TXNRD1 were not affected (see Chap. 1). This is consistent with the patient not exhibiting neurological phenotypes and the C65G allele was classified as a hypomorphic allele.

36.4 Concluding Remarks

At the very start of my career, as a junior group leader, a very distinguished member of my faculty quipped that my interest in selenoproteins in the brain was "mouseology", because no diseases existed in humans that affected selenoprotein expression in the brain. This situation has changed dramatically. Mutations in *GPX4*, *TXNRD1*, *TXNRD2*, *SECISBP2*, *SEPSECS*, and *TRNAU* have been found to affect brain selenoprotein expression, sometimes with devastating consequences. From my perspective, human genetics and stringent application of mouse genetics have come together to delineate the significance of selenoprotein expression in the brain. Although we do not yet know all the mechanisms, it is fair to say that selenoproteins are essential for brain development and function. Lack of specific selenoproteins or their impaired/reduced biosynthesis lead to neurodegeneration. It is safe to assume in the age of whole exome sequencing that many more rare diseases caused by selenoprotein deficiency will be discovered in the near future.

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Chapter 37
Interplay of Selenoproteins and Different Antioxidant Systems in Various Cancers

Petra A. Tsuji, Bradley A. Carlson, Byeong Jae Lee, Vadim N. Gladyshev, and Dolph L. Hatfield

Abstract Malignant tumors are known to require robust antioxidant systems to sustain their rapidly dividing cells and protect them from oxidative damage. The dietary trace mineral selenium, through its incorporation into selenoproteins such as thioredoxin reductase 1 (TXNRD1), glutathione peroxidase (GPX) 2 and the 15 kDa selenoprotein (SEP15), has been shown to play important roles in redox-regulation. Given that the functions of these selenoenzymes protect both normal and malignant cells from oxidative stress, these very same redox-regulatory processes are thought to result in both anti- and pro-tumorigenic effects at a tissue-specific and cellular level; thus, these selenoproteins are often referred to as having a "Dr. Jekyll and Mr. Hyde personality". Herein, we summarize the main findings with emphasis on TXNRD1 and SEP15, and their roles in the regulation of specific studies of lung, liver and colon cancers to illustrate the differences in the antioxidants involved, and the complexities of their interplay with other antioxidants or antioxidant systems. It should be noted that it remains to be established if any of the observed anti- and pro-tumorigenic effects of TXNRD1 and SEP15 are possibly tumor stage or grade-dependent.

Keywords 15 kDa selenoprotein • Carcinogenesis • Glutathione system • Glutathione peroxidase 2 • Liver cancer • Lung cancer • Oxidative stress • ROS • Selenoprotein interplay in cancer • Thioredoxin reductase 1 • Thioredoxin system

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37.1 Introduction

Selenium (Se) has been known for many years to serve as a chemopreventive agent that suppresses various cancers, largely from epidemiological and animal studies [1]. However, more recently, this element has also been shown to play major roles in promoting cancer in the form of selenoproteins ([2–7] and Chaps. 16, 19 and 38). The fact that Se was found to enhance the cancer process made perfect sense, as this element is one of nature's most potent antioxidants, and cancer cells are known to suffer from oxidative stress.

Numerous studies have shown that Se, in the form of thioredoxin reductase 1 (TXNRD1), is enriched in many cancer cells and tumors, and the inhibition of TXNRD1 might provide an avenue for therapy (see [8–14] and Chap. 16). These studies demonstrated that this selenoenzyme plays a role in driving or sustaining cancers. The molecular biology of Se's role in cancer promotion became more clearly defined, in part, when three selenoproteins, TXNRD1, the 15 kDa selenoprotein (SEP15) and glutathione peroxidase (GPX) 2, were shown to exhibit a Dr. Jekyll and Mr. Hyde personality in preventing and promoting malignancy (reviewed in [7, 15–17] and Chaps. 16, 19 and 38). All three of these selenoenzymes appear to take on an anti- or pro-carcinogenic identity depending on cancer type and stage of the cancer.

It should also be noted that GPX4 has, more recently, been shown to be a major player in cancer through the recognition of its role in a newly discovered phenomenon, designated ferroptosis (Chap. 43 and recent review [18]). Ferroptosis is an iron-dependent form of non-apoptotic cell death and GPX4 has been shown to be an essential regulator of ferroptotic cancer cell death.

In normal cells, a complex interplay exists among antioxidant selenoproteins, and/or between antioxidant selenoproteins and other antioxidants in combating reactive oxygen species (ROS) by maintaining a relatively stable equilibrium. Similarly, such complex interactions also exist in cancer cells, but usually at much enhanced levels that differ vastly from those of the corresponding normal cells from which they originated. These complex relationships also differ substantially among different tissues and cancer types.

Liver and lung tissues, and the different cancers that arise from them provide specimens for comparing how they utilize different antioxidants to maintain redox homeostasis in normal tissue, and how the antioxidants change in combating the enhanced ROS in malignancy. These normal and neoplastic liver and lung cells are also discussed herein. In addition, the interplay between two different selenoprotein antioxidants in colon cancer cells, wherein their individual loss reversed the cancer phenotype, but their collective loss restores cancer properties, is also discussed. Initially, however, elevated ROS levels, which are one of the hallmarks of cancer cells [19], and the manner in which cancer cells cope with oxidative stress, permitting them to grow at accelerated rates outdistancing neighboring, normal cells, is addressed. The underlying metabolic reasons governing how these selenoproteins maintain redox homeostasis to keep cells healthy, and how they are enriched or reduced in malignancy have also been described elsewhere in this book (Chaps. 16, 19 and 38).

37.2 ROS in Cancer Progression and Regression

Much attention has been dedicated to the role of ROS in cancer cells, since enhanced redox signaling and oxidant stress have been shown by many investigators to initiate and sustain cancers and, therefore, are considered prime targets in cancer therapy (reviewed in [11, 20–26]). The two major antioxidant systems in mammalian cells are the thioredoxin (TXN) and glutathione (GSH) systems, which numerous researchers have focused on, either individually [11, 27, 28] or collectively [23, 29–31], as constituents to encumber or reverse the malignancy process. One of the principal problems with down-regulating any antioxidant enzyme, or enzymes, or a system, is that the loss of expression of one or more of these components may induce expression or activity of other antioxidants, which can readily combat the enhanced oxidative stress generated by the malignancy, permitting the cancer cell to thrive (see below).

Other studies, however, have shown that ROS can, instead of initiating and promoting cancer, limit tumor growth and metastasis, illustrating further the complexity of ROS and oxidative stress in these processes. Harris et al. [29] demonstrated that the antioxidant GSH and TXN pathways play different roles by synergizing their efforts. The GSH pathway initiates the malignancy, and then the TXN pathway drives cancer progression. If the GSH pathway is inhibited prior to cancer initiation, the malignancy process can be retarded. However, once the cancer is initiated, its progression is then supported by the TXN pathway and inhibiting the GSH pathway no longer will retard tumorigenesis. Interestingly, inhibition of both pathways resulted in "a synergistic cancer cell death in vitro and in vivo" [29].

The requirement of inhibiting both pathways in retarding tumor growth in TXNRD1-deficient, tumor bearing mice was first demonstrated by Conrad and collaborators [30]. The varying roles of ROS and antioxidants in cancer initiation, progression and/or regression, illustrating the complexity of ROS and antioxidants in these processes, were detailed recently [31, 32]. A recent study that does not involve selenoproteins as antioxidants *per se*, should be mentioned as it further emphasizes the complexity of ROS in cancer ([33] and see review [34]). This study surprisingly found that ROS can retard metastasis in melanoma tumors, while only those malignant melanoma cells with enhanced antioxidant proficiency can actually accomplish metastasis.

37.3 Selenoprotein Roles in Normal and Malignant Lung and Liver Tissues

Lung and liver are two diverse tissues that rely on very different antioxidant systems to maintain redox stability. The corresponding cancers arising in both tissues utilize very different antioxidant systems to drive the respective malignancy. We initially examine the antioxidants primarily involving cancer cells and tumors in mice (see Sect. 37.3.1) and then discuss antioxidants primarily involving cancer cells and tumors in humans (see Sect. 37.3.2).

37.3.1 Selenoprotein Roles in Mouse Normal and Malignant Tissues

Selenoproteins participate in different roles in diverse tissues in promoting malignancy. For example, TXNRD1 is known to maintain redox homeostasis in normal hepatocytes, protecting them from oxidative damage and disease [35]. The loss of this selenoenzyme in hepatocytes, however, was found to greatly increase tumor incidence in liver of mice exposed to the liver carcinogen, diethylnitrosamine (DEN) [36]. The tumor increase appeared to be due to activation of NFE2L2, which in turn enhanced the expression of GPX2 and enzymes in the GSH pathway. These enriched enzymes apparently were responsible for providing the oxidative prowess to combat the enhanced ROS in driving the malignancy.

Other studies have examined the role of Se and selenoproteins in mouse hepatocarcinogenesis with varying genetic backgrounds. Mice encoding the hepatocarcinogenic driver genes, TGFα/c-Myc, were treated with DEN and placed on Se-deficient diets that were, or were not, supplemented with different levels of Se [37]. Interestingly, mice maintained on Se-deficient or highly supplemented Se (2.25 ppm Se) diets suppressed hepatocarcinogenesis compared to mice maintained on intermediate levels. The expression of most selenoproteins correlated with tumor formation in mice on the diets containing intermediate (adequate) levels, while mice on deficient and highly supplemented diets induced expression of detoxifying genes, inhibited cell proliferation and exhibited increased apoptosis [37].

An additional study exposed mice to DEN, wherein the mice encoded a mutant $tRNA^{[Ser]Sec}$ transgene, designated $Trsp^{tA37G}$, which produced reduced levels of non-essential, stress-related selenoproteins [38]. Tumor incidence increased in $Trsp^{tA37G}$ mice fed adequate levels of Se, whereas control, wild type mice fed Se-deficient or highly enriched Se diets were protected from tumor formation [38].

Overall, the above studies on tumorigenesis in mouse hepatocytes suggest a complex role of Se in chemically-induced or genetically driven hepatocarcinogenesis, which involve the interaction of selenoproteins, selenocompounds and chemical carcinogens. Furthermore, changes in dietary Se levels and/or selenoprotein expression in these mice may suppress or promote tumor formation, and the cell type and murine genotype also play roles in governing the malignancy process. TXNRD1 appears to play more of a protective role in hepatocytes guarding against tumorigenicity by maintaining redox homeostasis rather than a cancer promoting role. This may be due to TXNRD1-deficiency in hepatocytes being compensated for by induction of other antioxidant enzymes that can then drive tumor formation [36].

Several studies have suggested that tumor formation in mouse lung tissue and cells is highly dependent on TXNRD1 and the loss of this selenoenzyme is anti-tumorigenic. An earlier study clearly demonstrated that tumor formation in mouse lung cancer (LLC1) cells was virtually completely dependent on TXNRD1 [13]. Several of the cancer hallmarks [19] in TXNRD1-sufficient, LLC1 cells were reversed following targeted down-regulation of *Txnrd1*. Furthermore, tumorigenicity of TXNRD1-deficient LLC1 cells injected into the flanks of mice was dramatically

reduced compared to the corresponding TXNRD1-sufficient cells. The slower growing tumors arising from the TXNRD1-deficient cells were subsequently found to have lost the *Txnrd1* targeting vector and had re-expressed this selenoenzyme, demonstrating unequivocally that lung tumorigenesis in mice, at least regarding this cell line, depended on expression of TXNRD1 [13].

37.3.2 Selenoprotein Roles in Human Normal and Malignant Lung and Liver Tissues

Lung cancer is the number one cause of cancer deaths throughout the world and there are three types, non-small cell, small or oat cell, and metastatic. Each of these lung cancer types includes various forms which are considered to be different cancers. Adenocarcinoma (LAC) is the most common of the non-small cell forms representing about 35 % of all types. There are also several types of liver cancers, which comprise the sixth most common cancer and second leading cause of cancer deaths globally. Hepatocellular carcinoma (HCC) is the most prevalent form, representing about 75 % of all known human liver cancers. This chapter primarily examines LAC and HCC.

In a recent study, the development of lung tumorigenesis incidence in mice carrying a lung cancer gene was investigated. However, the resulting tumor size was increased considerably in those mice maintained on diets supplemented with the known antioxidants, N-acetylcysteine or vitamin E, compared to the littermate controls maintained on normal diets [39]. Low Se levels have also been linked to lung cancer development [40, 41], but questions and concerns have been raised whether Se intervention through dietary supplementation should be used as a strategy in lung cancer therapy [40]. It should also be noted that recurrence of non-small cell lung cancer in patients administered a Se supplement or a placebo manifested virtually no differences in cancer recurrence between the two groups that resulted in the trial being stopped early [42]. However, this trial was difficult to evaluate in light of beneficial or detrimental effects of Se in lung cancer due to its early termination [42].

The redox regulatory systems in HCC and LAC has been examined by comparing each respective tumor to its surrounding normal tissue to elucidate the changes that occurred to enrich the antioxidant capacity of the tumor to meet its needs for sustaining the cancer phenotype [43]. Very pronounced differences were observed in the TXN and GSH systems: TXNRD1 levels were elevated in both tumor types, while TXN levels were only slightly increased in HCC, but highly increased in LAC. Peroxiredoxin 1 (PRDX1), an enzyme within the TXN system, was upregulated dramatically in LAC compared to its surrounding normal tissue, and downregulated in HCC. Major differences were also observed in the GSH system between the two tumors and their respective normal tissues. These variations in antioxidants are summarized in Table 37.1. Interestingly, the role of PRDX1 is to protect against oxidative stress by hydroperoxides, such as hydrogen peroxide and

Table 37.1 Summary of changes in levels of redox components examined in tumor and normal surrounding tissues[a]

Antioxidant	Lung[b]	Liver[b]
TXNRD1	↑[c]	↑[c]
TXN	↑[d]	NS[d]
PRDX1	↑	↓
GPX1	NS	↓
GPX2	ND	NS
GPX4	NS	↑
GSR	NS	↓
GCLC	↓	↓
GSS	NS	↓
GLRX	NS	↓
GGT1	↓	↑
GSTA1	ND	↓
SOD1	NS	↓
CAT	NS	↓
G6PD	↑	↑
Ascorbic acid	NS	NS
Uric acid	NS	↓

[a]Data and table adapted from [43]

[b]↑ or ↓ indicate significant increase (↑) or decrease (↓); NS = not significant; ND = not detected

[c]Specific activity of TXNRD was approximately 1.5 times higher in normal lung than liver tissues, but TXNRD1 was reduced approximately by about half in lung tumor compared to liver tumor

[d]The level of TXN was enriched approximately six times in lung tumor compared to normal tissue by western blotting, while liver tumor was only slightly enriched in liver tumor compared to normal tissue

peroxynitrite, and is itself reduced by TXN [27]. This observation supports the proposal that enriched PRDX1 occurring in lung adenocarcinoma is reduced and maintained in the active state by increased TXN levels [43]. Overall, the data suggested that HCC has a much greater dependency on the TXN system and/or the GSH system to drive the malignancy, while LAC appears to depend largely on the TXN system to drive its malignancy. These findings strongly suggest that different therapeutic targeting strategies would be required to slow or reverse HCC or LAC (see also Concluding Remarks).

Upon targeted downregulation, TXNRD1-deficient human lung cancer A549 cells did not manifest reversal of their cancer properties to the same extent that LLC1 mouse cells did; however, the possibility that A549 cells may have retained sufficient TXNRD1 activity following its knockdown to drive the malignancy was considered [44]. Perhaps another possibility should be considered, in that TXNRD1-deficient A549 cells did not manifest reversal of their cancer properties like LLC1 cells because these two cancer lines are likely quite different from each other and may depend overall on different antioxidants to drive the cancer.

37.4 The Interplay Between TXNRD1 and SEP15 in Colon Cancer

Targeted removal of *Sep15* or *Txnrd1* in mouse colon cancer CT26 cells has been shown to result in reversal of several of the cancer properties such as anchorage-dependent and anchorage-independent growth and impaired ability to metastasize ([45] and see Chap. 19). It was anticipated that the simultaneous down-regulation of both these selenoproteins would result in cells more likely exhibiting a phenotype typically associated with normal (non-neoplastic) cells, since such cells were expected to lack the antioxidant ability to combat increased levels of ROS generated in more rapidly growing cells. Remarkably, the anti-cancer effects found in targeting SEP15 or TXNRD1 loss were reversed and the malignancy phenotype recovered when both genes were simultaneously down-regulated [45]. Various other genes were up- or down-regulated differently in SEP15/TXNRD1-deficient cells compared to their individually loss in CT26 cells, which underscored the complexity of these two selenoproteins in their regulatory roles in colon cancer. For example, interferon-γ-regulated guanylate-binding proteins, which are a family of GTPases that are important in providing protective immunity against viral and microbial pathogens, were highly expressed in SEP15-deficient and poorly expressed in TXNRD1-deficient cells. Members of the Wnt/β-catenin signaling pathway were enriched in TXNRD1 and SEP15-deficient CT26 cells. The data suggest that that these two selenoproteins are involved in quite different regulatory pathways in colon cancer cells, but ones that counter each other's regulatory pathways in colon cancer cells; and furthermore, provide new insights into the complexities of how different selenoproteins may interact when they both are under-expressed.

37.5 Concluding Remarks

In this chapter, we have focused largely on specific cancer studies in mice and humans involving selenoproteins to illustrate the differences in the antioxidants involved in different cancers and tumors, and the complexities of their interplay with other antioxidants or systems. There is, of course, a wealth of information on these topics in many other studies far too extensive to cover in a chapter of this size. For reviews on many other such studies, the reader is referred to several excellent reviews [11, 20–24, 26, 28].

Major efforts have been directed in understanding the underlying causes of enhanced antioxidant and/or ROS levels in cancer cells as a means of providing insights into how to slow or impede the cancer process. The reasoning for employing these approaches is to find avenues of inhibiting specific cancer cells and/or tumor growths. The fact that removal of TXNRD1 in hepatocytes enhances the expression of other antioxidants or antioxidant systems, which then drive the malignancy,

demonstrated that focusing on a single antioxidant or even antioxidant system to retard the malignancy will likely not be successful, as has been shown also for other cancers [29, 30]. Whether inhibition of both the GSH and TXN systems would impede a specific liver cancer type, e.g., HHC, remains to be determined. Focusing on reducing or enhancing ROS levels to impede liver cancer may be an alternative and fruitful avenue to pursue for therapy.

Lung cancer, e.g., LAC, appears to be far more dependent on TXNRD1 and the TXN system, as discussed above. Thus, inhibiting lung cancer (e.g., LAC) as a therapy by attacking TXNRD1 and/or the TXN system would seem a much better approach to pursue than with liver cancer (e.g., HHC).

Since the numerous types of human lung and liver cancers must all be considered as individual malignancies with different ROS and antioxidants driving them, specific therapies must be devised for each, and likely for each of the different stages during cancer development. Albeit the tumor study in mice which suggested that the malignancy was initiated by the GSH system and then sustained by TXNRD1 [29] demonstrated the interplay and complexities between the different antioxidants involved, the intricacies are likely far more multifaceted in many other cancers. There is still vast amount of research to be carried out to unravel the underlying molecular mechanisms and their many interactions in finding specific avenues in cancer therapy.

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Chapter 38
Glutathione Peroxidase 2, a Selenoprotein Exhibiting a Dual Personality in Preventing and Promoting Cancer

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Abstract GPx2 is mainly expressed in the intestine but also up-regulated in several cancer types. Being a selenoprotein and a target of the transcription factor Nrf2, it was first proposed to protect from tumor development. However, it turned out that the picture is much more complex. GPx2 is not only regulated by Nrf2, but also by β-catenin and the Wnt pathway, ΔNp63, and NKX3.1, which are pathways involved in the regulation of proliferative and survival processes. Accordingly, GPx2 was also found to enhance proliferation and to inhibit apoptosis. These are important functions required to maintain homeostasis in the healthy intestine. However, acting in cancer cells, they will promote the progression of the disease, as confirmed in several models of cancer and by a decrease of tumor development in *Gpx2* knockout mice. In contrast, if carcinogenesis is driven by inflammation, GPx2 rather acts protective. The recent identification of STAT3 as an additional transcription factor inducing GPx2 might explain the up-regulation of GPx2 in inflammation and point to a role of GPx2 in tissue regeneration and wound healing. Thus, in accordance with the different pathways regulating its expression, GPx2 can act anti- and pro-carcinogenic depending on the tumor model and stage of cancer. Whereas it can inhibit initiation, it supports tumor growth, if a cancer cell has been established.

Keywords Apoptosis • Cancer • Dual role • GPx2 • Inflammation • NKX3.1 • Np63 • Nrf2 • Proliferation • STAT • Transcriptional regulation • Wnt

38.1 Introduction

Eight glutathione peroxidases (GPx1-8) exist in mammals, of which five are selenoproteins in humans (GPx1-4 and 6) [1]. Although they are generally characterized as ‘antioxidant’ enzymes due to their capacity to reduce hydroperoxides, they appear to act in a specific context and also might have additional functions (see Chaps. 17 and 43).

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Selenium has been known for many years to have anti-carcinogenic functions. However, not all animal studies and clinical trials could confirm this function and not all GPxs contribute to cancer prevention [2]. Especially GPx2 was a surprise when initially found preventing, but then was observed to support cancer development depending on the model used. These paradoxical results point to a complex dual role of GPx2, which we try to explain by assessing its expression, localization, and transcriptional regulation, as well as by results obtained in *Gpx2* knockout (KO) mice or in *Gpx2* knockdown (kd) or overexpressing cells.

38.2 Structure and Substrate Specificity

GPx2 is a homotetramer, as are GPx1, 3, 5 and 6, and like all GPxs, it contains a conserved tetrad consisting of selenocysteine, Gln, Trp and Asn [3]. Whereas the tetrad has been experimentally verified as the catalytic center of GPx1, 3 and 4, this verification is lacking in GPx2 because the enzyme has never been isolated. Thus, substrate specificity or kinetics are not known. Hints of specificity were demonstrated indirectly: (i) kd of GPx2 in HT-29 cells reduced total GPx activity estimated with H_2O_2 as substrate by about 25 % [4]; and (ii), in accordance with its high ranking in the hierarchy of selenoproteins [5], re-supplementation of Se-deficient CaCo2 cells with selenium led to a re-expression of GPx2 first, which was later followed by GPx1. Re-appearance of GPx2 was associated with a particularly high total GPx activity measured with 13-hydroperoxy octadecadienoic acid (13-HPODE), whereas GPx activity measured with H_2O_2 increased with GPx1 re-appearance [6]. Thus, GPx2 reacts with H_2O_2 in principle, but obviously has a higher specificity for organic hydroperoxides.

38.3 Localization

GPx2 is expressed in epithelial cells lining the whole gastrointestinal tract, the breast, bladder, and lung [7–10]. Because GPx2 was first identified in the gastrointestinal system, it was suggested to act as a barrier against hydroperoxides produced during food digestion [8]. Levels of GPx2 are highest at crypt bases and gradually decline to the top of crypts in the colon or to the villi in the small intestine [9]. The location correlates with an active Wnt pathway, which regulates proliferation and differentiation of stem cells in the intestinal epithelium [11]. GPx2 is up-regulated in Barrett's esophagus, squamous cell carcinoma, lung adenocarcinomas, colorectal cancer, rat and human breast cancer, hepatocellular carcinoma, and prostate cancer (reviewed in [12]). High GPx2 expression has also been observed as part of a stem cell signature in human induced pluripotent stem cells (iPSCs) [13] and in mouse embryonic stem cells (ESC) [14]. Thus, the location of GPx2 appears to be connected to stem cell-like cells. Furthermore, GPx2 has recently been shown to be spatiotemporally expressed in mouse embryos, which indicates a possible role in organogenesis [15].

GPx2 levels are also high in Paneth cells [9]. Paneth cells belong to the secretory lineage, but in contrast to other differentiated intestinal epithelial cells (IEC) reside at the bottom of crypts in the small intestine. There, they are interspaced between intestinal stem cells (ISCs), which ensures a direct contact between both cell types. This is necessary because Paneth cells express Notch ligands on their surfaces, which interact with the respective Notch receptors on the next ISC [16]. In addition, Paneth cells secrete Wnt3 and epidermal growth factor (EGF), which are supposed to activate respective receptors on the surface of ISCs in a paracrine way. By doing so, Paneth cells essentially contribute to the microenvironment (niche) at the crypt base. As part of the innate immune response, Paneth cells also express and secrete enzymes with anti-bacterial activity to counteract the continuous inflammatory challenge by the intestinal microbiota [17]. In patients with Crohn's disease, Paneth cell function is often impaired and, therefore, is supposed to be involved in the etiology of the disease [17]. Interestingly, GPx2 is up-regulated in the intestine of patients with inflammatory bowel disease (IBD) [9]. Thus, its localization points to a role of GPx2 in inflammation, and proliferation and differentiation of cells.

38.4 Transcriptional Regulation

38.4.1 Nrf2

The nuclear factor (erythroid-derived 2)-like 2 (NFE2L2 or Nrf2) was the first transcription factor demonstrated to induce GPx2. The GPx2 promoter contains an Nrf2 responsive element (ARE), which could be activated by sulforaphane [18] and other natural Nrf2 activators [19] as well as the anti-inflammatory cycloprostane 15d-PGJ_2 [20] (Fig. 38.1). The analysis of Nrf2 KO mice revealed that Nrf2 is also essential for basal GPx2 expression in vivo in both lung [21] and colon [20]. An activation of Nrf2 was generally considered as beneficial, because it up-regulates the cellular defense system and supports cell survival [22–24], characteristics also ascribed to

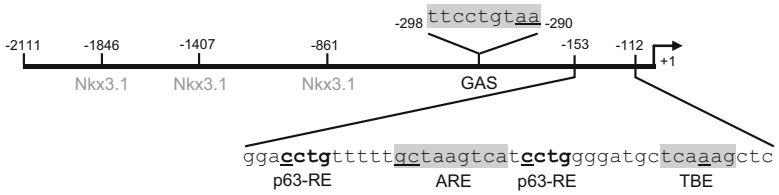

Fig. 38.1 Responsive elements located in the 5′-UTR of the human GPx2 promoter upstream of the ATG start codon. The antioxidant responsive element (ARE) is the binding site for Nrf2, while the TCF responsive element (TBE) is activated by Wnt signaling upon binding of TCF and β-catenin (both in *grey*). The responsive element for p63 and ΔNp63γ consists of two half sites (indicated in *bold*) flanking the ARE site. The interferon-γ-activated site (GAS) is activated by STAT transcription factors. The *underlined* bases were mutated to prove functionality and specificity of the respective binding elements. Putative Nkx3.1 binding sites were identified by in silico analyses using MatInspector. Numbers (−) indicate the up-stream base positions starting from the translational start ATG (+1)

GPx2. Nrf2 supports cell survival by activation and control of the cellular defense systems against oxidative and environmental stresses [24, 25]. Like GPx2, Nrf2 is up-regulated in many types of cancer [26, 27] resulting in the induction of the endogenous antioxidant defense system, inhibition of apoptosis, and the up-regulation of multidrug-resistance-associated proteins making cancer cells resistant to chemotherapy [28]. Consequently, not only healthy but also cancer cells survive (reviewed in [29]). The recently reported novel function of Nrf2 in proliferation of normal and cancer cells and differentiation of stem cells confirms this view [30].

38.4.2 β-Catenin

β-Catenin is the key player of the Wnt pathway which regulates the proliferation and differentiation of ISCs and contributes to a niche that also ensures the self-renewal capacity of the ISC [16]. The co-localization of GPx2 and the Wnt pathway, not only in healthy tissue, but also in dysplastic crypts [31], raised the question whether GPx2 is a Wnt target. This indeed turned out to be true [32, 33]. The GPx2 promoter contains a functional β-catenin/TCF responsive element, which could be stimulated by β-catenin (Fig. 38.1). *Vice versa*, an inducible deletion of β-catenin reduced GPx2 expression in isolated colonic crypt epithelial cells [33]. The findings point to a vital function of GPx2 in regulating the homeostasis of healthy intestinal epithelium. In addition, aberrant activation of the Wnt pathway promotes unlimited proliferation of stem cells resulting in tumor development. Thus, the regulation of GPx2 by the Wnt pathway can explain its up-regulation in tumors and its proliferation-supporting function (see below).

38.4.3 STATs

STATs are *S*ignal *T*ransducers and *A*ctivators of *T*ranscription, which regulate proliferation and differentiation of T-cells as well as of epithelial cells [34, 35]. In intestinal epithelial cells, especially STAT3 activation contributes to mucosal homeostasis and damaged tissue repair [36]. A search for binding sites for inflammation-related transcription factors within the *Gpx2* promoter revealed four putative responsive elements for STATs (so-called interferon-γ-activated sites (GAS)) from which the one next to the transcription start site was functionally active [20] (Fig. 38.1). STAT3 activity can be stimulated by several cytokines including interleukin (IL)-6 and IL-22. Accordingly, the *Gpx2* promoter was activated by both of them [20, 37] and by co-transfection with plasmids for constitutively active STAT3 [20]. *Vice versa*, mutation of the respective STAT binding site abolished activation. IL-22 was recently shown to be the main cytokine activating STAT3 in ISCs as part of the damage response [38]. Accordingly, IL-22 stimulation enhanced endogenous GPx2 expression in CaCo2 cells. During dextran sulfate sodium (DSS)-induced colitis, GPx2 activation in epithelial cells co-localized with high phospho-STAT3 and IL-22 levels [20]. Thus, the efficiency of GPx2 in regulating intestinal inflammation and wound healing might depend on a functional STAT3/IL-22 axis.

38.4.4 ΔNp63

ΔNp63 is the N-terminal splice variant of the p53 homolog p63 lacking the transactivation domain. It is highly expressed in undifferentiated basal epithelia and in tumors including those of bladder, prostate and colon [39, 40]. KO mice show early developmental defects resulting from an impaired capacity of stem and progenitor cells to proliferate and survive [39]. During a search for ΔNp63 targets, *Gpx2* was detected in the list of up-regulated genes and a novel functionally active ΔNp63γ binding site was identified in the *Gpx2* promoter [41] (Fig. 38.1). ΔNp63 inhibits p53-dependent stimulation of apoptosis and, thus, is essential for the survival and immortality of epithelial stem cells. Regulation of *Gpx2* by ΔNp63γ fits with its anti-apoptotic function (see below).

38.4.5 NKX3.1

NKX3.1 is a homeobox protein required for the development and differentiation of prostate epithelial cells [42]. Nkx3.1 mutant mice display a deregulated expression of redox-active enzymes among them a distinct down-regulation of GPx2 [43]. A direct regulation of GPx2 by NKX3.1 was demonstrated by Chip-coupled sequencing [44], and by the identification of a NKX3.1 binding site in the *Gpx2* promoter by a search in the MatInspector Program (Fig. 38.1) [45]. The regulation of GPx2 by NKX3.1 creates a link to its proposed role in differentiation.

Altogether, GPx2 is regulated by transcription factors regulating proliferation and survival of cells. The responsive elements for Nrf2, β-catenin and ΔNp63γ are located in very close proximity (Fig. 38.1) which might indicate that those transcription factors act in concert in regulating GPx2 expression. Until now, this has not been experimentally addressed. The consequences of being regulated by the introduced transcription factors in terms of tumor development are discussed below.

38.5 Functions of GPx2 During Tumor Development

38.5.1 Reduction of Hydroperoxides and DNA Damage

During tumor initiation, cells, most probably stem cells, acquire mutations [46]. These damaged cells have the potential to develop into tumors. One reason for such DNA damages is believed to be oxidative stress. Hydroperoxides derived from digestion of nutrients or produced during cellular metabolism can damage DNA and thereby initiate the progress of carcinogenesis [47]. Besides DNA repair mechanisms, especially death of mutated cells by apoptosis is the most efficient way to inhibit tumor development at this stage.

The increase in GPx2 expression in cancer has been considered beneficial at first glance. GPx2 was proposed to reduce DNA damage by reducing hydroperoxides which recently could be demonstrated in iPSCs [13]. A KO of GPx2 predisposed mice to squamous cell carcinoma (SCC) formation induced by UV light known to increase cellular H_2O_2 levels [48]. The beneficial role proposed for GPx2 was strongly connected to its regulation via Nrf2, which also was believed to protect from tumor initiation just by up-regulating protective and antioxidant enzymes. However, other studies do no support the beneficial effect of GPx2 in cancer prevention. Scavenging of hydroperoxides obviously is not the only function of GPx2 in the context of cancer.

38.5.2 Inhibition of Apoptosis

A constitutive KO of GPx2 (GPx2 KO) in mice results in normal development. Thus, the physiological function of GPx2 might not be essential or might be compensated, e.g., by other GPx isoforms. Surprisingly, GPx1 was highly expressed at crypt bases of GPx2 KO mice, the localization usually taken by GPx2 [49]. Accordingly, total GPx activity in response to H_2O_2 was even higher in *Gpx2* KO than in wild type (WT) mice. The histochemical investigation of the colon of *Gpx2* KO mice revealed a highly increased number of apoptotic cells at crypt bases, especially in mice on a diet moderately deficient in selenium [49]. Thus, GPx2 appears to protect ISCs and transit amplifying cells from apoptotic cell death. Up-regulated GPx1 obviously was not able to replace GPx2 in preventing apoptosis, pointing to a very specific role of GPx2 in the intestinal epithelium.

Blocking apoptosis, might be beneficial for maintaining the homeostasis of the healthy intestine. However, in terms of tumor development, it means that an important anti-cancer mechanism is impaired. In the azoxymethane (AOM) model, a mimic of spontaneous colorectal carcinogenesis, *Gpx2* KO mice indeed developed fewer pre-neoplastic lesions and adenomas than WT mice [31]. This was explained by the higher number of basal and AOM-induced apoptotic cells in colonic crypts of *Gpx2* KO mice. Thus, the efficient apoptotic removal of AOM-initiated cells in *Gpx2* KO mice appeared to prevent tumor formation [31] (Fig. 38.2). Such specific anti-apoptotic functions may be deduced from the transcriptional regulation of GPx2. Both, Nrf2 and STAT3 inhibit apoptosis by up-regulation of anti-apoptotic Bcl2 proteins [50, 51]. The ability of ΔNp63γ to counteract p53, to inhibit apoptosis, and to induce GPx2 led to the conclusion that ΔNp63γ inhibits p53-mediated oxidative-stress-induced apoptosis via up-regulation of GPx2 [41]. The high apoptotic rate in the intestine of *Gpx2* KO mice [49] supports this view.

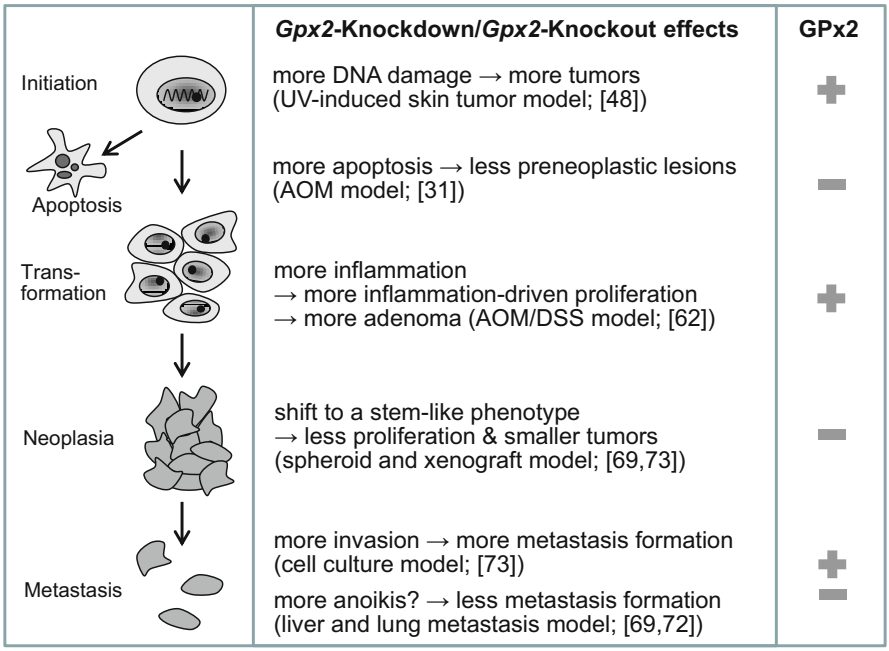

Fig. 38.2 Effects of a *Gpx2* knockdown or knockout on different stages of tumor development. + indicates a rather beneficial, anti-carcinogenic role of GPx2. – indicates an adverse, pro-carcinogenic role of GPx2

38.5.3 Role of GPx2 in Inflammation-Driven Carcinogenesis

Mice with a double knockout for *Gpx2*/*Gpx1* (DKO) develop chronic colitis [52], which could be prevented by one allele of *Gpx2* but not that of *Gpx1* [53]. Colitis was almost completely prevented when also *Nox1* was knocked out, indicating that Nox-1-derived reactive oxygen species, usually superoxide and H_2O_2, caused inflammation [54]. Chronic inflammation substantially contributes to the proliferation of established cancer cells and, thus, is part of a tumor-promoting microenvironment [55]. GPx2 is up-regulated in the colon of patients with intestinal bowel disease (IBD) [9, 56] and in the lungs of mice after induction of allergic airway inflammation [57]. Also, in experimental models of colitis, such as DSS application [20, 58], infection of recombinase-activating gene-2 (Reg2) KO mice with *Helicobacter hepaticus* [59], as well as in mucin 2 (*Muc2*) KO mice [60], GPx2 expression was increased. In the latter model, the IL-22-STAT3 pathway was activated and responsible for maintaining homeostasis under conditions of a compromised mucus layer [61]. This might explain the up-regulation of GPx2 in this model. In mice with acute DSS-induced colitis, epithelial STAT3 was activated and GPx2 was induced up to fourfold [20]. In the regenerative phase, however, GPx2

induction appeared to be independent of STAT3 and was specifically located in regenerative crypts with high proliferation indicated by high ki67 levels. Possibly, GPx2 was induced in ISCs in response to damage and expression was maintained to further enhance proliferation until wound closure is achieved.

In both models, i.e., the AOM/DSS model of inflammation-triggered carcinogenesis and the allergic airway inflammation model, *Gpx2* KO mice developed a significantly more severe inflammation than WT mice [57, 62]. F4/80 (or EGF-like module-containing mucin-like hormone receptor-like 1) is a marker for macrophages and was used to characterize the inflammation status of the colon. Even in unstressed *Gpx2* KO mice, the number of intra-epithelial F4/80 positive cells was enhanced indicating a latent inflammatory state in the absence of GPx2 [31]. Accordingly, *Gpx2* KO mice developed more colonic adenoma than WT mice in the AOM/DSS model [62]. This indicates an anti-inflammatory effect obviously unique for GPx2, since no other selenoprotein was up-regulated in the inflamed colon [20]. Based on these observations together with the results obtained with DKO mice (see above), a major function of GPx2 in the prevention and/or resolution of inflammation can be concluded (Fig. 38.2).

The underlying mechanism is so far mainly explained by the removal of hydroperoxides produced in the inflammatory state to prevent non-specific oxidation and, thus, damage to cells and tissues. However, there is increasing evidence that reactive oxygen species—mainly H_2O_2—are not only harmful, but are also essential for multiple signaling cascades [63]. H_2O_2 and other hydroperoxides can be sensed and transduced by peroxiredoxins [63, 64] and probably also by GPxs. Moreover, GPxs regulate lipoxygenases. GPx4 reacts with 12/15 lipoxygenase products to stimulate AIF-mediated cell death [65]. Lipoxygenases as well as cyclooxygenases (COXs), need a certain peroxide tone to become active. In human colitis patients, GPx2 and COX2 are co-localized in areas where inflammatory cells were accumulated [4] with so far unknown consequences. Interestingly, silencing of *GPX2* in HT29 cells led to a significantly enhanced COX2 expression in both, control and IL-1 stimulated cells, as well as COX2-mediated migration and invasion [4]. Whether GPx2, apart from inhibiting COX2 and prostaglandin E synthase (PGES) expression as well as PGE_2 production [4], senses COX- or LOX-products should be investigated. It is tempting to speculate that it might be one of the so far unidentified selenoproteins involved in the Se-depending switch from PGE_2 to 15d-PGJ_2 production and, thus, from pro- to anti-inflammatory mediators so far only found in macrophages [58, 66].

38.5.4 Role of GPx2 in Neoplasia and Tumor Proliferation

In most tumor types, GPx2 is transiently up-regulated in the early stage of cancer, which had been initially shown in colon cancer [9]. Comparing GPx2 in pre-neoplastic (IEC-1) and neoplastic (SCC-25 and COLO-16) cells with normal (HEK) cells showed that this selenoprotein was highest in pre-neoplastic (40-fold greater than control) and less markedly but still up-regulated in neoplastic cells (tenfold

over control) [67]. In colon cancer patients, three different colon cancer subtypes (CCS) have been identified, CCS 1-3. While CCS1 and CCS2 are well-characterized as chromosomal-instable and microsatellite-instable cancers, CCS3 tumors are poorly differentiated, have a high recurrence rate, and express genes involved in matrix remodeling and epithelial-mesenchymal transition [68]. Based on transcriptomic profiles, GPx2 expression was clearly enhanced in CCS1 tumors in comparison to those in CCS2 and CCS3 tumors [69] (see also Sect. 38.5.5). Also in urothelial carcinoma, GPx2 levels were lower in more aggressive tumors with a high tumor grade than in tumors with a low tumor grade [70].

The putative essential and mainly beneficial functions of GPx2 in the healthy intestine, inhibition of apoptosis and supporting proliferation, may be reversed, if these functions are maintained in established cancer cells (Fig. 38.2). A kd of GPx2 in rat and human mammary carcinoma cell lines inhibited proliferation of these cancer cells [71]. In addition suppression of GPx2 by siRNA in hepatocellular carcinoma cells inhibited cell proliferation and enhanced apoptosis [72]. HT-29 cells encoding a stable shRNA-mediated *GPX2* kd formed substantially fewer colonies than WT cells and developed much smaller tumors in a xenograft model [69, 73]. Similarly, silencing of *Gpx2* in castration-resistant prostate cancer cells resulted in growth inhibition due to a cyclin B1-dependent G2/M arrest and an inhibition of tumor growth, when transplanted into castrated mice [74]. The obvious growth supporting action of GPx2 can easily explain why prostate cancer patients with low GPx2 expression in biopsy specimen had a significantly longer prostate specific antigen-recurrence-free and overall survival time than those with a high GPx2 expression [74].

A *GPX2* kd in HT-29 cells or in cancer cells obtained from tumor specimens of colon cancer patients resulted in a higher hydroperoxide tone and loss of the differentiation potential [69]. This was observed in adherently growing cells as well as in a 3D cell culture, so-called colonospheres. These spheres display cellular heterogeneity and are characterized by proliferating differentiated tumor cells and more stem cell-like, quiescent cells. When injected into immune compromised mice, *GPX2* kd colonospheres form slow-growing, small, and undifferentiated tumors mainly consisting of stem cell-like cells. *Vice versa*, GPx2 over-expression stimulated multi-lineage differentiation, proliferation, and tumor growth [69]. This shows for the first time that GPx2 can stimulate cell differentiation, a function also required for its postulated role in the development of the mucosal epithelium.

38.5.5 Metastasis

The high number of stem cell-like cells in patient-derived colonospheres with *GPX2* kd would imply an increased aggressiveness and capacity to form metastasis. Unexpectedly however, tumor cells with low GPx2 expression completely lack the ability to form metastases in mouse liver [69]. Also, suppression of GPx2 by siRNA in hepatocellular carcinoma cells reduced the number of metastatic nodules in lungs when inoculated into nude mice [72]. The most likely explanation is that *Gpx2* kd cells are more prone to

anoikis after detachment from the tumor or sphere extracellular matrix. This eventually kills the floating tumor cells before they are able to form metastasis, a view supported by an increase of floating melanoma cells after reduction of cellular oxidation state by the glutathione precursor N-acetyl-cysteine (NAC) [75]. This way, GPx2 might support the survival of floating tumor cells resulting in a higher probability for metastasis formation (Fig. 38.2). The main reason for a shorter relapse-free survival time after resection of the tumor is metastasis formation. In line with metastasis facilitated by GPx2 is the observation that a low tumor-resident GPx2 expression was significantly correlated with a longer relapse-free survival time of colon cancer patients with the aggressive subtype CCS3 (see above), most probably by reducing metastasis formation [69].

The GPx2-mediated stimulation of metastasis is in contrast to previous hypotheses only at first glance (reviewed in [2]). In the two studies described above, cancer cells have been directly injected into the blood stream or spleen. Subsequently, the up-stream event of migration and invasion through the basal lamina to be in contact with the blood stream cannot be covered. Previous conclusions about an inhibition of metastasis by GPx2 have been drawn from an enhanced invasion through a matrigel membrane and an enhanced wound closure in a scratch assay of *GPX2* kd cells compared to control cells [73]. However, this also appears to depend on the cell line used, because opposite effects were observed in a rat hepatocellular carcinoma cell line (L2) with high metastatic capacity [72]. Thus, it appears that the role of GPx2 also differs during the individual steps of metastasis formation. Much more work has to be done to clarify this lesser investigated phenomenon.

38.6 Concluding Remarks

All physiological functions of GPx2 known so far are required for cell survival. Reduction of hydroperoxides can prevent oxidative stress which, if unhampered, can damage DNA, initiate mutations, and finally lead to carcinogenesis. At this very early state, GPx2 appears to be anti-carcinogenic by its removal of hydroperoxides, inhibition of inflammation, dampening the production of pro-inflammatory mediators, and enhancing wound healing. Usually, damaged cells are eliminated by apoptosis. The anti-apoptotic function of GPx2 is needed for the survival and self-renewal of stem cells in the intestine to maintain the homeostasis of the intestinal epithelium. In cancer cells, however, it inhibits the removal of initiated cells. Also the support of cell proliferation is physiologically required but turns into the contrary, if it is acquired by cancer cells.

Having this in mind, contrasting results from human studies and animal experiments will become clearer. GPx2 will act preventive at very early stages of cancer or if carcinogenesis is driven by inflammation. If the cancer cell is initiated and apoptosis needed to eliminate the initiated cell, the presence of GPx2 rather has adverse effects instead. In already established cancers, GPx2 supports proliferation and drives tumor grow. During metastasis, GPx2 appears to prevent detachment of cells from the extracellular matrix but also appears to equip floating cancer cells with a better capability to survive.

Similarly, a beneficial health effect of selenium itself might be observed only in early stages of cancer development. A follow-up of the Linxian study [76], in which the effect of a mixture of selenium, vitamin E and β-carotene on gastric cancer was tested, revealed that the preventive effect of the mixture, which was attributed mainly to selenium, was much more efficient in individuals younger than 55 years of age, and was almost without effect in individuals older than 55 years. Reduction of gastric cancer was even reversed by increased aging. In view of the generally observed increase in tumor development with age, GPx2 as well as dietary selenium may inhibit tumor initiation at a young age but support tumor growth of already initiated cells as discussed in [29] and previously postulated in 1986 [77].

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Chapter 39
Selenoproteins in Cardiovascular Redox Pathology

Diane E. Handy and Joseph Loscalzo

Abstract Redox-active selenoproteins, such as the mammalian glutathione peroxidases and thioredoxin reductases, are essential components of anti-oxidant defence systems that limit the damaging accumulation of oxidants and maintain the pool of reduced cellular thiols. Accumulating evidence from epidemiological and experimental studies indicates that these key redox-active proteins are essential to the cardiovascular system: their deficiencies are detrimental for cardiovascular health and their excess can be beneficial in protecting against cardiovascular injury and the development of atherogenesis. In this chapter, we will highlight the role of selenoproteins in cardiovascular development, vascular and cardiac function, thrombosis, and atherogenesis.

Keywords Atherosclerosis • Cardiomyopathy • Coronary artery disease • Endothelial dysfunction • Glutathione peroxidase • Ischemia reperfusion injury • Selenoproteins • Stroke • Thioredoxin reductase • Thrombosis

39.1 Introduction

In human populations, a cardiomyopathy, Keshan disease, is endemic in provinces of China with low selenium in the soil [1]. Decreased expression of selenoproteins is characteristic of this disease, and replacement of selenium in the diet increases selenoprotein expression and is a successful preventive treatment. Experimental evidence confirms a role for individual selenoproteins in complex cardiovascular diseases, such as atherosclerosis and stroke, primarily through modulating the damaging effects of reactive oxygen species (ROS).

The selenocysteine-containing glutathione peroxidases (GPx) and thioredoxin reductases (Txnrds) are components of the glutathione (GSH)- and thioredoxin (Trx)-based systems, respectively, two major redox systems that eliminate

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intracellular hydrogen and lipid peroxides and maintain the reduced state of protein thiols. In the GSH system, GPx enzymes detoxify peroxides by using GSH as a reductant, and the NADPH-dependent- (non-selenoprotein) GSH-reductase maintains the GSH/oxidized glutathione (GSSG) balance. In contrast, the NADPH-dependent Txnrd selenoproteins maintain the redox state of Trx proteins, cofactors for the peroxiredoxin enzymes that reduce hydrogen peroxide. Reduced Trxs have other functions that are crucial for proliferation and survival, including binding to the apoptosis signal-regulating kinase (ASK1) to prevent apoptosis [2]. Importantly, hydrogen peroxide levels and the Trx and GSH systems regulate the cysteine- (Cys)-oxidation state of redox-sensitive protein thiols to modulate protein function and cell signaling. Excess reducing equivalents, however, can lead to reductive stress that can decrease protein thiol oxidation, decrease cell signaling, and attenuate cellular proliferation [3] (and, paradoxically, can promote increased oxidative stress in some disease states [4]). Thus, selenoproteins have an essential role in modulating both the beneficial and damaging effects of ROS.

This chapter provides an overview of the effects of selenoproteins, especially the GPxs and Txnrds, on cardiovascular function. Of the selenocysteine-containing GPxs, GPx1, GPx3, and GPx4 have been shown to modulate cardiovascular function. GPx1 was the first Sec-containing protein to be identified ([5] and Chap. 17). It is ubiquitously expressed, found intracellularly, primarily in cytosolic and mitochondrial compartments, and exists as a tetramer. GPx3 is also a tetramer but it is a secreted glycoprotein, often referred to as plasma GPx. Kidney is the major source of GPx3 production. In mice, knockout of either the *Gpx1* or *Gpx3* gene is non-fatal, although these deficiencies contribute to cardiovascular dysfunction. The GPx4 protein or phospholipid GPx, exists as a monomer, and its deficiency is lethal, causing ferroptosis, a form of cell death that involves an accumulation of lipid hydroperoxides and iron overload ([6] and Chap. 43). In the mouse, lack of either cytoplasmic Txnrd1 (in *Txnrd1*$^{-/-}$ mice) or mitochondrial Txnrd2 (in *Txnrd2*$^{-/-}$ mice) results in embryonic lethality, and the *Txnrd2*$^{-/-}$ mice have altered cardiac development. Other selenoproteins, such as MsrB1, a methionine-sulfoxide reductase, as well as the endoplasmic reticulum (ER) selenoproteins SelK and SelS, may also contribute to redox homeostasis in the cardiovascular system [7], although, less is known about the functions of SelK and SelS.

39.2 The Role of Selenoproteins in Regulating Vascular Tone

Endothelial dysfunction can be characterized by a decrease in bioavailable nitric oxide (NO) and a subsequent loss in normal endothelium-dependent vasorelaxation responses to flow or to agonists, such as acetylcholine [8]. Excess ROS may reduce bioavailable NO directly (for instance by the reaction of NO with superoxide to form peroxynitrite) or it can decrease the availability of cofactors necessary for the activation of endothelial NO synthase, which produces NO. Importantly, loss of bioavailable NO contributes to platelet activation, proliferation of vascular smooth

muscle cells, and pro-inflammatory activation of the endothelium. In mouse knockout models, both heterozygous (*Gpx1*$^{+/-}$) and homozygous (*Gpx1*$^{-/-}$) *Gpx1*-deficient mice [9–11] have endothelial dysfunction, consistent with a role for GPx1 in vascular homeostasis. Endothelium-independent responses to NO generators, such as sodium nitroprusside, are preserved in *Gpx1*-deficient mice, indicating that smooth muscle responses to NO are intact in these mice. We found that plasma and aortic levels of the isoprostane, $iPF_{2\alpha}$-III, were increased by GPx1 deficiency [9, 10], whereas compensatory treatment with L-2-oxothiazolidine-4-carboxylic acid to increase intracellular thiol pools restored vasorelaxation responses in *Gpx1*-deficient mice, and lowered $iPF_{2\alpha}$-III levels. Other studies have shown that compared to normal carotid arteries, those from *Gpx1*$^{+/-}$ mice have diminished vasodilatory responses to acetylcholine at low doses of the ROS-generating angiotensin II (AII) [11], whereas carotid arteries from mice overexpressing GPx1 were resistant to dysfunction under conditions that altered function in wild type vessels. Similarly, in a mouse model of hyperhomocysteinemia, we found that GPx1 activity was reduced [12], in part, by mechanisms that decrease Se-dependent expression of GPx1 [13, 14]. Furthermore, the hyperhomocysteinemia-induced decrease in GPx1 activity also caused endothelial dysfunction [15], whereas overexpression of GPx1 in the context of hyperhomocysteinemia preserved bioavailable NO and restored normal endothelium-dependent vasodilation [12]. Similarly, in hypertensive subjects, GPx1 activity was inversely correlated with endothelium-dependent vasodilation responses, illustrating the importance of GPx1 in modulating vascular function in humans [16].

In *Gpx3*$^{-/-}$ mice, circulating levels of cGMP, a marker of NO production, are decreased, and vascular beds show endothelial dysfunction [17], illustrating the importance of endogenousextracellular antioxidant enzymes to the endothelium. Interestingly, pharmacological inhibition of Txnrd in isolated aortic rings also impaired relaxation and reduced cGMP [18]; however, concurrent with Txnrd inhibition, both the endothelium-dependent and -independent responses were suppressed. This observation may be due, in part, to the excess S-nitrosylation of proteins caused by Txnrd inhibition that may attenuate vascular responses.

Paradoxically, in some vascular beds, hydrogen peroxide may modulate vasodilation in response to arachidonic acid. Thus, *excess* GPx1 was found to decrease vasodilatory responses to low micromolar hydrogen peroxide in isolated cerebral vessels [19]. These studies suggest the effects of ROS on vascular function may depend on many local factors, including the pathways regulating vasodilation, as well as ROS levels.

39.3 The Role of Selenoproteins in Inflammation and Atherogenesis

Endothelial dysfunction and oxidative stress are thought to promote atherogenesis; yet in the context of a high fat diet in mice, GPx1 deficiency on a C57Bl/6 background did not promote atherogenesis. Rather, *Gpx1*$^{-/-}$ mice had decreased severity of aortic sinus lesions [20], possibly due, in part, to compensatory upregulation of

glutaredoxin-2, a redox-active enzyme that can preserve protein thiol reducing potential [21]. In the context of ApoE deficiency, however, lack of GPx1 increased atherogenesis in response to a Western diet [22], and in combination with streptozotocin-induced diabetes mellitus [23]. Notably, compared to *Apoe*$^{-/-}$ mice, *Apoe*$^{-/-}$/*Gpx1*$^{-/-}$ mice had excess aortic ROS production, enhanced NADPH-stimulated ROS production, and enhanced mitochondrial ROS generation [22]. Other studies have found that ebselen, a GPx-mimic, decreases aortic lesion formation in diabetic *Apoe*$^{-/-}$ mice, illustrating a role for oxidant stress in atherogenesis in the *Apoe*$^{-/-}$ mice [24]. Ebselen has a broader substrate specificity than GPx1 and can effectively reduce membrane phospholipids that are normally reduced intracellularly by GPx4.

Consistent with the importance of GPx1 in cardiovascular disease, red blood cell GPx1 activity was found to be a strong predictor of future cardiovascular events in individuals with coronary artery disease, with the lowest tertile having a greater than threefold increase in cardiovascular disease risk compared with those in the highest tertile of activity [25]. Similarly, in coronary artery disease patients, homocysteine and GPx1 activity are predictors of cardiovascular disease, and the combination of lowest GPx1 activity and highest plasma homocysteine conferred the greatest risk [26].

Given these findings, many studies have analyzed polymorphisms in the *GPX1* gene to determine whether they associate with disease phenotypes. A common polymorphism involving a T for C substitution that results in a leucine (Leu) amino acid substitution for proline (Pro) at position 198 (Pro198Leu) in the GPx1 protein has been identified. The Leu variant has been reported to cause decreased expression of GPx1 under conditions where selenium is limited [27], and this variant may contribute to risk in Keshan disease. Similarly, in a case-control study of coronary artery disease patients in China, the presence of the Leu allele was associated with increased disease risk [28], and the Leu allele was associated with increased vascular disease burden in Japanese type 2 diabetic subjects [29, 30]. Additional studies have examined variants in other selenoprotein genes for their association with cardiovascular disease. In particular, variants in the *SELS* gene show an association with coronary heart disease in a Finnish population [31].

Excess GPx4 was also found to lessen atherogenesis in *Apoe*$^{-/-}$ mice [32]. This protective effect correlated with a reduction in lipid peroxidation in the aorta without any effect on plasma lipid levels. In isolated mouse aortic endothelial cells, overexpression of GPx4 reduced endothelial production of hydroperoxides and decreased adhesion molecule expression in response to oxidized phospholipids, correlating the effects on lipid oxidation with a decrease in inflammatory responses.

Other studies in endothelial cells suggest that GPx1 modulates pro-atherogenic gene expression in response to intracellular oxidants generated during cyclic stress [33] or following cytokine or endotoxin exposure [34, 35]. In fact, GPx1 deficiency alone was found to promote up-regulation of adhesion molecules in human microvascular endothelial cells [34]. Additionally, deficiency of GPx1 enhanced inflammatory signaling through NFkB and MAPK pathways [35, 36], and excess expression of adhesion molecules caused by GPx1 deficiency promoted monocyte binding to endothelial cells [14], an early step in atherosclerotic lesion formation.

Selenoprotein K (SelK) may also potentiate macrophage foam cell formation, an essential step in the development of atherosclerotic lesions. SelK is an ER membrane protein that modulates receptor-mediated calcium flux in immune cells. *Selk*$^{-/-}$ macrophages were found to have a defect in the uptake of oxidized LDL that was caused by a decrease in CD36 palmitoylation, a reversible Cys residue-specific modification that occurs in the ER which is necessary for the stability and cellular localization of CD36 to lipid rafts [37]. Although the exact role of Selk in CD36 palmitoylation is not understood, transfer of bone marrow from *Selk*$^{-/-}$ mice into the atherogenic-susceptible *Ldlr*$^{-/-}$ mice attenuated atherosclerotic plaque development.

Trx1 and Trxnrd1 also regulate inflammatory responses, in part, due to their effects on the oxidation state of redox-sensitive Cys residues in proteins, including those that modulate the transcriptional activity of NFkB [38]. Although activation of NFkB can promote inflammatory pathways, its activation is also necessary for cell survival and the expression of a number of anti-apoptotic genes. Txnrd1 also has an essential role in limiting apoptosis by maintaining Trx1 in a reduced state to inhibit apoptotic signaling; however, little is known on how these crucial roles of Txnrd1 modulate atherogenesis.

39.4 The Role of Selenoproteins in Vascular Remodeling and Angiogenesis

In-stent restenosis is the re-narrowing of a blood vessel following balloon angioplasty and stenting to open a vessel obstructed by atheroma. This re-narrowing of the vessel involves vascular smooth muscle cell (VSMC) proliferation and neointimal hyperplasia. In a mouse model of balloon angioplasty and stenting, GPx1 was shown to have a predominant role in modulating vascular remodeling. Thus, in *Apoe*$^{-/-}$/*Gpx1*$^{-/-}$ mice, neointima formation was enhanced following balloon angioplasty and stenting [39]. Furthermore, there was a corresponding decrease in endothelial cell regeneration following the balloon injury. Interestingly, in this study, enhanced vascular smooth muscle cell growth was associated with changes in protein thiol modifications that increased glutathiolation of SHP-2, a tyrosine phosphatase, to attenuate its activity. This modification was linked to enhanced kinase activity of ROS1 which, in turn, promoted VSMC proliferation and migration. Deficiency of GPx-1 in the *Apoe*$^{-/-}$/*Gpx1*$^{-/-}$ mice also corresponded with reductive stress following balloon/stenting characterized by an accumulation of excess GSH compared to stented *Apoe*$^{-/-}$/*Gpx1*$^{+/+}$ mice; however, the balloon/stenting procedure simultaneously increased oxidative stress in the *Apoe*$^{-/-}$/*Gpx1*$^{-/-}$ mice, suggesting that both reductive and oxidative stress may contribute to the pathology in these animals. In balloon angioplasty-stent patients, variants in *ROS1* and *GPX1* were found to be independently associated with in-stent stenosis, consistent with the role identified for these proteins in the mouse model system. Similarly, previous studies from Japan reported an association of the Leu allele of *GPX1* with an increased risk of restenosis following stenting [40].

GPx1 was also found to modulate endothelial cell growth as shown in our hind limb-ischemia study of angiogenesis. In this study, GPx1 deficiency attenuated neovascularization in response to ischemic injury, in part, due to deficiencies in endothelial progenitor cells and their increased sensitivity to ROS-induced apoptosis [41].

39.5 The Role of Selenoproteins in Stroke and Thrombosis

We found a causal relationship between rare forms of familial deficiency of GPx3 and childhood cerebrovascular thrombotic disease [42, 43]. Although the underlying genetic defects in these families are unknown, the dominantly inherited defect reduces plasma GPx3 activity approximately 50 % in affected patients. Concurrent with a decrease in plasma GPx3, NO failed to block platelet P-selectin expression and platelet aggregation in studies with normal gel-filtered platelets mixed with GPx3-deficient plasma. These findings suggest that modest alterations in circulating GPx3 can alter platelet homeostasis, thereby contributing to thrombosis and stroke. Additional studies in our laboratory found that *GPX3* gene expression was transcriptionally upregulated by hypoxia [44], suggesting that increased production of GPx3 in response to lower oxygen tension, as in ischemic stroke, may guard against ROS-induced damage during reoxygenation. In our subsequent analysis of human thrombotic disorders, we have identified a variant haplotype (H_2) in the *GPX3* gene promoter that correlated with reduced transcriptional activity under normoxic and hypoxic conditions [45]. Furthermore, we found that this haplotype is a strong, independent risk factor for cerebral venous thrombosis [46] and that it is associated with increased risk of arterial ischemic stroke in the young [45]. Independent studies in a German cohort confirmed that the H_2-haplotype was a risk factor in arterial ischemic stroke in children [47]. This latter study found no association between *GPX3* genotypes and thromboembolic or cerebral sinovenous thrombosis in children. Other studies have found an association of the rs713041 (T718C) polymorphism in the 3′ untranslated region of the *GPX4* gene with risk of cerebral stroke in patients of Russian origin with essential hypertension [48].

To study further the role of GPx3 in maintaining the balance between hemostasis and thrombosis, we developed a *Gpx3* mouse gene knockout. Consistent with altered platelet function in GPx3 deficient patients, we found attenuated bleeding times, elevated soluble P-selectin (a marker of platelet and endothelial activation), and increased platelet aggregation in response to ADP infusion in an in vivo model of platelet function as well as increased ADP-activation of platelets in in vitro platelet assays [17]. Furthermore, these mice have an insufficiency of NO: circulating levels of cGMP are decreased, and vascular beds manifest endothelial dysfunction. To determine whether alterations in platelet function would result in stroke injury, we used the middle cerebral artery-ischemia-reperfusion (MCA I/R) model. We found that $Gpx3^{-/-}$ mice were more sensitive to cerebral injury following MCA I/R, with increased infarct size and greater neurological impairment. Clopidogrel, a platelet inhibitor, significantly reduced stroke volume and improved neurological

function, suggesting that platelet activation contributed to the extensive injury caused by lack of GPx3. Use of MnTBAP, an antioxidant, was similarly able to reduce brain injury following MCA I/R, indicating a role for oxidative stress in the underlying dysfunction caused by GPx3 deficiency.

GPx4 also has an important role in endothelial homeostasis that was only uncovered by using conditional endothelial-cell specific *Gpx4*$^{-/-}$ mice [49]. On normal chow diets, replete with vitamin E, the lack of endothelial GPx4 had no effect on endothelial function. Ex vivo experiments with dissected blood vessels revealed significant deficiencies in angiogenesis that were proposed to be due to the lack of vitamin E in the ex vivo culture conditions. Subsequent experiments performed in vivo with diets deficient in vitamin E showed a role for endothelial-specific GPx4 in thrombosis: absence of endothelial GPx4 resulted in thromboembolic death, elevated systemic blood pressure, increased lipid peroxidation, and evidence of endothelial cell death in multiple organs. These studies suggest a role for the redox state of the endothelial cell in thrombogenesis and that vitamin E is sufficient to compensate for GPx4 deficiency.

A significant amount of the injury in the MCA stroke model is due to the ROS caused by the reperfusion following the ischemic event. In brain, GPx1 deficient neurons were found to be more susceptible to apoptosis following MCA I/R [50]. In addition, lack of GPx1 resulted in increased oxidative stress and enhanced activation of NFkB [50, 51]. Furthermore, in *Gpx1*$^{-/-}$ mice, cerebral injury was exacerbated by vascular dysfunction that limited microvascular blood flow following ischemia [52]. Injury following MCA occlusion was attenuated by treatment with ebselen, which decreased infarct size and improved vascular function in *Gpx1*$^{-/-}$ mice [52]. Similarly, transgenic mice overexpressing GPx1 had significantly less cerebral injury following MCA I/R than wild type mice [53]. Overall, these findings suggest a critical role for redox balance in neuronal protection in response to cerebral ischemia-reperfusion. Interestingly, it has been reported that ebselen may also improve neurological outcomes following stroke in human subjects [54]; however, these early studies have not been confirmed.

39.6 Cardiovascular Development and Selenoproteins

Tissue specific knockdown of the *Trsp* gene encoding the tRNA for selenocysteine illustrated the importance of selenoproteins in endothelial cell development and cardiac muscle function [55]. In the endothelial specific knockout mice, the lack of *Trsp* was embryonically lethal, whereas the myocyte specific knockout caused perinatal lethality with severe myocarditis. *Txnrd2*$^{-/-}$ mice die at E13 due to defects in hematopoiesis as well as cardiac development [56]. Cardiac-specific *Txnrd2* knockout confirmed that Txnrd2 was essential for normal cardiac function as it caused fatal dilated cardiomyopathy and morphologically abnormal cardiomyocytes, whereas cardiac-specific *Txnrd1* knockout mice have no cardiac dysfunction [57]. As discussed in the following section, deficiency of cardiac Txnrd2 in adult mice

augments cardiac injury in a model of ischemia-reperfusion [58]. Consistent with an important role for Txnrd2 in cardiac function, two novel *TXNRD2* mutations that have amino acid substitutions in the flavin-adenine dinucleotide binding domain of Txnrd2 were found in patients with dilated cardiomyopathy; however, these mutations caused only a moderate phenotype [59].

Rare premature-truncation mutations of *GPX4* in humans were found to cause autosomal recessive Sedaghatian-type spondylometaphyseal dysplasia, a lethal syndrome characterized by severe developmental defects in cardiac, central nervous, and skeletal systems that lead to perinatal death [60].

39.7 Cardioprotection and Selenoproteins

Although the lack of selenium is detrimental to cardiac function, dietary supplementation with excess selenium has not shown a clear beneficial effect on cardiovascular health [61]. Nonetheless, experimental and clinical studies support the concept that many selenoproteins are cardioprotective and that they mitigate cardiac injury to hypertrophic stimuli, myocardial infarction, and other cardiomyopathies (reviewed in [62]).

In in vivo models of cardiac hypertrophy, several selenoproteins, including MsrB1, GPx3, GPx4, and Txnrd1, were upregulated, suggesting that these proteins may play a role in lessening oxidative injury following hypertrophic stimulation [63]. Additional studies implicate other selenoproteins in protection against oxidative injury by using transgenic and knockout gene model systems. Thus, GPx1 deficiency augmented AII-induced left ventricular hypertrophy, increasing myocyte cross-sectional area and intraventricular septal thickness, and decreasing cardiac fractional shortening [64]. AII is a vasoactive peptide that promotes hypertension, vascular remodeling, and cardiac hypertrophy, in part, via AII-receptor 1-mediated activation of NADPH oxidases to increase superoxide generation. Although the mechanistic basis for increased cardiac hypertrophy and dysfunction is not fully known, it is likely that it is related to excess ROS caused by loss of the GPx1 antioxidant enzyme.

In mouse cardiac I/R injury models of myocardial infarction, the presence of GPx1 has been found to preserve cardiac function, as hearts from transgenic mice overexpressing GPx1 are more resistant to myocardial ischemia reperfusion injury than those from non-transgenic controls. Consistent with this finding, we found that aged $Gpx1^{+/-}$ mice have structural abnormalities in the myocardial vasculature and diastolic dysfunction following myocardial I/R [9]. Our subsequent studies found increased susceptibility to myocardial injury following I/R in hearts from male, but not female, $Gpx1^{-/-}$ mice [65]. These findings may be attributed, in part, to other compensatory antioxidant mechanisms in female $Gpx1^{-/-}$ mice that preserve pools of reduced ascorbate and augment the conversion of nitrate to nitrite. In a separate study, excess reactive oxygen generation in (male) $Gpx1^{-/-}$ murine hearts subjected to ischemia-reperfusion injury correlated with diminished mitochondrial function,

characterized by increased damage to mitochondrial DNA, decreased levels of mitochondrial protein expression, and reduced NADH and ATP generation [66]. Thus, I/R injury generates excess ROS, in part, via increased mitochondrial output of oxidants, and the damaging effects of mitochondrial oxidants may be augmented by lack of GPx1. In support of a crucial role of GPx1 in mitochondrial ROS-flux, the absence of GPx1 has been shown to upregulate mitochondrial production of hydrogen peroxide [66].

To study the role of mitochondrial oxidants on I/R injury, a transgenic mouse was engineered to overexpress specifically a rat mitochondrial form of GPx4 [67] and used in the Langendorff model of global no-flow I/R injury. In these studies, overexpression of GPx4 resulted in improved contractile function characterized by improved rates of contraction, developed pressure, and peak systolic pressure compared to nontransgenic hearts. These functional improvements may be the result of decreased mitochondrial damage, as there was less overall lipid peroxidation in mitochondria and electron transport complexes had preserved function in hearts from *Gpx4* transgenic mice. Similarly, overexpression of the mitochondrial form of GPx4 decreased cardiac contractile dysfunction in a streptozotocin and hyperglycemia-induced mouse model of diabetes, in part, by lessening oxidative injury to preserve mitochondrial structure [68]. Overall, these findings suggest that excess GPx4 in mitochondria effectively removes harmful oxidants to improve cardioprotection. Conversely, *Gpx4* haploinsufficient mice ($Gpx4^{+/-}$) fed a high fat, high sucrose diet developed obesity and had augmented levels of oxidant stress, cardiac fibrosis, cardiomyocyte hypertrophy, and cardiac mitochondrial dysfunction compared to wild type mice on the same diet [69].

To determine the role of Txnrd2 in myocardial I/R injury,α-MHC-restricted Cre expression induced by tamoxifen was used to eliminate Txnrd2 from cardiomyocytes of adult $Txnrd2^{flox/-}$ mice [58]. These $Txnrd2^{-/-ic}$ mice had increased cardiac dysfunction following I/R injury with elevated mitochondrial ROS, increased mitochondrial dysfunction and swelling, and increased cell death. Pretreatment with antioxidants such as N-acetylcysteine lessened cardiac dysfunction, improving mitochondrial function and decreasing cell death. These data suggest that impairment of Txnrd2 can potentiate cardiac dysfunction by mechanisms involving excess ROS. Consistent with the role for ROS in Txnrd2 dysfunction, in cell-based studies, the specific knockout of Txnrd2 was shown to lessen cellular GSH and decrease cell survival, phenotypes that were also improved by N-acetylcysteine pretreatments.

39.8 Concluding Remarks

Redox-active selenoproteins, especially GPx1, GPx3, GPx4, and Txnrd2, all have proven beneficial roles in the cardiovascular system, in part, via their roles in preventing or mitigating oxidative stress (Fig. 39.1). Both GPx1 and GPx4 are known to be cardioprotective and anti-angiogenic. To date, little is known about the cardiac-specific functions of GPx3; however, this secreted protein plays an

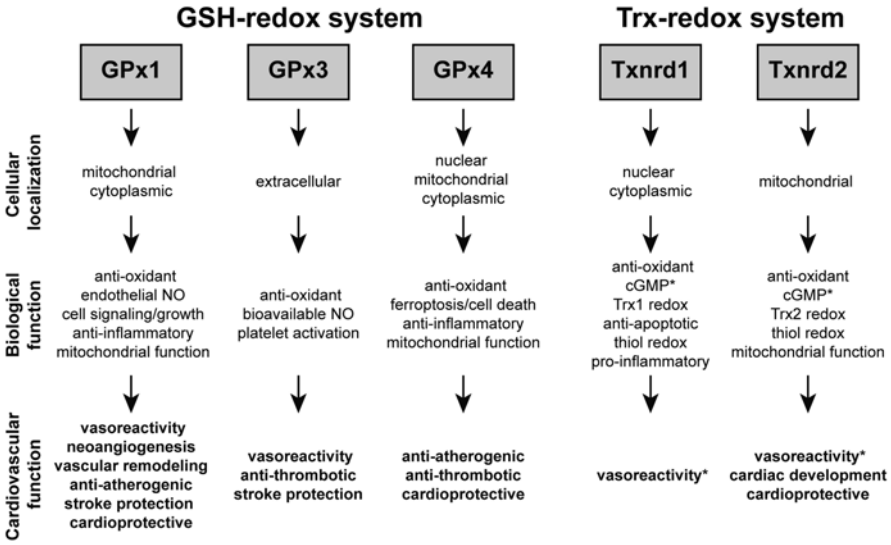

Fig. 39.1 Overview of the role of selenoproteins of the GSH and Trx systems in cardiovascular function. Selenoproteins of the two major redox systems, via their extracellular and intracellular effects on redox and oxidants, contribute to various cellular functions that, in turn, influence cardiovascular health and disease. Many of these functions have been elucidated through the use of knockout and overexpression systems in cells grown in culture as well as in vivo murine models (as reviewed in the text). Some of these findings have been confirmed in human subjects. *The effects Txnrd on vascular reactivity and cGMP production were determined in the presence of a general Txnrd inhibitor, therefore it is unclear whether both Txnrd1 and Txnrd2 contribute to these functions

important role in maintaining vascular homeostasis, preventing thrombosis, and protecting against stroke. Txnrd2 function appears to be crucial for normal cardiac development and protects against stress, possibly due to its critical role in regulating mitochondrial function. Studies with a Txnrd inhibitor show that this system is crucial for optimal cGMP production in blood vessels, although it is unclear how both Txnrd1 and Txnrd2 contribute to this function. Txnrd1 may also contribute to cardiovascular health by preventing apoptosis; however, its function may also enhance the activity of activated pro-inflammatory transcription factors. Similarly, SelK, an ER specific protein, is known to play a role in immune cell function but its presence may be pro-atherogenic in macrophages. Studies in human subjects suggest a role for many of these selenoproteins in cardiovascular disease, highlighting the importance of understanding the mechanisms by which these selenoproteins regulate the underlying disease processes.

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Chapter 40
What Do We Know About Selenium Contributions to Muscle Physiology?

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Abstract Early observations of selenium (Se) deficiencies in human and livestock revealed the importance of this trace element for normal muscle function. However, the molecular and cellular dysfunction connecting low selenium diets to muscular diseases remain elusive. Importantly, mutations in the *SEPN1* gene encoding selenoprotein N (SEPN1) were shown to cause an inherited muscular disease in humans. Therefore, it is expected that understanding the role of SEPN1 and the related pathophysiology will unveil the participation of Se in molecular processes essential for muscle function and pave the way for targeted therapeutics. However, the functional characterization of SEPN1 is still lacking. Analysis of its activity in cellular and animal models pointed to its involvement in oxidative stress defense and in control of calcium (Ca^{2+}) handling. A link between the activities of SEPN1 and of the Ca^{2+} transporters, RyR1 and SERCA, was shown, but the enzymatic reaction catalyzed by SEPN1 has not been characterized.

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40.1 Introduction

Se deficiency is a contributing factor to multiple pathophysiological conditions, including heart and neuromuscular disorders. Its importance for skeletal or cardiac muscular function was revealed by early observations establishing a link between low Se diet and different muscular disorders in farm animals and further reported in several human muscular syndromes. Se supplementation was beneficial against myopathies in livestock in many cases. However, in the human forms of muscular diseases, the protective role of Se remains more controversial. Altogether, this situation suggests a complex connection between Se supplementation and other environmental or genetic factors in the development of muscle disorders. Molecular studies, including the identification of the selenoproteome, provide the basis for the understanding of the role of Se in muscular function and it is expected that a more specific mechanistic comprehension will provide the rationale for the design of therapies. Importantly, the identification of mutations in the gene coding for the selenoprotein, SEPN1, as the causative factor for different forms of central muscular diseases, provide the first direct evidence for the importance of Se in muscle function and maintenance. Understanding the molecular and physiological role of SEPN1 is a key step to decipher the link between Se and muscle function.

40.2 Se Sources and Forms in Muscles

Different forms of Se have been characterized in muscle mainly present as selenomethionine and selenocysteine (Sec) inserted into proteins. Other forms of organoselenium compounds have been identified, but their antioxidant activity is poorly characterized. Recently, it has been shown that red muscle of tuna contains large amounts of an organic Se compound that was identified as 2-selenyl-N,N,N-trimethyl-L-histidine, a Se analog of ergothioneine that was called selenoneine [1]. Selenoneine is believed to be a strong radical scavenger that binds to the heme group of hemoglobin and myoglobin and protect them from auto-oxidation under hypoxic conditions [2], but its physiological activity remains largely elusive. The Sec containing enzymes are known to constitute the principal biological active form of Se in muscle as in many organs. Animal studies show that different forms of supplemented Se (selenite, selenate, selenomethionine) can largely influence muscle Se concentration [3–7]. From these studies, it was concluded that for muscle, organic Se sources are more efficient for Se absorption, and have different outcomes on Sec incorporation into selenoproteins. Selenoprotein gene expression was

mapped in skeletal and cardiac muscles from different animals according to Se dietary concentration and forms [8, 9]. A transcriptomic experiment was conducted to analyze regulated genes from liver and gastrocnemius muscle in mice fed either Se-deficient diet or diets supplemented with different organic or inorganic forms of Se. Nineteen differentially expressed genes were identified, among them 12 corresponding to selenoproteins [10].

Several studies demonstrated a hierarchy in the distribution of Se between different tissues, making certain tissues more susceptible to Se depletion [11]. Brandt-Kjelsen and collaborators [12] performed a pulse chase experiment in chicken using a ^{75}Se isotope tracer from labeled wheat to measure Se turnover in a whole organism. The uptake of ^{75}Se was assessed in all organs, showing the lowest activity in breast muscle. One week after the diet shift to nonradioactive Se, the excretion of the labeled tracer was followed and Se retention determined in individual organs. The shortest half-lives (4 days) were observed in metabolic organs such as the liver, kidney and pancreas, whereas strong retention was measured in muscle (half-live 12 days), brain and lung (13 days) [12]. These results suggest a low metabolic Se turnover in muscle tissue. Dissection of the process at the molecular level provided a more complex interaction. In the Se deprived rodent, muscle glutathione peroxidase Gpx1 activity decreased to 6 % of Se-adequate level, while Gpx4 activity was not significantly decreased. Muscle was more affected by Se depletion in case of dietary Se deficiency compared to other less susceptible organs, such as testis or brain [9].

40.3 Muscular Dysfunction Associated with Se Deficiencies in Livestock

Nutritional muscular dystrophy (also called nutritional myopathy, rigid syndrome or white muscle disease) is an acute, degenerative disease of cardiac and skeletal muscles caused by a dietary deficiency of Se, sometimes combined with lack of vitamin E. Such disorders have been described in different animal species with different presentations. In lambs, when cardiac muscle was affected, animals displayed respiratory distress, cardiac arrhythmias and sudden death, since primary skeletal muscle affection leads to signs of muscle weakness, back stiffness, postural instability and disabled walking. Skeletal muscle degeneration is characterized by a pale discoloration of affected muscles and intramuscular edema. The white streaks seen in cardiac and skeletal muscle bundles correspond to bands of coagulation necrosis, fibrosis and calcification [13].

Se deficiency was classified as a prevalent determinant, as it was observed that animals raised in regions with low Se content in the soil, or born from mothers with low Se status during gestation, are the usual pre-conditions for the disease in lambs [14]. In addition, Se supplementation appeared to be more efficient than vitamin E in preventing nutritional muscular dystrophies [15–17]. The Se deficiency was correlated with a reduced expression of a subset of selenoproteins in liver and muscles [18].

In pigs, a higher frequency of Mulberry heart disease, another muscle pathology related to vitamin E/Se deficiency was described in animals fed torula yeast diets containing low levels of Se. Mulberry heart disease has been experimentally reproduced in animals with diets deficient in vitamin E and Se [19, 20]. The common manifestation is sporadic and sudden death of young rapidly growing pigs, often accelerated by exercise. This condition is characterized by deposition of fibrin strands in the myocardium, leading to necrosis and hemorrhages. Supplementation with vitamin E and Se resulted in a reduced incidence of the lesions including decreased myocardial degeneration and skeletal muscle wasting [21].

Inadequate vitamin E level combined with sulfur amino acid deficiency also results in severe muscular dystrophy and exudative diathesis in young chicks [22]. This disease is characterized by degeneration of breast and leg muscle fibers, pancreatic atrophy, liver necrosis and severe edema of the subcutaneous tissues. Initial studies involving the effects of diet on muscular dystrophy showed that Se and vitamin E supplementation reduced the incidence of muscular dystrophy in chicks [23]. In addition, exudative diathesis was replicated in broiler chicks fed a corn-soy diet produced in a Se-deficient area of China. In these conditions, several selenoprotein genes were down-regulated in muscle and liver tissues [8, 24], inducing increased oxidative stress that consequently triggered the activation of p53-dependent and p38 MAPK/JNK/ERK signaling pathways [8, 25].

From the analysis of these animal disorders, it is still not clear whether the pathogenic mechanism results from a general increase in an oxidative stress linked to a general decreased expression of selenoproteins or in the dysfunction of a specific pathway, due to lower activity of a selective subset of selenoenzymes. Interestingly, it was reported that most of the affected animals manifest a high metabolism level and a rapid growth rate, suggesting a link between the Se-related activity and the energetic or metabolic status of the affected animals. Many studies conducted in animals arrived at a conclusion of a cooperative effect of Se and vitamin E supplementations to alleviate muscle disorders suggesting the convergence to a common antioxidant process. In a recent study, Fujihara and Orden [26] showed that in rats, higher vitamin E consumption resulted in a decreased Se concentration in various organs of the body, indicative of a compensatory interrelationship between the two compounds.

40.4 Muscular Dysfunction Associated with Selenium Deficiencies in Humans

Keshan disease is a cardiomyopathy characterized by multiple necrotic lesions, inflammatory areas and calcification throughout the myocardium [27, 28]. This disorder has been described in individuals with low Se status in different areas of eastern China, due to low Se concentrations in the soil (for reviews see, [29–31]). Experimental approaches conducted in mice concluded a dual etiology for the disease: i) a lack in Se; and ii) an infection by an otherwise non-virulent strain of the

enterovirus *Coxsackie*. Increased oxidative stress in the Se-deficient host induces mutations in the genome of the benign or mildly pathogenic viral strain, turning it into a cardiovirulent one [32].

Prolonged parenteral nutrition is another cause for severe Se deficiency linked to skeletal muscle disorders [33–35]. The myocardic morphological features reported in such conditions at autopsy are similar to those described in Keshan disease. Low plasma Se concentrations and lower erythrocyte Gpx activities were associated with muscle pain and weakness. Accordingly, affected patients responded positively to Se administration [36]. The benefits of Se administration were also tested in patients with myotonic dystrophies, since it was observed that their Se levels in blood decreased with the evolution of the disease [37]. However, no conclusive evidence of beneficial effects of Se treatment were obtained [38, 39]. Similarly, no improvement on muscle function was reported for Se or vitamin E supplementation to patients with Duchene muscular dystrophies [40–42]. From these analyses Se does not appear as a contributing factor for the two human muscular dystrophies, but one should mention that in all these studies, Se was provided as sodium selenite, a non-organic form, and increased Se concentration in the plasma was not addressed systematically. Several studies reported a significant lower serum Se level in elderly persons with sarcopenia, a muscle disorder with symptoms such as poor muscle strength or low skeletal muscle mass [43–45]. However, no supplementation trials have been conducted to validate the importance of Se as a contributing or aggravating factor in this pathology.

In conclusion, other than Keshan disease and the deficiency during parenteral nutrition, Se did not constitute a contributing, nor an aggravating factor for muscular dystrophies, despite the previously reported link between Se concentration and the severity of the symptoms in most cases.

40.5 Selenoproteins and Congenital Muscle Disorders

Mutations in *SEPN1*, the gene coding for SEPN1, were initially associated with an inherited muscular disease in humans, which is the rigid spine form of congenital muscular dystrophy [46]. Subsequently, the same and additional SEPN1 mutations were found in other pediatric muscular disorders, e.g., the classical form of multi-minicore disease [47], desmin-related myopathy with Mallory body-like inclusions [48], and the congenital myopathy with congenital fiber type disproportion [49]. These disorders share common symptoms characterized by an early onset phenotype including muscle weakness from infancy, predominantly affecting neck and trunk muscles and generalized muscle atrophy, leading to spine rigidity, severe scoliosis and potentially lethal respiratory insufficiency [50, 51]. Insulin resistance was also described in a few patients [49]. This clinical pattern, supported by muscle magnetic resonance imaging, is an essential basis for the diagnosis of SEPN1-related myopathies. In contrast, the spectrum of histopathological abnormalities associated with the disease is unusually large. It includes an increase in the

endomysial extracellular matrix, changes in the number and size of type 1 fiber, and/or the presence of intracellular lesions such as areas of sarcomere disorganization termed minicores or protein aggregates. Hence, four neuromuscular disorders historically distinguished as independent clinical entities based on the histological defects described in muscle biopsies from patients are now recognized as one pathological entity, the SEPN1-related muscular dystrophy or SEPN1-RM. All *SEPN1* mutations identified to date are autosomal recessive (for a list see [52]), including nonsense or missense, consistent with a loss of SEPN1 function. In particular, several of the identified mutations affect the Sec insertion machinery, such as mutations affecting the UGA codon that codes Sec or the Sec Insertion Sequence (SECIS) element and selenocysteine redefinition element, *cis* sequences required for efficient Sec insertion and protein expression [53, 54]. No clear-cut genotype-phenotype correlations have been established.

Some mutations in the gene coding for the Sec-specific translation cofactor, SECISBP2, have also been associated with muscle weakness. Nonsense mutations were identified in three patients with multisystemic disease characterized by abnormal thyroid hormone metabolism, delayed bone maturation, delayed motor development, muscle weakness, scoliosis and mildly reduced lung vital capacity [55, 56]. The radiological distribution of muscle involvement was similar to that in SEPN1-RM, and in two cases, reduced levels of SEPN1 were detected in cultured fibroblasts from patients. However, reports of other mutations in the *SECISBP2* gene were reported in patients with growth retardation and thyroid defects, but lacking muscular symptoms ([57–59] and Chap. 44). Loss of function of *SECISBP2* affects the translation of several selenoproteins and it has been shown that *SECISBP2* can discriminate the different SECIS elements, resulting in the hierarchical binding to SECIS elements from different selenoprotein mRNAs and selective outcomes for different selenoprotein expression [60, 61]. This observation implies that the nature of the amino acid mutated in *SECISBP2* might differentially affect the set of depleted selenoproteins, including or not SEPN1, in connection with the severity of the muscular symptoms.

40.6 SEPN1-Related Myopathy Pathophysiology: Translational Research and Therapeutic Implications

SEPN1-RM is the first inherited disease identified that is due to mutations in a selenoprotein gene. Thus, understanding the physiological role of SEPN1 and the pathophysiology of SEPN1-RM emerged as a model paradigm to appreciate and therapeutically target other selenoproteins involved in human health and disease. Yet, the pathogenesis of SEPN1-RM remained undetermined for long time and no specific treatment was available for this potentially lethal disorder.

Studies of an ex vivo model of SEPN1 deficiency provided the first indication that SEPN1 plays an important role in cell defense against oxidative stress and redox homeostasis in human skeletal muscle cells [62]. Primary cultured myotubes

from patients with *SEPN1* null mutations showed a significant increase in basal intracellular oxidant activity and global oxidized protein content compared to controls, including excessive oxidation of the contractile proteins actin and myosin heavy chain II which potentially contributes to mechanical dysfunction. Accordingly, the amount of oxidized proteins was globally increased in fibroblasts from patients. Increased protein oxidation was also described in mice with a deleted *Sepn1* gene, an animal model for SEPN1-RM. Adult *Sepn1*$^{-/-}$ mice display no phenotype under standard laboratory conditions [63, 64], but only develop skeletal muscle alterations after inducing oxidative stress, e.g., when submitted to a forced swim test (FST). This test generates physical activity in a general stress context, with unusual stimulation of back and neck muscles to maintain the head above water. After 3 months of FST, mutant mice developed a strong kyphosis and were less mobile than their wild type littermates. At the tissue levels, a switch toward smaller and slower fibers was observed in paravertebral muscles, and tubular aggregates were detected in muscle fibers from older mutant mice [63]. In addition, analysis of the *Sepn1* mouse knockout model showed that SEPN1 deficiency leads to abnormal lung development, raising the possibility that the respiratory syndrome observed in patients with *SEPN1* mutations may have a primary pulmonary component in addition to the weakness of respiratory muscles [64].

On the other hand, SEPN1-deficient human myotubes showed increased resting cytosolic Ca^{2+} concentration, reduced sarcoplasmic reticulum Ca^{2+} load and caffeine-induced Ca^{2+} release. These observations suggested that in the absence of SEPN1, the reactive oxygen and nitrogen species generated by muscle cells regulate intracellular Ca^{2+} concentration via modulation of redox-sensitive Ca^{2+} channels (the ryanodine receptor RyR1 and/or the Ca^{2+} ATPase SERCA and/or the IP3 receptors), favoring Ca^{2+} release or leaking from the endoplasmic reticulum [62]. Accordingly, in myofibers from *Sepn1*$^{-/-}$ mice, the Ca^{2+} release channels RyR showed lower sensitivity to caffeine [64]. Furthermore, zebrafish studies support the role of SEPN1 as a RyR modifier [65], and it has been recently confirmed that SEPN1 contributes to maintaining the reduced state of SERCA [66]. In this later publication, the authors reported that SEPN1 activity enhanced SERCA2 activity and protected SERCA2 luminal cysteines (Cys) against the hyper-oxidizing conditions elicited by the endoplasmic reticulum oxidoreductin 1, ERO1.

Finally, there is evidence to suggest that SEPN1 is also involved in modulation of cell survival pathways. Patient-derived primary fibroblasts and muscle cells devoid of SEPN1 show a higher susceptibility to H_2O_2 induced oxidative stress, manifested by a severely increased cell death rate [62]. In addition, adult skeletal muscles from the *Sepn1* knockout mouse showed a reduced pool of muscle progenitor cells (satellite cells), associated with defective muscle regeneration [67].

Altogether, the pathophysiological data mentioned above point to redox homeostasis as a primary pathogenic defect that could potentially be targeted to develop therapies for this so-far incurable infantile disease. Indeed, cultured primary cells from patients with *SEPN1* mutations have been used to evaluate the ex vivo effect of antioxidants drugs. Interestingly, pre-treatment with N-acetyl cysteine (NAC) rescued the cell phenotype, improving significantly myoblast survival upon H_2O_2

exposure and rendering the cell death rate of *SEPN1*-mutant fibroblasts identical to that of control, unchallenged cells. In addition, NAC normalized the levels of oxidized proteins in *SEPN1*-mutant myotubes [62]. Pre-treatment of the *SEPN1*-deficient cells with the flavonoid fisetin or the carotenoid astaxanthin prior to H_2O_2 exposure had a partial or null protective effect. These findings demonstrate that NAC is an effective ex vivo treatment of SEPN1 deficiency. Remarkably, they also establish that not all types of antioxidant drugs have the potential for therapeutic replacement of a particular selenoprotein function. The specificity of NAC consists in its Cys-donor activity, which could partially replace the hypothetical thiol exchanger activity of Sec in SEPN1. However, this molecule also has other numerous biological roles [68]. NAC is one of the rare antioxidant drugs approved for human use, has been shown to inhibit muscle fatigue in healthy adults and has no serious side effects when administered at the established dosage. This, together with its ex vivo efficiency on *SEPN1*-defective cells and recent data showing an in vivo effectiveness in the *Sepn1* KO mouse model [69], paves the way to a first therapeutic trial of SEPN1-RM in human patients, which is currently under preparation.

In summary, our current knowledge shows SEPN1 as a key protein at the crossroads of cell stress, redox signaling, Ca^{2+} homeostasis and cell survival pathways. More importantly, it identifies SEPN1-RM as one of the rare monogenic human condition primarily due to antioxidant defense failure, and the only structural myopathy primarily due to oxidative stress and antioxidant protection failure. As such, it represents an useful model paradigm for more complex, multifactorial disorders (such as cancer, cardiovascular disorders, diabetes or aging) in which selenoproteins have been implicated. Therefore, finding a specific pharmacological treatment for this rare disease could also open interesting perspectives to human health at large.

40.7 Selenoprotein N Expression, Phylogeny and Predicted Functions

The human *SEPN1* gene is located on chromosome 1p35-36 and is composed of 13 exons, the UGA selenocysteine codon resides within exon 10. This gene codes for two alternative transcripts and the shorter isoform, in which exon 3 is spliced-out, is predominantly expressed. This third exon consists of an Alu sequence that contains an additional in frame UGA codon, and accordingly is found only in primates. It was reported that only the shorter isoform is translated into protein. Therefore, it was assumed that that the UGA codon located within exon 3 of the larger isoform is not reprogrammed by the SECIS element into a Sec codon, but rather recognized as a premature stop codon [70]. Inclusion of the Alu-derived exon may be important for regulating *SEPN1* activity in muscle, since qPCR analysis revealed a tissue-specific increase in its inclusion level between humans and chimpanzees [71]. In agreement with the non–coding nature of the longer transcript, the *SEPN1* transcript was not found on the list of the protein-coding Alu exons established on the basis of high-throughput proteomic and ribosome profiling data [72].

The major transcript of *SEPN1* codes for a 556 amino acid protein with a predicted mass of 62 kDa, but migrating at a higher apparent molecular weight due to protein modifications. Glycosylation of the protein was experimentally validated using deglycosylation assays [70]. SEPN1 is an endoplasmic reticulum (ER) resident protein. The ER targeting sequence located between residues 30–49 consists of a di-arginine motif and a stretch of hydrophobic amino acids located at the N-terminus of the protein [70]. Protease protection experiments allowed the characterization of SEPN1 topology. SEPN1 contains a single transmembrane domain located at the N-terminus and it corresponds to a type II transmembrane protein with a single membrane-spanning domain at the N-terminus. Most of the protein, including the predicted active site, is localized within the lumen of the ER (unpublished data). This topology is in agreement with the amino acid charge distribution flanking the hydrophobic membrane signal, the so-called "positive inside rule".

In several instances, prediction of selenoprotein redox activity has been derived from protein sequence analysis and alignment. Bioinformatics searches using SEPN1 protein sequence revealed no significant homology to any other known protein. In SEPN1, aside from the N-terminal transmembrane domain, motif prediction searches identified a typical EF-hand motif, corresponding to a Ca^{2+} binding domain. Selenoprotein activity may also be deduced from the sequence context of the Sec residue, which constitutes a landmark of the catalytic center. SEPN1 harbors a SCUG predicted catalytic site, reminiscent of the thioredoxin reductase GCUG motif. This similarity suggests a reductase activity. However, the classical animal thioredoxin reductases contain two additional important domains: the FAD and the NADPH binding domains. These two domains, essential for reduction of the N-terminal thiol active site and electron transfer to the C-terminal selenenylsulfide bond [73], are absent in SEPN1. Lack of this second redox active center might be compensated by interactions with partners that remain to be identified. In addition, the highly accessible selenolate active site at the C-terminus of thioredoxin reductase is supposed to confer a broad substrate specificity to these enzymes, from small molecules such as selenite, lipid hydroperoxides, and dehydroascorbate, to proteins such as thioredoxin, protein disulfide isomerases or glutathione peroxidases. In contrast, the localization of the active site in the central part of SEPN1, therefore possibly less accessible, might reflect a higher selectivity of SEPN1 for its substrate(s). Moreover, SEPN1 and the thioredoxin reductases are located within different cellular compartments, the endoplasmic reticulum and cytoplasm, respectively, whose display are significantly different redox potentials [74]. The endoplasmic reticulum contains the most oxidative systems to introduce disulfide bounds into secreted proteins [74]. The redox level of the compartment directs the oxidative status of the enzyme reactive center, and therefore the nature of the catalytic reaction, oxidation versus reduction. In addition, the SCUG catalytic motif of thioredoxin reductase was shown to be involved in two consecutive reactions; on one hand the reduction of thioredoxin, and on the other hand, the oxidation of a N-terminal two Cys redox motif (Cys59-Cys64 of thioredoxin reductase), which can accept electrons from the protein-bound FAD [73]. Whether SEPN1 is also involved in a similar electron transfer or catalyzes one reaction and then regenerated by another cellular redox

system is currently not known. Interestingly, the SEPN1 C-terminal domain between positions 389–566, which contains the Sec residue, shares similarity with the UAS domain, a structural motif of the thioredoxin-like superfamily [75, 76]. This domain is present in proteins such as the FAS-associated factor 1 (FAF1) or the UBX domain-containing proteins, UBDX7 and UBDX8. In FAF1 and UBDX8, the UAS domain was shown to bind long-chain unsaturated fatty acids and to mediate polymerization of the proteins [77]. Moreover, the thioredoxin-like fold of SEPN1 was modeled based on its homology with other oxidoreductases and it revealed that the SCUG redox motif is correctly positioned for catalysis [75, 76].

Orthologs of SEPN1 were identified in many animal species, both vertebrate and invertebrate, including sponges, annelids, echinoderms or some arthropods. However, based on the available sequenced genomes, the SEPN1 coding gene was not detected in protists, nematodes, or crustacean and hexapode lineages. Protein sequence comparison revealed strong conservation among animal species, notably two blocks of amino acids, the second one flanking the Sec residue is likely to form the active catalytic pocket [52, 78].

The *SEPN1* gene had already evolved in ancestral metazoans, which are organisms lacking organized muscle structures. This phenomenon suggested that SEPN1 possesses an original function unrelated to muscle differentiation and maintenance. Moreover, analysis of *SEPN1* expression pattern in human or mouse tissues demonstrated that SEPN1 expression is not specific to the muscle tissues, but present ubiquitously in all tissues examined [79]. Together, the expression pattern and phylogenic distribution of SEPN1 suggested that this selenoprotein may have additional functions that remain to be characterized. Another interesting feature revealed by the sequence comparison of SEPN1 homologs from different animal species, is that there exists no SEPN1 with a Cys instead of the Sec residue. Most selenoproteins have Cys orthologs or paralogs, and Sec forms a selenenylsulfide bond resulting in a redox motif equivalent to the Cys containing ones in non-selenoproteins oxidoreductases [80]. Hence, this observation suggests that the presence of a Sec residue is strictly required for SEPN1 catalytic activity, a situation that may be important to address the unique role of this unique amino acid in selenoenzymes, once this activity is characterized.

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Chapter 41
Tissue-Specific Regulation of Thyroid Status by Selenodeiodinases

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Abstract The iodothyronine deiodinases activate (Type 1 and 2 deidoinases or DIO1 and DIO2) or inactivate (DIO1 and the Type 3 deiodinase or DIO3) thyroid hormone depending on which iodine is removed from the iodothyronine molecule. These enzymes are selenoproteins, containing the rare amino acid selenocysteine (Sec) in their active center. Sec insertion sequence binding protein 2, SECISBP2, is an important component of the selenoprotein synthesis machinery, and its binding to the Sec insertion sequence (SECIS) element is crucial for Sec incorporation at the UGA codon to override termination. In patients with impaired selenoprotein synthesis due to SECISBP2 mutations, abnormal serum thyroid hormone profiles due to alterations in deiodinase activity are found. The deiodinase enzymes can also play important roles in the modulation of T3 within specific tissues. One example of this is the concerted regulation of *Dio2* and *Dio3* expression after muscle injury in mice which tightly controls intracellular T3 levels in muscle stem cells during muscle regeneration. Thus, expression of the deiodinase selenoproteins regulates both circulating and intracellular levels of active thyroid hormone independently of the hypothalamic-pituitary axis with significant physiologic consequences.

Keywords Deiodinase • Muscle • SECISBP2 • Thyroid hormone • Thyroxine • Thyroid hormone receptor

41.1 Introduction

Thyroid hormone, 3,3′,5-triiodothyronine or T3, regulates a variety of processes including growth, development, and metabolic rate. In order to produce these effects, T3 enters the nucleus and binds to thyroid hormone receptors, which in turn bind to

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specific DNA sequences in T3-responsive genes, regulating their transcription in both a positive and negative manner [1]. Accumulating evidence also suggests that T3 may have some non-genomic effects [1]. The thyroid gland predominantly produces thyroxine (T4), which has low biological activity, with ~10-fold lower affinity for thyroid hormone receptors than T3 [2]. Thus, the enzyme-catalyzed removal of an outer-ring iodine from T4 by DIO1 and DIO2 to produce T3 is an essential step in thyroid hormone action. Conversely, DIO3, and under some conditions DIO1, can inactivate T3 and T4 by the elimination of an inner-ring iodine, generating the biologically inactive T2 or reverse T3 (rT3), respectively. Accordingly, the iodothyronine deiodinases modulate T3 action by regulating both its production and degradation [3, 4].

41.2 Deiodinases Are Integral Membrane Selenoproteins Containing a Thioredoxin- (TRX)-Fold that Are Regenerated by a Peroxiredoxin-Like Mechanism

All three deiodinases are homodimeric integral membrane proteins containing one transmembrane domain [5–8]. DIO1 and DIO3 are found in the plasma membrane, while DIO2 is located in the endoplasmic reticulum [9, 10]. Nuclear localization has also been recently reported for both DIO2 and DIO3 [11, 12]. While sequence identity between the deiodinases is less than 50 %, all share a conserved active center of ~15 amino acids containing the rare amino acid Sec (Fig. 41.1) [4]. *DIO2* also has an additional UGA codon positioned seven amino acids before a UAA stop codon, and while ^{75}Se labeling studies indicate Sec is incorporated at this site, mutational studies suggest that this second UGA plays no role in DIO2 enzyme function [13].

Insight into the structure of the deiodinase enzymes has been obtained through *in silico* protein modeling using hydrophobic cluster analysis, and was further recently refined by X-ray crystallography of the catalytic domain of DIO3 [14, 15]. Using these methods it was determined that deiodinases share a common overall structure with a single transmembrane segment in the N-termini, and several clusters of α-helices or β-strands composing deiodinase globular domains [15, 16]. These are

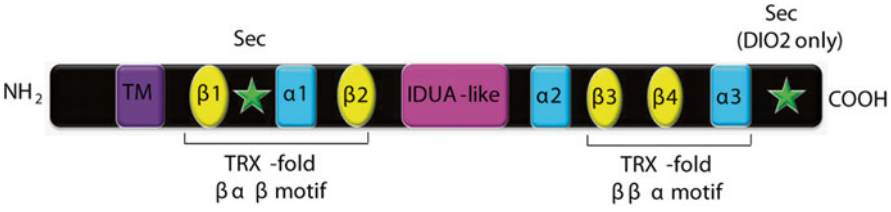

Fig. 41.1 General deiodinase structure. Deiodinases are integral membrane proteins containing one transmembrane domain (TM), and a TRX fold, defined by βαβ and ββα motifs, interrupted by an intervening element similar to α-L-iduronidase (IDUA-like). All deiodinases contain a Sec (Sec, indicated by a *star*) within the first TRX βαβ motif. DIO2 has an additional Sec, located seven amino acids before a TAA stop codon

arranged to form a TRX-fold, defined by βαβ and ββα motifs, which is also found in many other thiol oxidoreductases, including the glutathione peroxidase (GPx) selenoproteins [17]. Uniquely, the βαβ and ββα motifs within the canonical TRX-fold of the deiodinases are locally interrupted by intervening elements forming a compact protrusion with sequence homology to α-L-iduronidase (IDUA), a lysosomal enzyme that cleaves α-linked iduronic acid residues from glycosaminoglycans (Fig. 41.1) (also referred to as the deiodinase-specific insertion D-loop) [15, 16]. This structure, taken in context of the homodimeric nature of the deiodinases, leads to a model that predicts the active center to be formed by the β1- α1- β2 motifs of the TRX-fold and one of the IDUA intervening elements, with the Sec residue being contained in this pocket where iodothyronine binding occurs. Analysis of crystallized DIO3 further predicts that dimerization will displace an autoinhibitory region between α2/β3 from the iodothyronine binding site, and that the Sec residue is found at the same position as the peroxidatic cysteine (Cys) of TRX-fold containing thiol reductases such as TRX reductase, peroxiredoxins, and GPx [15]. Notably, mutations in the active center change kinetic properties of deiodinases, thus confirming the relevance of this model and further suggesting conformational changes during catalysis [15, 16]. In this regard, amino acid 128 of DIO1 is a serine, while proline is found in the corresponding position of DIO2 [16]. This position is predicted to modulate local rearrangements required for the resolution of the selenenyl-iodine intermediate within the deiodinase, and the presence of a bulky proline residue in this position would shield the selenenyl-iodide intermediate from the uncompetitive inhibitor propylthiouracil (PTU) [15]. Accordingly, the serine-containing DIO1 is normally PTU sensitive with ping-pong kinetics of substrate catalysis. However, a Ser128Pro modification of the DIO1 enzyme results in resistance to PTU and a change to sequential kinetics, making DIO1 more similar to DIO2. Furthermore, the corresponding substitution of Ser for Pro in the equivalent position of DIO2 made it more similar to DIO1, with the mutant DIO2 now being sensitive to PTU and displaying ping-pong kinetics [16, 18].

The crystal structure of the DIO3 catalytic domain further elucidates a path for proton transfer to substrate during deiodination that explains the previous negative effects of mutations of conserved histidine and glutamic acid residues in DIO1 and DIO2 [15, 16, 19]. Solving the DIO3 crystal structure identified similarity to other TRX-fold containing proteins of the atypical 2-Cys peroxiredoxin family [15]. This suggested a peroxiredoxin-like mechanism for the regeneration of the oxidized deiodinase enzyme, wherein the selenenyl-iodide intermediate is resolved by formation of an intramolecular selenenyl-sulfide intermediate, that would further react with an intramolecular Cys to form a disulfide bond which could be more easily be reduced by endogenous thiols [15].

Endogenous thiols such as glutathione, TRX, and glutaredoxin are required for deiodinase activity in vitro [15, 20, 21]. Within the cell, levels of these co-factors are depleted by the oxidative stress induced by the loss of the selenoproteins glutathione peroxidase or TRX reductase, and thus this represents an indirect way that selenium deficiency might decrease deiodinase function [22]. In this regard, deiodinase function has been shown to be negatively regulated by oxidative stress which

can be partially overcome by selenium supplementation [23, 24]. Additionally, the peroxide-reducing enzyme Peroxiredoxin 3 (PRX3) is a DIO3-interacting protein involved in the reducing network necessary to regenerate the oxidized DIO3 enzyme after catalysis [25]. Since oxidized PRX3 is in turn regenerated by TRX, a decrease in the selenoprotein thioredoxin reductase and consequent decrease in PRX3 regeneration could be another indirect mechanism by which deiodinase function could be regulated by selenium [26].

41.3 SECISBP2 Mutations Result in Altered Deiodination and Thyroid Hormone Profiles

As with all selenoproteins, UGA encodes for the insertion of Sec during translation of the deiodinases [27–29]. UGA is normally read as a signal for termination, and in order for this codon to specify Sec incorporation, additional components are required to facilitate translational read-through of selenoproteins such as the deiodinases [4, 30]. In fact, it was the expression cloning of the DIO1 enzyme that allowed the recognition that eukaryotic selenoprotein mRNAs require a stem-loop structure in their 3′-untranslated region for successful Sec incorporation, termed the SECIS element [27, 31]. In brief, deiodinase synthesis requires the SECIS element that recruits the binding protein SECISBP2 [32]. SECISBP2 in turn interacts with an elongation factor, EFsec, promoting the insertion of Sec from a specific tRNA (Sec-tRNA^{Sec}) by the ribosome at the UGA codon [33, 34]. Sec incorporation is not very efficient, and if the UGA codon in *DIO1* is mutated to a Cys codon, up to 400-fold more DIO1-protein was produced in transfected cells; however, catalytic efficiency is also reduced in parallel [35]. Thus, all the factors necessary for selenoprotein production are needed for deiodinase synthesis, and changes in their availability could seriously disrupt thyroid physiology. This concept has recently been highlighted by the identification of patients with abnormal thyroid hormone profiles caused by defects in deiodinase synthesis as a result of mutations in SECISBP2 [36].

The first patient found with a SECISBP2 mutation was identified due to growth retardation, with the affected individual falling below the third percentile of growth at 14 years of age [36]. Thyroid hormone is known to profoundly influence growth and development, and additional studies identified abnormal thyroid profiles in the proband and three out of seven of his siblings, with an elevated circulating TSH and T4, while serum T3 was below the normal range [36]. Of note, TSH is negatively regulated by T3, both at the level of transcription and secretion, and this feedback loop requires both circulating T3, as well as T3 produced by the deiodination of T4 by DIO2 in the pituitary and hypothalamus [37]. In this regard, T4 administration is more potent in decreasing serum TSH levels, and studies have shown that conversion of T4 to T3 within the thyrotropes of the pituitary *via* DIO2 is necessary to produce this effect [38]. Further, in mice with either a global or thyrotrope-specific deletion of *Dio2*, serum TSH can be suppressed by T3, but not T4 treatment [39–41].

With this in mind, a key piece of information in discovering the basis of the deficit in thyroid hormone metabolism in the above patients was that T3 administration suppressed TSH in all family members, wherein T4 was ineffective in affected individuals. This indicated that the affected individuals might have a deficit in DIO2 (and potentially DIO1, see below)-mediated T4 to T3 conversion. Using cultured skin fibroblasts, it was determined that affected individuals had considerably lower DIO2 enzyme activity [36]. Notably, *DIO2* mRNA levels of these patients were not decreased, suggesting that the lower DIO2 activity was due to a post-transcriptional defect. The parents of the proband belong to the same Bedouin tribe, and linkage analysis identified homozygosity for a missense mutation in the RNA binding domain of SECISBP2 in affected individuals. Further studies revealed that the selenoproteins GPx1 and selenoprotein P were also decreased in these individuals, suggesting a global defect in selenoprotein synthesis. The DIO1 enzyme also converts T4 to T3, further contributing to circulating levels of T3, and thus impaired expression of this selenoprotein could contribute to the abnormal thyroid hormone profiles found in these patients. DIO1 is found in less accessible tissues such as liver, kidney and thyroid, and DIO1 activity has not been evaluated directly in any of these patients. A concomitant decrease in DIO1 in these patients is supported by a mouse model where *Secisbp2* was deleted in liver, leading to over a sixfold decrease in hepatic DIO1 activity [42].

Since this initial report, several other patients have been identified with different mutations in *SECISBP2* that also inhibit selenoprotein synthesis [43–46]. All patients have had abnormal thyroid function tests, with serum T3 concentrations being either low, or in the low range of normal, high serum T4, and either elevated or inappropriately normal serum TSH, since TSH should be decreased when T4 is elevated. Thus, an impairment of T4 to T3 conversion by DIO2 in the hypothalamic-pituitary axis, and potentially defects in the production of T3 by DIO1 containing tissues, appears to be a hallmark of this syndrome. Notably, the linear growth of these patients was improved by T3 administration; however, not surprisingly, selenium supplementation did not normalize serum thyroid hormone profiles [36, 44, 47]. Interestingly, a detailed characterization of these patients indicated that their symptoms cannot be solely attributed to hypothyroidism caused by impaired T4 activation *via* the deiodinases, suggesting these phenotypes are due to deficiencies in other selenoproteins [43, 44].

41.4 Local Control of Thyroid Hormone Concentrations by DIO2 and DIO3 Is Critical for Tissue Specific Regulation of Thyroid Hormone Action

It has been estimated that normally ~80 % of the daily circulating T3 produced in humans is from T4 deiodination, while ~20 % is derived from direct thyroidal secretion [4]. In rats, about ~60 % is derived from deiodination and ~40 % from thyroidal

secretion. The deiodinases also allow for intricate regulation of intracellular T3 concentrations in a tissue-specific fashion while circulating T4 and T3 remain unchanged [48]. This becomes important when specifically-timed changes in T3 concentrations are required in specific tissues for developmental processes or after injury, since it would be impossible to produce such subtle changes solely through modulation of the levels of circulating T3.

DIO2 and DIO3 are the main players in the local regulation of T3 within tissues, while DIO1 contributes principally to circulating T3 levels, especially during hyperthyroidism [3, 4] (Fig. 41.2). As mentioned, DIO2 is a T4 activating enzyme, producing T3 by removing one iodine from the outer ring of T4. The DIO2 enzyme is found in the endoplasmic reticulum, and thus T3 produced from DIO2 is thought to preferentially supply T3 to the nucleus [9, 37, 49]. DIO2 levels are regulated post-translationally, with increased catalysis of T4 to T3 leading to increased ubiquitination and degradation of the DIO2 protein, which also contributes to the tight regulation of T3 production by this selenoenzyme [50]. One of the best-characterized functions of DIO2 is its role in mediating a thermogenic response of brown adipose tissue (BAT) [51–53]. Thus, when rats are acutely cold exposed, DIO2 activity

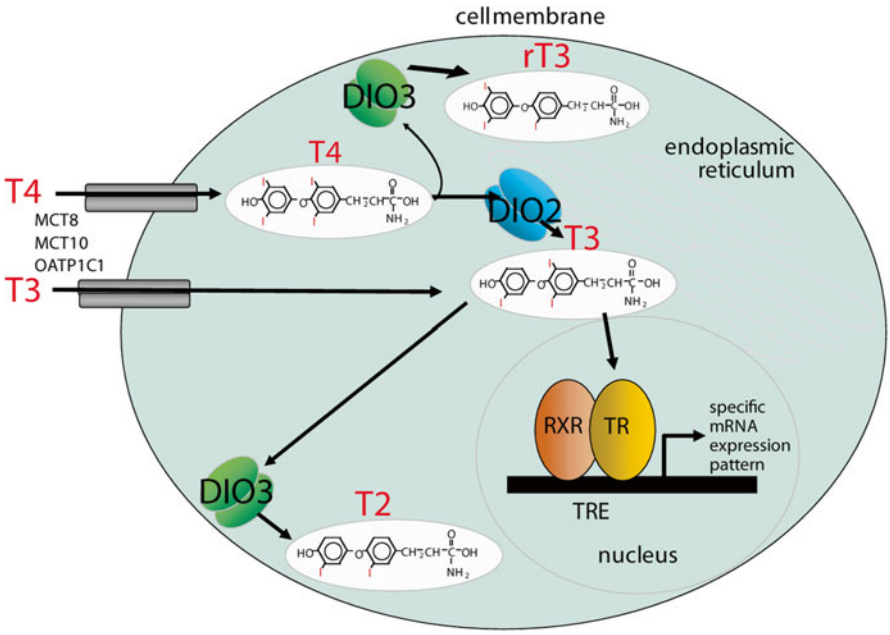

Fig. 41.2 DIO2 and DIO3 can fine tune tissue thyroid hormone concentrations. T3 or T4 enters the cell *via* specific T3 transporters such as Monocarboxylate transporter 8 (MCT8), Monocarboxylate transporter 10 (MCT10), or Organic anion transporter 1C1 (OATP1C1). In cells expressing DIO2, T4 can be converted to T3 while in those expressing DIO3, it can be inactivated to rT3. DIO3 can also inactivate T3 to 3, 3′-T2. It is unlikely that DIO2 and DIO3 are expressed in the same cell (as shown), but may be present in different cells within the same tissue, or in the same cell at different times

increases in BAT, increasing the amount of T3 in this tissue, while circulating levels remain unchanged [51]. The net result is that thyroid hormone receptor occupancy increases from ~50 % at room temperature to ~95 % in cold exposed BAT, while receptor occupancy levels in other tissues remain virtually unchanged. These changes facilitate a program of gene expression in BAT that allows adaptation to cold exposure [54]. In line with this, *Dio2* knockout mice have an impaired thermogenic response to cold exposure, only surviving by compensatory shivering [55].

Conversely, the thyroid hormone signal can be reduced at the tissue/cellular level *via* inactivation of T4 and T3 by DIO3. DIO3 preferentially removes an iodine from the inner ring of thyroid hormones, thus converting T4 and T3 to the biologically inactive reverse T3 (rT3) and 3, 3′ T2, respectively. The DIO3 enzyme is located in the plasma membrane and is recycled through the early endosomes, inactivating thyroid hormones before they are able to access the nucleus and occupy thyroid hormone receptors [10, 56, 57]. A striking example of the downstream effects of DIO3 can be found in basal cell carcinomas (BCCs) [58]. In these tumors, *DIO3* over-expression is driven by aberrant activation of sonic hedgehog. Notably, when *DIO3* expression is blocked or excess of T3 provided, BCC cell proliferation rates are reduced and tumor formation is impaired.

The use of *Dio2* and *Dio3* global and tissue-specific knockout mice has uncovered many diverse physiological roles of local T3 production and inactivation. These include: maturation and function of the thyroid axis, brown adipose activation during cold exposure, chondrocyte differentiation, development of the auditory and vision systems, optimal bone strength and mineralization, energy metabolism and control of glucose secretion, cardiac function and response to ischemia, testis development, and muscle regeneration [39–41, 55, 59–71]. To illustrate the importance of DIO2 and DIO3 in regulating local T3 supply, we will focus on the role of DIO2 and DIO3 in one model system, regeneration of skeletal muscle after injury (see below).

41.5 DIO2 and DIO3 Play Key Roles in Skeletal Muscle Regeneration During Injury

Both hyper- and hypothyroidism have been recognized for decades as having severe adverse effects on muscle function [70–74]. However, until recently, it was thought that most of the T3 in skeletal muscle was derived from circulation [75]. New data have revealed the programmed local inactivation of thyroid hormone by DIO3 and a subsequent DIO2-mediated T4 activation is essential for normal muscle repair [70, 71]. Satellite cells are the muscle stem cell equivalents, forming the new myoblast precursors after injury [76]. During muscle regeneration, muscle satellite cells proliferate, followed by differentiation into myoblasts and fusion to form myofibers (Fig. 41.3) [76]. *Dio2* mRNA is expressed in muscle, and low DIO2 activity can be measured in adult mice [77]. *Dio2* mRNA and activity are higher in differentiating

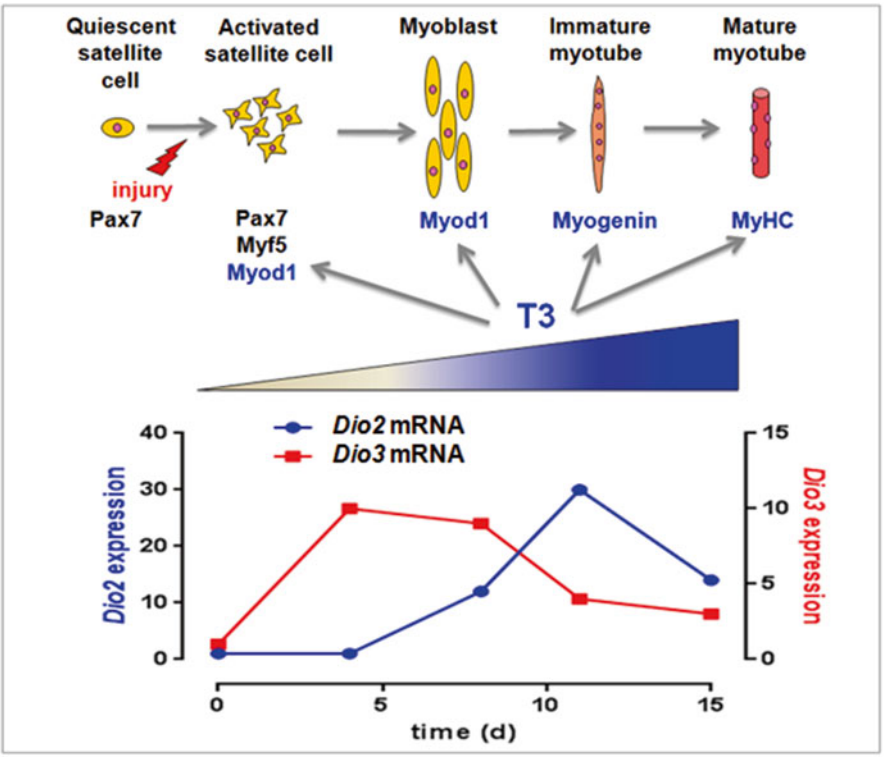

Fig. 41.3 Changes in *Dio3* and *Dio2* regulate T3 concentration leading to satellite cell differentiation during regeneration. After skeletal muscle injury (*red* lightning bolt), Pax7-expressing satellite cells become activated and proliferate, while *Dio3* expression is increased leading to low intracellular T3 (*lower blue* shading in the *triangle*). Conversely, as *Dio3* falls, *Dio2* expression increases leading to increased T3 (*higher blue* shading in the *triangle*), and the T3-dependent expression of transcription factors such as Myf5, Myod1, Myogenin, and Myosin Heavy Chain (MyHC), which promote transition to myoblasts, immature myotubes, and finally mature myotubes. Adapted from [70, 71]

versus proliferating cultures of mouse primary muscle precursor cells, and increase prior to a subsequent increase in the T3-responsive gene *MyoD*, the master regulator of the myogenic developmental and regeneration program [70]. Further studies blocking *Dio2* induction in vitro via RNAi inhibited muscle differentiation, and this was overcome by T3 supplementation [70].

In vivo, muscles of *Dio2* knockout mice displayed mild hypothyroidism, with the expression of T3-responsive genes such as MyoD, Myogenin, Troponin 2, and SERCA2 all being decreased [70]. The regeneration potential of *Dio2* knockout mouse muscle was further evaluated using a model where the anterior tibialis muscle was wounded by cardiotoxin injection. This caused localized muscle damage, which was followed by a robust regenerative response in wild type animals, with DIO2 activity and *Dio2* mRNA expression peaking in the injured tissue at 8–11 days

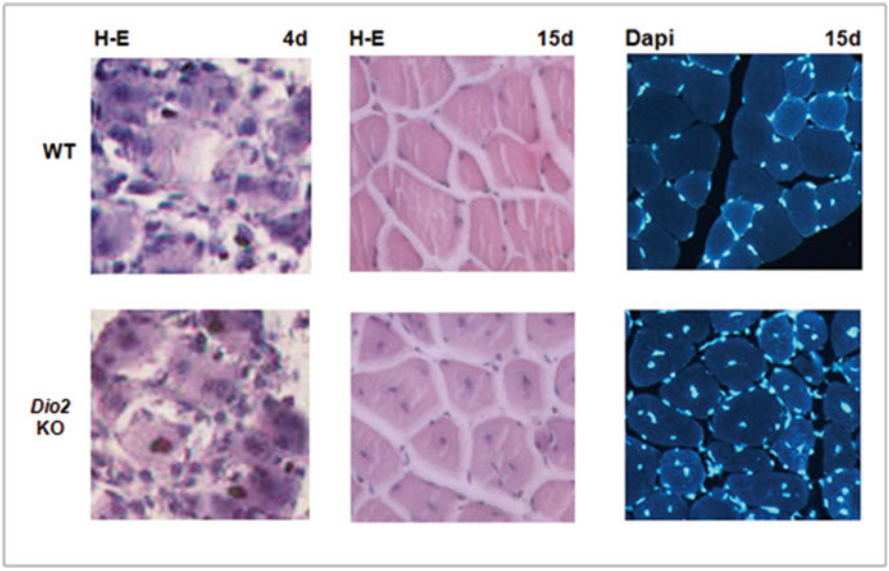

Fig. 41.4 *Dio2* knockout mice exhibit impaired muscle regeneration after injury. Mice were injured by cardiotoxin injection of their anterior tibialis muscle at day 0. No difference was observed between wild type and *Dio2* knockout mice by H&E staining 4 days after injury. However, at 15 days post-injury, *Dio2* knockout mice exhibit a significant delay in regeneration, as shown by the increased numbers of centrally localized nuclei characteristic of immature myofibrils visualized by either H&E staining or with the nuclear stain DAPI. The figure was modified from Dentice et al. [70]

after injection (Fig. 41.3). Notably, wild type and *Dio2* knockout mice displayed no difference in the extent of injury at 4 days after injection (Fig. 41.4). However, *Dio2* knockout mice had significantly delayed regeneration at 15 days post-injury when this process is complete in wild type mice, with a large number of centrally nucleated immature myofibers (Fig. 41.4). Cellular proliferation rates were also assessed using 5-bromodeoxyuridine (BrdU), which is incorporated into newly synthesized DNA in place of thymidine. Fifteen days post-injury, the number of BrdU labeled nuclei found in muscle of *Dio2* knockout mice was twice that of wild type mice, indicating a greater percentage of cells still replicating. These results illustrate the importance of muscle DIO2 during regeneration after injury.

In many other systems, local thyroid hormone content is tightly regulated by reciprocal changes in DIO2 and DIO3 [3]. This also holds true in the muscle injury model, where the increase in DIO2 after injury is preceded by elevated *Dio3* mRNA, and DIO3 protein and enzymatic activity, peaking 3–5 days post-injury (Fig. 41.3) [71]. Co-immunofluorescence analysis showed DIO3 staining in PAX7-positive progenitor cells. However, in mice where the *Dio3* gene was conditionally deleted in PAX7+ satellite cells 4 days after cardiotoxin-induced injury there was a notable reduction in PAX7-positive cells due to massive apoptosis [71]. At 14 days post-injury, when regeneration is complete in a wild type mouse, there was a dramatic

reduction in the numbers of regenerating fibers. Using the pp6 primary satellite cell model, *Dio3* loss was found to lead to aberrant T3-dependent induction of *FoxO3* and a premature expression of genes associated with muscle differentiation which led to apoptosis [71].

Taken together, these experiments indicate the local increase of T3 production mediated by DIO2 in satellite cells is critical for proper muscle regeneration, and further, that DIO3 is required prior to this increase to protect the satellite cell from premature T3-dependent induction of differentiation (Fig. 41.3). These exciting observations suggest that modulation of the thyroid status of muscle precursor cells by manipulation of deiodinases could have therapeutic application for patients with muscle disease.

41.6 Concluding Remarks

One key feature of all the deiodinase enzymes is Sec in their active center. Thus, deiodinases are subject to all the constraints that will also regulate selenoprotein synthesis. An example of this can be found in patients with mutations in SECISBP2 that exhibit abnormal serum thyroid hormone profiles due to deficits in synthesis of DIO2. Another important role of deiodinases is the local regulation of thyroid hormone activation and inactivation such as during muscle differentiation and regeneration. These examples highlight the permissive role of selenium in normal thyroid hormone action.

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Chapter 42
The Role of Selenoproteins in Resolution of Inflammation

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Abstract Inflammation is an evolutionarily conserved physiological response mounted with an intention to resolve the insult inflicted on the biological system. This process is extremely well orchestrated, where many cells, including macrophages, play key roles in facilitating discreet steps leading to the successful resolution and wound healing. As a result of oxidative stress during the initial phase of inflammation, cellular redox homeostasis holds utmost importance in shaping and dictating the cellular response, where selenoproteins play a critical role. Our studies in mice that lack macrophage expression of selenoproteins, via the deletion of *Trsp*, have demonstrated selenoprotein expression to be key in their polarization from a pro-inflammatory cell type towards an anti-inflammatory phenotype, which aids in resolution of inflammation and wound healing. Such a transition is accompanied by changes in cellular metabolism, particularly that of arachidonic acid, which leads to the formation of many bioactive oxylipids, including cyclopentenone prostaglandins (CyPGs). These endogenous metabolites differentially modulate signaling cascades and transcription factors to effect the switch in macrophage polarization. The ramifications of selenoprotein-dependent macrophage polarization in the resolution of inflammation in rodent models involving gut inflammation by chemical injury and parasitic infections are discussed here. In addition, the anti-carcinogenic role of macrophage-derived CyPG, produced in a selenoprotein-dependent manner, to impact the viability of cancer stem cells in hematologic malignancies will be discussed. Finally, we describe a new role for selenoproteins in the tissue microenvironment in shaping efficient stress erythropoiesis that is critical to resolve anemia that follows inflammation.

Keywords Cyclopentenone prostaglandins • Eicosanoid • Erythropoiesis • Helminth infection • Leukemia stem cells • Macrophage polarization • Macrophage selenoproteins • Prostaglandin J_2

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42.1 Overview of Inflammation and Its Resolution

Inflammation is a physiological response to cellular insult by diverse stimuli initiated and driven by external signals and endogenous chemical mediators. Acute inflammation is characterized by leakage of serum proteins and leukocytes from blood to the extravascular tissue. These tightly regulated events result in predominance of neutrophils in the inflamed area, which are gradually replaced by monocytes that differentiate into macrophages. As a result of phagocytic activity, the entrapped foreign material is degraded by hydrolytic and proteolytic enzymes along with reactive oxygen and nitrogen species (RONS) in the phagolysosomes leading to digestion of the invading pathogen. Therefore, the acute inflammatory process often operates to protect the host. However, a collateral activity of acute inflammation is the local tissue damage and amplification of acute inflammatory signals that result in chronic inflammation [1]. Such prolonged inflammation contributes to the pathogenesis of many diseases including cancer, neurodegenerative diseases, anemia, gastrointestinal, cardiovascular, and autoimmune disorders [2].

It is evident that endogenous anti-inflammatory mediators reverse vascular changes, inhibit leukocyte migration and activation, while promoting safe removal of leukocytes by apoptosis and subsequent phagocytosis by macrophages resulting in resolution post-acute inflammation [1]. Research in recent years has uncovered new endogenous lipid mediators that have potent immunomodulatory and anti-inflammatory effects in the form of lipoxins, resolvins, protectins and CyPGs. The bioactivities of these endogenous specialized pro-resolving mediators (SPMs) include decreased neutrophil influx and leukocyte extravasation and counter regulation of pro-inflammatory cytokines such as tumor necrosis factor-α (TNFα) in mice [3, 4].

Exacerbated production of RONS contributes significantly to inflammatory disease states. Particularly, hydrogen peroxide and peroxynitrite have the ability to interact with many cellular lipids and proteins to elicit pathways of inflammation [5]. These have been shown to modulate intracellular second messengers to regulate upstream kinase pathways involving signal transducers and activators of transcription (STAT)-3, p38, JNK, Akt, and NF-kB to shape the inflammatory response [6]. Therefore, to regulate oxidative stress in particular and inflammation in general, biological systems have evolved with diverse antioxidant based anti-inflammatory or pro-resolving strategies in a variety of immune cells [7].

Macrophages are innate immune cells that exist throughout the body [8]. They are embryonically derived and maintained in tissues, as well as differentiated from infiltrating bone marrow-derived monocytes during inflammation [9, 10]. During inflammation, macrophages serve three major functions: i) antigen presentation; ii) phagocytosis; and iii) immunomodulation through production of various cytokines, lipid mediators and growth factors. Depending on the stimulus, macrophages adopt different activation states. IFN-γ, TNFα, granulocyte macrophage-colony stimulating factor (GM-CSF), lipopolysaccharide (LPS), and other Toll-like receptor (TLR) ligands promote classically activated (M1) macrophages, which are considered

pro-inflammatory because of their production of RONS [11, 12]. Interleukin (IL)-4, IL-13, and IL-10 promote alternatively activated (M2) macrophages, which are considered anti-inflammatory because of their production of arginase-1 (Arg-1) [11–13]. Arg-1 competes with inducible nitric oxide synthase (iNOS) for the substrate L-arginine to produce L-ornithine and urea instead of nitric oxide, and, therefore, helps reduce inflammatory RONS [14]. This classification of macrophages represents two ends of a spectrum with poorly defined intermediates [11]. The M2 macrophages play a critical role in the initiation, maintenance, and resolution of inflammation that involves cessation of neutrophil influx and macrophage-mediated clearance of debris and apoptotic bodies (efferocytosis). Efferocytosis results in generation of SPMs, which play a key role in resolution of inflammatory process [15] in addition to driving the polarization of macrophages towards an M2 phenotype [16]. Of relevance to this discussion is the endogenous production of cyclooxygenase (COX)-derived CyPGs in macrophages that also contribute to the resolution of acute inflammation [17], where selenoprotein expression is pivotal.

42.2 Modulation of Arachidonic Acid Metabolism in Macrophages by Se

Macrophages produce an array of arachidonic acid (ARA)-derived prostaglandins (PG) in response to diverse stimuli, including the TLR4 ligand such as LPS. Upon activation, ARA esterified within the membrane phospholipids is mobilized by a calcium-dependent phospholipase A_2. ARA, thus released, is rapidly metabolized to a wide variety of bioactive eicosanoids by COX, lipoxygenases (LOX), and cytochrome P450 (CYP) enzymes. Studies related to metabolites of eicosanoids in inflammation have mainly focused on the signaling pathways activated by PGs that are produced by COX-1 and the inducible COX-2. The initial step of PG synthesis involves formation of PGH_2 from ARA by COX isozymes. COX-2 in macrophages functionally couples to downstream synthases such as microsomal PGE synthase-1 (mPGES-1), and thromboxane synthase (TXAS) to form PGE_2 and TXA_2, respectively, which possess pro-inflammatory properties. COX-1 (as well as COX-2) functionally couples with PGD synthases (H-PGDS and L-PGDS) to form PGD_2 from PGH_2 [18–20]. Unlike PGE_2 and TXA_2, PGD_2 undergoes spontaneous dehydration and isomerization to downstream metabolites, Δ^{12}-prostaglandin J_2 (Δ^{12}-PGJ_2) and 15-deoxy-$\Delta^{12,14}$ PGJ_2 (15d-PGJ_2), collectively called "CyPGs". Being potent Michael reaction electrophiles, CyPGs have wide ranging effects. 15d-PGJ_2 inhibits TNFα-stimulated expression of adhesion molecules in primary human endothelial cells as well as suppresses pro-inflammatory pathways, including NF-kB, AP1, and STATs in macrophages by 15d-PGJ_2 [21]. Previous work showed that Se plays an important role in preferential production of CyPGs in macrophages by differential regulation of expression of mPGES1, TXAS, and H-PGDS to favor

shunting of ARA towards H-PGDS pathway during inflammation [22], appropriately termed eicosanoid class switching, where incorporation of selenium (Se) into selenoproteins was pivotal. As mentioned later, disruption of such a highly orchestrated class switching process by non-steroidal anti-inflammatory drugs (NSAIDs), led to the reversal of anti-inflammatory and anti-carcinogenic effects of selenoproteins, including a block in the polarization of macrophages towards the M2 phenotype. Together, these studies suggest that selenoprotein-dependent modulation of ARA metabolism via the COX pathway in immune cells could impart some of the anti-inflammatory and/or pro-resolving effects of Se.

CyPGs formed covalent adducts with a reactive cysteine thiol in the active site of IkB kinase-2 (IKK2) leading to the inhibition of NF-kB in macrophages only when selenoproteins were expressed [23]. CyPGs also suppressed NF-kB through ligand-dependent activation of peroxisome proliferator activated receptor (PPAR)γ [24, 25]. Such an activation of PPARγ also required expression of selenoproteins that was key in the polarization of macrophages towards an M2 phenotype [26]. The ability of Se to regulate the expression of pro-inflammatory genes was associated with the inhibition of acetylation of histone and non-histone proteins. Se-dependent CyPG production was key to inhibiting the activity of histone acetyltransferase (HAT) p300 suggesting a role for Se and eicosanoid class switching in epigenetic regulation of gene expression [27, 28].

Recent studies have begun to dissect the role of selenoproteins in inflammation through mechanisms that involve the degradation of pro-inflammatory PGE_2. Selenoprotein expression was required to up-regulate the expression of 15-PG-dehydrogenase (15-PGDH), an enzyme that oxidizes lipid mediators with a preference towards PGE_2, specifically in the gut, to anti-inflammatory 15-keto-PGE_2 [29]. Such a metabolic inactivation of PGE_2 facilitated an anti-inflammatory response promoting resolution, as seen in a murine model of dextran sodium sulfate (DSS)-induced ulcerative colitis. Furthermore, pharmacological inhibition of 15-PGDH blocked the protective effect of Se supplementation in the DSS model confirming the importance of metabolic inactivation of pro-inflammatory PGE_2 to initiate pro-resolution responses. Se supplementation improved the disease activity index and decreased M1 markers, while up-regulating M2 markers such as IL-10, Fizz1, and Arg-1 in the colonic tissue to promote effective resolution of gut injury and restore epithelial barrier integrity.

42.3 Resolution of Gastrointestinal Inflammation During Helminth Infection

Nippostrongylus brasiliensis is a rodent helminth that has a similar life cycle to the human hookworms, *Ancylostoma duodenale* and *Necator americanus*, which are classified as soil-transmitted helminths (STH). STH infections are considered a neglected tropical disease and affect approximately two billion people worldwide

according to the World Health Organization. Depending on the severity of infection, helminths can cause physical, nutritional, and cognitive disabilities in infected individuals. The most widely implemented method for controlling STH infections is through periodic administration of anthelmintic drugs. Current treatments focus on reducing severity of infection and transmission rather than curing. However, there is increasing concern for anthelmintic drug resistance [30]. Discovery of alternative therapies to eradicate STH infections is essential.

It is well established that *N. brasiliensis*, among other STHs, promote a pronounced T-helper-2 (Th-2) cell response, characterized by increases in anti-inflammatory cytokines, such as IL-4 and IL-13, which impact polarization of macrophages [31–35]. M2 macrophages have been shown to promote resolution and are key components in immunity to parasitic helminths [34, 36, 37]. Selenoproteins act synergistically with IL-4, in vitro, to decrease expression of prototypical markers of pro-inflammatory M1 macrophages such as iNOS, IL-1β, and TNFα [13], while increasing the expression of Arg-1, Ym-1, and Fizz-1, characteristic of pro-resolution and reparative M2 macrophages [13]. Treatment of bone marrow-derived macrophages harvested from mice, on Se-deficient (<0.01 ppm) and Se-adequate (0.08 ppm as sodium selenite) diets, with the hematopoietic PGD_2 synthase inhibitor, HQL-79, significantly reduced the effect of Se on Arg-1 activity [13]. Additionally, treatment of these cells with either GW9662, a PPARγ antagonist, or leflunomide, a STAT6 antagonist, led to a significant decrease in M2 macrophage markers [13]. These studies suggest a synergistic effect of Se supplementation and IL-4 signaling to increase M2 macrophages (Fig. 42.1).

To examine the effect of Se in vivo, mice were infected with 500 L3 stage *N. brasiliensis* larvae and maintained on Se-deficient, Se-adequate, or Se-supplemented (0.4 ppm as sodium selenite) diet [26]. Se-deficient mice had a significantly higher parasite burden compared to Se-adequate and Se-supplemented mice. Additionally, increases in dietary Se levels increased M2 and decreased M1 in the jejunum of infected mice [26]. Infection of $Trsp^{fl/fl}Cre^{LysM}$ mice, which are deficient in selenoproteins in monocytes and macrophages, had a significantly higher parasite burden compared to $Trsp^{fl/fl}Cre^{WT}$ mice despite being on Se-supplemented diets. Loss of *Trsp* also led to a decrease in M2 macrophage markers and a corresponding increase in M1 macrophage markers in the jejunum compared to wild type mice [26].

To determine if selenoproteins were exerting this effect through manipulation of the ARA pathway, helminth infected mice on Se-adequate or Se-supplemented diets were treated with indomethacin, a non-selective COX inhibitor. Mice on indomethacin had a significantly higher parasite burden and had significantly less M2 macrophages in the intestine compared to vehicle-treated control mice. Treatment with exogenous 15d-PGJ_2 rescued the Se-supplemented phenotype of indomethacin-treated mice [26]. Finally, treatment of Se-adequate infected mice with GW9662 led to a significant increase in parasite burden and decrease in M2 macrophages compared to controls [26]. These results suggest that optimal Se levels are required for endogenous production of PGJ_2 metabolites, which act through PPARγ, to increase both M2 macrophage and *Hpgds* expression to efficiently clear *N. brasiliensis* infections (Fig 42.1).

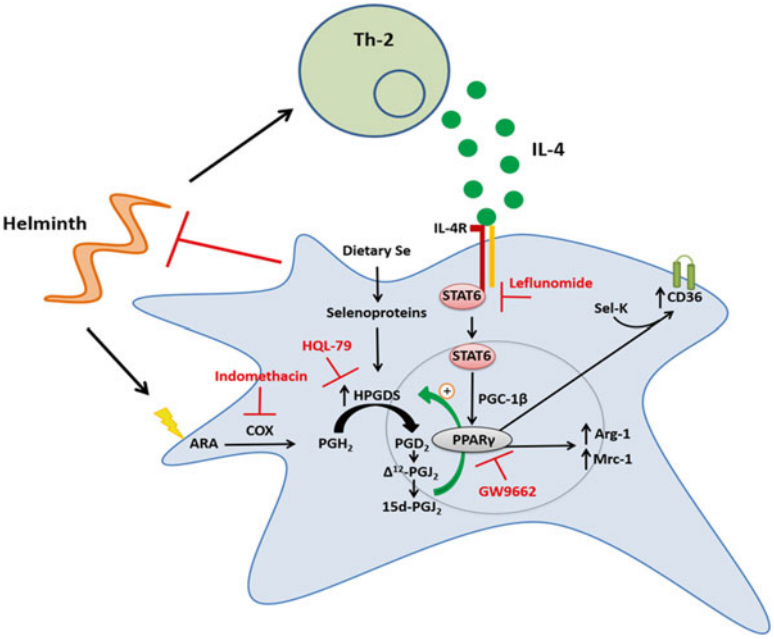

Fig 42.1 Role of selenoproteins in macrophage polarization and helminth clearance. Helminth infections induce a potent Th-2 response, leading to release of IL-4, as well as providing a stimulus to mobilize arachidonic acid (ARA) in macrophages. Se, through selenoproteins, drives eicosanoid class switching to produce CyPGs. Both PGJ_2 and IL-4R signaling converge on PPARγ to create a positive feedback loop and increase Hpgds, Arg-1, Mrc-1, and CD36 expression to induce macrophage polarization. M2 macrophages play an important role in the enhanced clearance of *N. brasiliensis*. Inhibition of either ARA metabolism (with indomethacin or HQL-79) or PPARγ signaling (with leflunomide or GW9662) led to a loss of M2 macrophages and increased helminth burden

In addition to *N. brasiliensis*, the clearance of *Heligmosomoides polygyrus* (also referred to as *Heligosomoides bakeri*), a common parasitic nematode found in rodents, was also affected by host dietary Se levels [38, 39]. Interestingly, the course of primary infection was unaffected by diet. Instead, Se-deficient mice had a delayed worm clearance and increased fecundity during a secondary challenge [39], compared to *N. brasiliensis* infection, where Se levels affected primary infection [26]. Se-deficiency was associated with a reduced local Th-2 response and reduced M2 macrophages [39]. Understanding novel mechanisms for the treatment of STHs is a global health issue that needs to be addressed, particularly in light of increasing development of parasitic resistance to current anthelmintic drugs [30]. Adjuvant therapy with Se supplementation in combination with anthelmintic regimens provides a novel treatment of STH infections, where regulation of intracellular pathways of resolution in innate immune cells is key to driving robust host immune responses.

42.4 Macrophage Regulation of Inflammation and Resolution in Leukemia

Cancer progression involves the active recruitment of circulating monocytes and macrophages into tumors, thereby altering the tumor microenvironment. In return, signals from tumor and stromal cells can effectively alter macrophage phenotypes. Based on the expression of prototypical markers, tumor-associated macrophages (TAMs), which resemble M2-polarized macrophages are thought to be critical modulators of the tumor microenvironment. The anti-inflammatory and pro-resolving functions of TAMs may support tumor cell growth. Studies have suggested some correlation of TAM accumulation in tumors to poor clinical outcome; however, the role of Se in this process is unknown. The conundrum is that macrophage polarization is part of a continuum broadly classified as M2 macrophages. Intermediates within this spectrum may actually have opposing effects on the tumor microenvironment.

While the literature is replete on the pro-apoptotic role of Se in a large number of cancer cells lines, the effect of Se on primary cancer stem cells (CSCs) is not clear. CSCs represent a chemoresistant population that proliferates, leading to disease relapse following chemotherapy. The major reason current chemotherapies fail to eliminate the CSC population is that these cells have a unique metabolic profile and are resistant to therapeutic regimens. Se acts as an oxidative regulator and metabolic switch in several pathways. Given that the WNT/β-catenin pathway is often dysregulated in CSCs [40, 41], the effect of Se on this pathway remains of great interest.

The role of Se in CSCs has been studied through the use of rodent models of experimental leukemia. In models of chronic myelogenous leukemia (CML), Se supplementation of mice selectively induced apoptosis of leukemia stem cell (LSC) populations through the production of PGD_2-derived endogenous CyPGs [42]. As seen in the case of Se-dependent effect on helminth clearance, NSAID treatment blocked the effect of Se-supplementation in CML mice, implicating an essential role of the COX pathway. Furthermore, in vitro studies involving culturing of LSCs with conditioned media from Se-supplemented primary BMDMs led to the apoptosis of LSCs, while BMDMs treated with various NSAIDs blocked the pro-apoptotic effect. These studies clearly indicated a cross talk between endogenous lipid mediators derived from macrophages, presumably with M2-like characteristics, with LSCs leading to apoptosis. Analysis of LSCs undergoing apoptosis indicated exacerbated RONS and activated p53, a tumor suppressor protein. Addition of macrophage (Se-supplemented at 250 nM) conditioned media, lipid extract of the macrophage conditioned media, or purified CyPGs all increased p53-dependent apoptosis, while conditioned media from Se-deficient macrophages did not cause apoptosis of LSCs [42].

In addition to its role in apoptosis, p53 is also a central regulator of the cell cycle and cellular metabolism [43]. Se supplementation at 0.4 ppm in CML mice led to

the activation of p53-regulated genes in LSCs [42]. This was accompanied by increases in the expression of p53-inducible glycolysis and apoptosis regulator (*TIGAR*), which has been reported to lower fructose-2,6-bisphosphate that blocks glycolytic activity [44]. Furthermore, Se also increased the expression of phosphate-activated mitochondrial glutaminase (*Gls2*) that may lead to increased glutamate and α-ketoglutarate for the TCA cycle [45] to facilitate energy production by oxidative phosphorylation in LSCs. Consistent with this observation, Se treatment of LSCs also increased the expression of cytochrome c oxidase assembly protein (*Sco2*), to potentially increase mitochondrial respiration leading to the reversal of the Warburg phenomenon (Fig. 42.2). These effects were seen upon Se-supplementation in vivo as well as supplementation of LSCs co-cultured with macrophages, suggesting a cross talk between the two cell types.

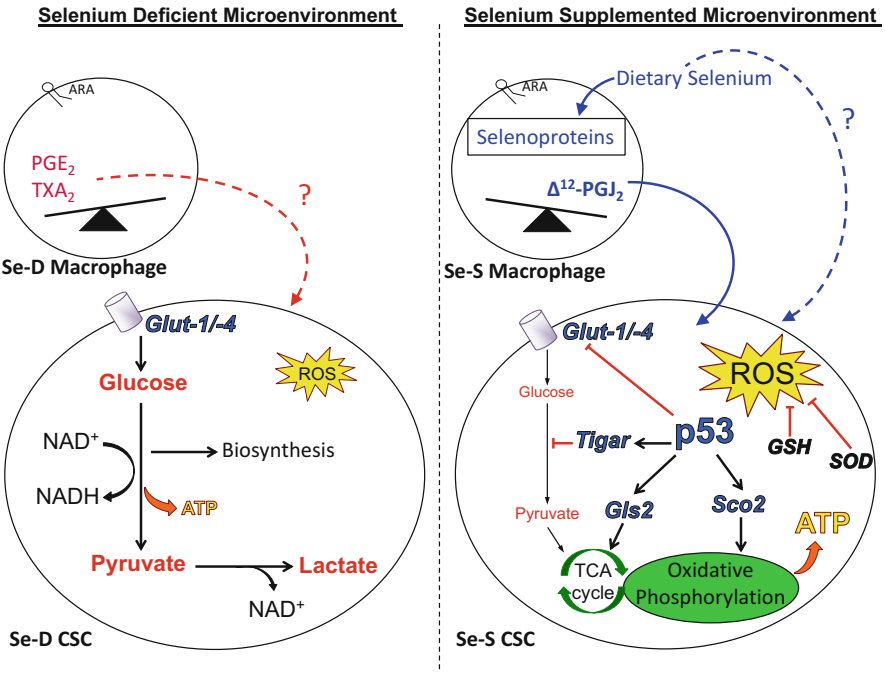

Fig. 42.2 The role of Se in the cross talk between macrophages and CSCs as seen in leukemia. In a Se-deficient microenvironment (shown on the *left*), as a result of the Warburg effect in cancer stem cells (CSC) in CML leukemia, where glycolysis is the main source of energy, pro-inflammatory M1 macrophages produce mostly PGE_2 and TXA_2 from ARA via the COX pathway. Following Se-supplementation (shown on *right*), selenoproteins in the M2 macrophage affect the production of Δ^{12}-PGJ_2. Δ^{12}-PGJ_2 activates tumor suppressor protein, p53, and p53-dependent expression of Tigar, Sco2, and Gls2 upregulate the TCA cycle and oxidative phosphorylation. Glut-1 and Glut-4 are downregulated after p53 induction, which lowers glucose uptake by the cell. As a compensatory mechanism, antioxidant gene expression is increased; however, the increase in not sufficient to control RONS. CSCs are unable to compensate this metabolic shift and undergo apoptosis

42.5 Selenium and Selenoproteins Maintain Redox Status in Erythropoiesis

Inflammation is often associated with defects in erythropoiesis. During anemia of inflammation, changes in cellular metabolism by hypoxia is accompanied by oxidative stress, which is effectively buffered by redox mechanisms in erythrocytes. Studies have suggested an inverse causal link between low serum Se and pathological erythropoiesis [46, 47]. In sickle cell anemia (SCA) patients, Se and GPX1 activity were significantly lower than in nonanemic control subjects, which correlated with increased oxidative stress in SCA patients [48]. Low serum Se was also associated with anemia among elderly population in the United States, and increase in Se was associated with a reduced risk of anemia [49]. Se deficiency was thought to contribute to anemia associated with pulmonary tuberculosis, cardiomyopathy, and among dialysis patients [50–52].

For many years, researchers have been developing animal models to unveil the mystery between Se and erythropoiesis. In a chicken model, Se-deficient chicken erythrocytes showed altered transcription in selenoproteins and some pro-inflammatory cytokines. GPXs, thioredoxin reductase (TXNRD1), selenoprotein P (SEPP1), and selenophosphate synthetase 2 (SEPHS2) were highly expressed regardless of experimental groups, suggesting a critical role of these selenoproteins in chicken erythrocytes [53]. Though long-term dietary Se deficiency in rats showed normal hematopoietic parameters [54], during pathological erythropoiesis, dietary Se supplementation manifested protective effects. Pups from pregnant rats treated with methimazole, an antithyroid drug, exhibited symptoms of anemia and signs of increased oxidative stress, which could be alleviated by Se supplementation [55]. Se deficient mice exhibited anemia due to defects in erythropoiesis at an early erythroid progenitor stage that was accompanied by several markers of oxidative stress, such as methemoglobin and oxidative degradation of hemoglobin [56]. As a result, Forkhead box O3 (FoxO3a) transcription factor, a key redox regulator during erythropoiesis, was activated in Se deficient murine erythroblasts to upregulate transcription of several antioxidant genes, including *Gpx1* [56]. These studies suggest the indispensable role of Se in maintaining redox balance during erythropoiesis.

The primary role of selenoproteins in regulating erythropoiesis was revealed by a conditional selenoprotein knockout mouse model (*Trsp*$^{fl/del}$:*Mx1-Cre*) [57]. Knockout mice exhibited symptoms of anemia, and highly upregulated the expression of oxidative stress-associated genes, such as NAD(P)H quinone oxidoreductase1 (*Nqo1*), glutamate-cysteine ligase catalytic subunit (*Gclc*), heme-oxygenase 1 (*Hmox1*), and *Txnrd1*, indicating perturbation in redox balance. Immature erythrocytes and damaged/degraded mature erythrocytes accumulated in peripheral blood and bone marrow of knockout mice, corresponding with increased serum bilirubin levels, strongly arguing the importance of selenoproteins in protecting erythrocytes from hemolysis. Furthermore, *Nfe2l2*$^{-/-}$*Trsp*$^{-/-}$ double knockout exacerbated the

phenotype, with marked accumulation of RONS in erythroid cells. Importantly, in the absence of selenoproteins, Nrf2 played a key role in the expression of *Nqo1* and other antioxidant genes.

The function of specific selenoproteins involved in erythropoiesis is currently unclear. Recent research has identified selenoproteins to be directly involved in erythropoiesis [58–61]. Surprisingly, even though GPX1 has been demonstrated to be a key antioxidant enzyme involved in the protection of erythrocytes and hemoglobin from oxidative breakdown [62, 63], erythropoiesis in *Gpx1*$^{-/-}$ mice was nearly normal [64, 65]. However, lack of *Gpx4* led to embryonic lethality, but whether erythroid cells were affected has remained unclear [62, 66]. Similarly, deletion of thioredoxin 1 (*Trx1*), *Trx2*, and *Txnrd1* also led to embryonic lethality [67–69]. It is not clear if erythrocytes and erythropoiesis were affected in these models. Ubiquitous knockout of mitochondrial *Txnrd2* was embryonic lethal at E13 and the *Txnrd2*$^{-/-}$ embryos were severely anemic. However, the underlying mechanisms have remained unclear [70]. Therefore, it appears that besides Gpx1, Txnrd2 also plays a critical role in supporting erythropoiesis by potentially maintaining redox homeostasis in erythroid cells. Further studies are essential to delineate the effects of selenoproteins and their role in various stages of erythropoiesis, where signals from the microenvironment, including macrophage-derived mediators of inflammation and resolution, are likely to serve as key factors.

42.6 Concluding Remarks

Development of mouse models that lack selenoprotein expression in specific immune cells, such as macrophages and T-cells, has significantly aided in the enhanced understanding of the immune modulatory role of selenoproteins in diseases. Of specific interest is the role of selenoproteins in these cell types in mitigating inflammation to further resolve the injury to enhance wound healing responses, which is critical to reestablish homeostasis. The role of individual selenoproteins in this process remains to be elucidated. In particular, their ability to skew eicosanoid pathways, in addition to other pathways of cellular metabolism, could possibly identify new activities for selenoproteins. Based on the studies using in vivo models, it appears that supranutritional levels of Se are essential to effectively resolve inflammation in most instances. Thus, it remains to be seen if small molecular weight metabolites, including selenocompounds, may play a protective role in the resolution process.

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Chapter 43
Glutathione Peroxidase 4 and Ferroptosis

José Pedro Friedmann Angeli, Bettina Proneth, and Marcus Conrad

Abstract Glutathione peroxidase 4 (Gpx4) is one of eight members of the mammalian glutathione peroxidase family of enzymes. Gpx4 is unique due to its capacity to efficiently reduce phospholipid hydroperoxides. Additionally, it has been recognized that Gpx4 governs a novel form of non-apoptotic cell death, named ferroptosis. Ferroptosis was initially described to be induced by small molecules in specific tumor types and in engineered cells overexpressing oncogenic RAS. Recently, its relevance in non-transformed cells and tissues was highlighted expanding the importance of this cell death pathway in several pathophysiological contexts, encompassing immunity, neurodegenerative diseases and tissue damage upon ischemia/reperfusion scenarios. Importantly, regulation of selenoprotein biosynthesis emerges as an important factor in control of ferroptosis. In this chapter, we present an updated view on the ferroptotic process as well as review the implications of how selenoprotein expression might impact on ferroptosis *per se*.

Keywords Ferroptotic signaling • Ischemia/reperfusion injury • Lipid peroxidation • Liproxstatin-1 • Mevalonate pathway • Neurodegeneration • Non-apoptotic cell death • PHGPx • Phospholipid hydroperoxide glutathione peroxidase • Regulated necrosis

43.1 Introduction

Glutathione peroxidase 4 (Gpx4) is one of eight members of the glutathione peroxidase family of enzymes. Specifically, mammalian Gpx1, Gpx2, Gpx3 and Gpx4 are selenoproteins, whereas Gpx5, 7 and 8 are Cys-containing paralogs. Additionally, GPX6 is a selenoprotein in humans, but a Cys-containing enzyme in rodents and some other mammals [1]. Glutathione peroxidases (Gpxs) are most commonly known for a shared enzymatic mechanism involving a conserved catalytic tetrad, consisting of selenocysteine (Sec), asparagine (Asn), glutamine

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(Gln) and tryptophan (Trp) [2]. Their catalytic activity is responsible for reducing H_2O_2 and organic hydroperoxides to H_2O and the corresponding alcohols, respectively. The catalytic consumption of peroxides is driven by glutathione (GSH) which serves as electron donor and is then recycled by GSH reductase at the expense of NADPH. Nevertheless, it is now recognized that GSH is not the sole physiological reducing substrate for Gpxs. Some alternative substrates have been shown to be thioredoxin and/or glutaredoxins for Gpx3, protein disulfide isomerase for Gpx7 and 8 [3], and, under special conditions, Gpx4 can use "unspecific" thiols acting as a non-selective thiol peroxidase [4].

Gpx4 was initially characterized in 1982 by Ursini and colleagues and shown to be an enzyme efficiently protecting liposomes and biomembranes from peroxidative degradation in the presence of GSH [5]. Gpx4 presents substantial overall homology to the monomeric Gpx7 and 8, which, however, differ from Gpx4 by the presence of Cys instead of Sec. Compared to other Gpxs, the group of monomeric Gpxs is the least homologous to the most studied "classical" or "cytosolic" Gpx1. Gpx4 differs from other Gpx family members in terms of its monomeric structure, relatively low substrate specificity, and its necessity for mouse development [6].

Analysis of the *Gpx4* gene identified three distinct isoforms which differ by the use of three individual promoters [7]. Each isoform presents a different subcellular localization and a particular tissue-specific expression. Alternative transcription initiation at exon 1 and exon 1b (Ea) generates either the short cytosolic form (20 kDa) or the mitochondrial form (23 kDa) with an N-terminal mitochondrial leader sequence. The alternative exon (Ea) gives rise to a 34 kDa isoform of Gpx4 containing a nuclear targeting sequence. The nuclear form of Gpx4 (nGpx4) was previously referred to as sperm nuclei-specific Gpx (snGpx) [8]. The cytosolic form of Gpx4 is ubiquitously expressed, whereas the mitochondrial and the nuclear forms are predominantly expressed in testis [8–10].

Based on genetic studies in mice, it is now well accepted that the isoform of Gpx4 conferring the cytoprotective function and the one essential for mammalian development is the short (also referred to as cytosolic) form [11]. Specific ablation of the short form is not possible as it is part of both the nuclear and mitochondrial forms. Alternatively, specific ablation of the mitochondrial and the nuclear isoforms is feasible due to their exclusive transcription initiation sequences and consequently the presence of unique N-terminal extensions, i.e., the mitochondrial targeting sequence and the nuclear localization sequence/DNA binding motifs, respectively. Deletion of either the nuclear or the mitochondrial form gives rise to viable offspring, however, the respective knockout (KO) mice exhibit defects in male gametogenesis including male infertility [12, 13]. Additional studies crossing transgenic mice overexpressing the cytosolic or the mitochondrial form on a *Gpx4* KO background demonstrated that only the cytosolic form is able to rescue the lethal phenotype of the mouse *Gpx4* null mutation [11].

Several studies have reported a pro-survival function of Gpx4 in protecting against apoptosis (reviewed in [14]). More recently, Gpx4 overexpression has been shown to prevent tumor necrosis factor α-induced cell death in quiescent Jurkat T lymphocytes in a mechanism involving decreased formation of truncated

phospholipids [15]. These in turn were generated by the oxidative degradation of phosphatidylcholine through NADPH oxidase 4-derived H_2O_2. It was also found that Gpx4 has an essential function in protecting against cardiolipin oxidation, thus negatively regulating the intrinsic apoptotic pathway [16]. This notion has been further reinforced by in vivo studies showing that overexpression of Gpx4 protects liver from diquat-induced damage by decreasing cardiolipin oxidation and subsequently inhibiting the release of pro-apoptotic factors such as cytochrome C and SMAC/DIABLO. Interestingly, using conditional gene disruption and robust oxi-lipidomics analysis, it was shown that upon Gpx4 deletion in the kidney of induced Gpx4 KO mice several oxidized products of cardiolipin accumulate and that this process precedes the oxidation of any other class of phospholipids [17].

43.2 Gpx4: Beyond Apoptosis

It is now increasingly clear that cells can engage in a plethora of cell death pathways that are not limited to apoptosis. Among the cell death paradigms that have attracted growing interest from the scientific community are necroptosis, ferroptosis/oxytosis, parthanatos and cyclophilin-D mediated necrosis (for a comprehensive review see [18, 19]). Among these novel cell death paradigms, ferroptosis has gained momentum because of to its putative implication in several pathological conditions, spanning from ischemia/reperfusion injuries in various tissues to neurodegenerative diseases.

The term "ferroptosis" was first coined in 2012 by Stockwell and co-workers [20] (Fig. 43.1), where they identified the mechanism of action of the small molecule erastin, which had been discovered to be synthetically lethal to isogenic cancer cell lines carrying mutant forms of RAS [21]. Erastin was shown to deprive cells from GSH via abrogation of cystine uptake mediated through inhibition of the system x_C^- antiporter [20]. This antiporter takes up one molecule of cystine (the oxidized form of Cys) at the expense of the release of one molecule of glutamate [22]. Interestingly, inhibition of system x_C^- can also be achieved by high extracellular concentrations of glutamate (>1 mM). These observations are reminiscent of previous work demonstrating that glutamate can be neurotoxic by a mechanism that also involves GSH depletion and subsequent calcium-dependent activation of lipoxygenases, culminating in a form of cell death, named oxytosis [23]. At present, oxytosis and ferroptosis are hardly distinguishable, and more studies are warranted to determine if they in fact represent the same phenomenon.

Of note, a recent study has provided provocative evidence that ferroptosis may be involved in antitumor actions of the tumor suppressor p53 through its ability to inhibit transcription of *SLC7A11*, which is the substrate-specific subunit of system x_C^- [24]. Nonetheless, ferroptosis and oxytosis share similar features: Cys deprivation (by decreased cystine uptake) and GSH depletion. The crucial function of GSH is further supported by studies showing that GSH depletion using the γ-glutamylcysteine synthetase (γ-GCS) inhibitor, L-buthionine sulfoximine (BSO),

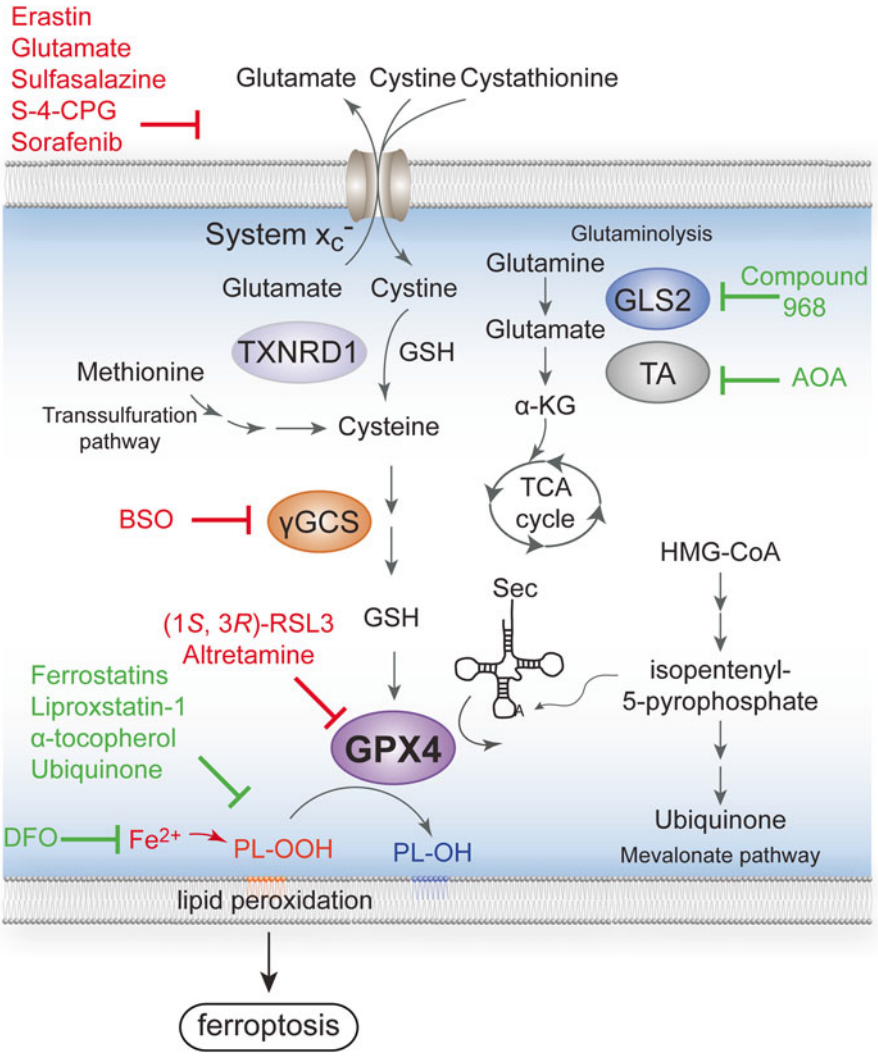

Fig. 43.1 Gpx4 is the key regulator of ferroptotic cell death. Availability of cysteine either through the cystine/glutamate antiporter (System x_C^-), the neutral amino acid transporter (ASCT, not shown) or the transsulfuration pathway feeds glutathione (GSH) biosynthesis. Gpx4 is unique and a central node in ferroptosis as it directly reduces lipid hydroperoxides (PL-OOH) to the corresponding alcohols (PL-OH). Expression of Gpx4 is indirectly dependent on the mevalonate pathway (see text). Besides iron-dependent lipid peroxidation, glutaminolysis was identified as a source of detrimental oxygen radicals. Inducers of ferroptosis are shown in *red*, whereas ferroptosis inhibitors in *green*. *α-KG* α-ketoglutarate, *AOA* aminooxyacetate, *BSO* L-buthionine sulfoximine, *DFO* deferoxamine, *γ-GCS* γ-glutamylcysteine synthetase, *GLS2* glutaminase 2, *HMG-CoA* 3-hydroxy-3-methylglutaryl coenzyme A, *S-4-CPG* (S)-4-Carboxyphenylglycine, *TA* transaminase, *TCA* tricarboxylic acid, *TXNRD1* thioredoxin reductase 1

is able to induce cell death in a manner similar to ferroptosis/oxytosis. The recognition that GSH is essential to prevent this form of cell death came imbued with the intrinsic question: whether there is a GSH dependent enzyme responsible for this pro-survival effect. Definitive answers to this question were given in 2014, when two groups independently provided unequivocal evidence that Gpx4 is the rate limiting enzyme supporting the pro-survival function of GSH, at least in certain defined contexts. Specifically, while investigating new molecules able to elicit ferroptosis, Stockwell and collaborators were able to identify a sub-class of ferroptosis inducers that are able to trigger cell death with ferroptotic characteristics without impacting on GSH levels [25]. Target characterization of their mechanism of action culminated with the discovery of these small molecules as the first Gpx4 inhibitors. Concomitantly, using Gpx4 conditional KO cells, our group was able to demonstrate that cell death induced by Gpx4 deletion can be prevented by ferroptosis inhibitors and that this process proceeds without impacting on GSH levels [17].

Analogous to its function in preventing the accumulation of phospholipid hydroperoxides, the inactivation of Gpx4, either through genetic or pharmacologic means, leads to increased levels of oxidized phospholipids. At present it is, however, not clear if lipid peroxidation is just an executioner of the cell death process by causing loss of physicochemical properties of cellular membranes that will trigger loss of barrier function. Alternatively, lipid hydroperoxides may represent defined and meaningful signals that are transduced by protein sensors engaged downstream, thus activating defined signaling cascades. Additionally, the assumption that Gpx4 inhibition is associated with an elevated level of lipid peroxidation that is required for cell death progression may support the notion that cells constantly oxidize their lipidomes for a yet-to-be defined physiological purpose, a process that, in turn, is finely tuned by Gpx4. Therefore, it is of utmost importance to understand when and how these lipids are being modified. Early work has proposed that lipoxygenases would fulfill the criteria of best candidates to perform such tasks [26, 27], a notion that was further supported by pharmacological means [28]. Nonetheless, recent studies have questioned their importance and provide evidence of off-target effects for most lipoxygenase inhibitors [17].

On the other hand, it has become evident that iron metabolism plays a critical role in ferroptosis [20]. Studies have revealed several molecules involved in iron metabolism that are able to impact on the ferroptotic process. Specifically, iron-bound transferrin (Tfn) taken up via the transferrin receptor (TfnR) is now accepted as an important supplier for the iron pool that can be mobilized during ferroptosis [29]. Moreover, iron responsive element binding protein 2 (IREB2), F-box and leucine-rich repeat protein 5 (FBXL5) have been shown to impinge on this system presumably by stabilizing *Tfn* and *TfnR* mRNAs by IREB2 [30]. The effect of IREB2 is then counteracted by FBXL5 which actively marks IREB2 for proteosomal degradation [31]. Yet, how exactly iron impacts on the cell death process is far from being clear, as it is not yet known whether iron *per se* via mobilization of the so-called labile iron pool promotes unrestrained damage to lipids and other bio-molecules or, alternatively, supports the function of a critical iron-dependent enzyme.

It has recently been proposed that the process of glutaminolysis is essential for ferroptosis and that this can be efficiently prevented by inhibiting the conversion of glutamine to glutamate [29], a process that can be bypassed by supplying α-ketoglutarate. Interestingly, α-ketoglutarate is a substrate of α-ketoglutarate-dependent dioxygenases, a family of iron-dependent enzymes required for the oxygenation of a wide array of substrates which have also been shown to be an important source of mitochondria-derived H_2O_2. In addition to the contribution of glutamine metabolism to ferroptosis, recent reports have started to demonstrate that lipid metabolism is also a critical node defining sensitivity to ferroptosis. A study using insertional mutagenesis in haploid KBM7 cells was able to identify acetyl-CoA synthase long chain family member 4 and lysophosphatidylcholine acyltransferase 3 as important regulators of cell death induced by the Gpx4 inhibitor RSL3 [32]. Yet, how they modulate ferroptosis is still unclear. At present, it is hypothesized that these proteins modulate the content of arachidonic acid-containing phospholipid species that are modified during ferroptosis. Nonetheless, which and how these phospholipids are oxidatively modified remains unresolved.

The recognition that Gpx4 is a key regulator of ferroptosis raises the question of how Gpx4 activity/expression levels are modulated. Importantly, mechanisms governing transcription/translation of selenoproteins may shed light on some of the processes that could impact on Gpx4. Specifically, early work has shown that the Sec-specific tRNA, designated $tRNA^{[Ser]Sec}$, may undergo post-translational modifications, which directly affects translational efficiency. Among the critical posttranscriptional modifications of $tRNA^{[Ser]Sec}$ is the Trit1-mediated isopentenylation of adenosine at position 37 [33], thus creating isopentenyladenosine (i^6A) [34]. This modification is important for ensuring maximal translation efficiency at the UGA codon. A critical feature of this system is that isopentenyl pyrophosphate, the precursor for isopentenylation, is generated via the mevalonate pathway, thus linking the cholesterol biosynthetic pathway with selenoprotein expression [35] (Fig. 43.1). Such an assumption is supported by earlier studies employing some unusual side-effects of statins which are widely used 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors. Prolonged treatment of hypercholesterolemic patients with these molecules was shown to be associated with an impaired synthesis of selenoproteins thus leading to excessive oxidative damage and possibly myopathy [36]. Importantly, it was demonstrated that treatment with statins directly impairs Gpx4 function [37]. These studies reconcile early findings showing increased muscular waste in animals treated with the statin, cerivastatin, by a mechanism that could directly be involved in muscle tissue repair that was recently suggested to be dependent on a Gpx4-mediated repair mechanism [38].

Another unique feature of selenoproteins that could be expanded to Gpx4 is the conversion of Sec to dehydroalanine (DHA). This oxidative modification is known to irreversibly inactivate the enzyme in a selenium-dependent manner. Despite the recognition that Cys residues can also be found in normal tissues in the form of $Cys–SO_2H$ [39], and some of them are converted to DHA [40], DHA is not frequently found in positions corresponding to Cys residues. This difference is likely attributable to the greater strength of the C–S bond (272 kJ/mol) compared to the

C–Se bond (234 kJ/mol). The specific characteristic of irreversible inactivation of Gpx4 may be relevant in conditions where the immune system may induce ferroptosis in malignant cells via the neutrophil oxidative burst by inactivating this selenoenzyme, thus again highlighting an important antitumoral role of selenoproteins and Gpx4.

43.3 In Vivo Relevance of Ferroptosis

At present, the in vivo implications of ferroptosis are scarce and its study is not trivial due to the absence of robust biomarkers. Currently, the implication and importance of ferroptosis in vivo can be inferred mostly based on genetic studies using models of putative regulators, namely xCT (SLC7A11), γ-GCS and Gpx4. Investigations using KO animals for targeting the aforementioned ferroptosis regulators have allowed us to draw important conclusions on which level these players act and when perturbed lead to engagement of this cell death modality. For instance, studies using *xCT* KO animals taught us that system x_C^- is dispensable for embryo development and KO animals are fully viable showing no overt phenotype [41], unless they are stressed by the redox cycling compound paraquat [42], or by ischemia/reperfusion injury in the kidney [43]. Therefore, it becomes obvious that conditions which modulate xCT activity or expression, at least in vivo and in non-transformed cells, cannot be regarded as an efficient pathological mechanism eliciting ferroptosis. This comes in contrast to the in vitro phenotype, whereby xCT inhibition efficiently triggers ferroptosis [20]. This is a phenomenon that is easily explained by the fact that under cell culture conditions Cys is only available in the form of cystine [22], which is in stark contrast to the in vivo situation [41]. Yet, xCT inhibition has been repeatedly proposed as a putative druggable target for inducing ferroptosis in tumors [24, 44]; therefore, caution should be taken when proposing that inhibition of xCT in vivo decreases tumor burden due to increased ferroptotic rates in cancer cells.

On the other hand, *Gpx4* KO animals provided the most valuable insights into which organs are susceptible to this form of cell death (Table 43.1). Specifically, the whole body KO of *Gpx4* is embryonic lethal at E7.5 [6], which is in agreement with the whole body KO of *γ-GCS* [45], thus supporting Gpx4 as the critical enzyme responsible for the pro-survival function of GSH. Recently, two studies independently provided definitive proof that the active site of Gpx4 (Sec) is indeed essential for early embryo development as mice homozygous for Sec-Ala or Sec-Ser mutations invariably died similar to systemic *Gpx4* KO mice [46, 47].

With the implementation of conditional KO for *Gpx4*, the specific study of the role of Gpx4 in particular tissues was made possible [28]. The first conditional KO of *Gpx4* was generated using the *CamKIIα*-Cre mice, which deletes Gpx4 in neurons. These animals were born at normal Mendelian rates, but after 2 weeks following birth, they developed ataxia, seizures, neurodegeneration in the CA3 region of the hippocampus and premature death. These manifestations highlighted

Table 43.1 Knockout (KO) mouse models for Gpx4

Year	Tissue (Cre line)	Major outcome	Ref.
2003	Systemic	Embryonic lethality at E7.5	[6, 57]
2005	Nuclear form (*nGpx4*$^{-/-}$)	Fully viable, but impaired sperm chromatin condensation	[12]
2008	Ubiquitous (Cre-Deleter)	Embryonic lethality at E7.5	[28]
2008	Systemic	Early embryonic lethality	[58]
2008	Neurons (*CamkIIα-Cre*)	Ataxia, seizures, neurodegeneration in CA3 region of hippocampus; premature death around 2 weeks after birth	[28]
2009	Mitochondrial form (*mGpx4*$^{-/-}$)	Fully viable but exhibit male infertility due to malformation of mitochondrial capsules of sperm	[13]
2009	Overexpression of cytosolic form in *Gpx4*$^{-/-}$ background	Fully viable but exhibit male infertility due to lack of mGpx4 expression	[11]
2009	Spermatocytes (*Pgk2-Cre*)	Male infertility due to atrophy of germinal epithelium, morphologically aberrant, dysfunctional spermatozoa	[59]
2010	Neurons (*CamkIIα-Cre*)	Seizures, ataxia and loss of parvalbumin-positive interneurons strongly resembling loss of *Trsp*	[48]
2012	Photoreceptor cells (*Crx-Cre*)	Complete loss of photoreceptor cells by P21	[52]
2013	Endothelium (*Cdh5*(*PAC*)*-CreER*^{T2})	Vitamin E-deprived KO display many thromboembolic events causing various clinical pathologies, e.g., heart failure, renal and splenic micro-infarctions or paraplegia	[56]
2013	Inducible full body deletion (*ROSA26-CreER*^{T2})	Early death 2 weeks after KO induction; loss of neurons in the hippocampus	[51]
2013	Skin (*K14-Cre*)	In perinatal mice, epidermal hyperplasia, dysmorphic hair follicles and alopecia	[53]
2014	Neurons (*Tα1-Cre*)	Cerebellar hypoplasia and Purkinje cell death	[49]
2014	Inducible full body deletion w/o brain (*ROSA26-CreER*^{T2})	Renal tubular cell death, acute renal failure and death at ~12 days upon KO induction, which cannot be rescued by breeding on a 12/15-lipoxygenase (*Alox15*) KO background	[17]
2015	Motor neurons (*Thy1-cre/ERT2,-EYFP*; SLICK-H)	Death of motor neurons and rapid onset and progression of paralysis and death	[50]
2015	T cells (*CD4-Cre*; *ROSA26-CreER*^{T2})	CD8+ T cells fail to maintain homeostatic balance in periphery; CD8+ and CD4+ T cells fail to expand and to protect mice from acute lymphocytic choriomeningitis virus and *Leishmania major* parasite infections	[55]
2015	Systemic (Sec-Ala mutation)	Early embryonic death, which cannot be rescued by breeding on an Alox15 KO background	[46]
2015	Systemic (Sec-Ser mutation)	Early embryonic death of homozygous mice, but impaired male fertility in heterozygous mice	[47]
2015	Hematopoiesis (*ROSA26-CreER*^{T2})	Anemia caused by receptor interacting protein kinase 3 (RIP3)-dependent necroptosis in erythroid precursor cells.	[54]

the importance of this cell death pathway for neuronal populations. Subsequently, studies using an alternative neuron-specific deletion strain, i.e., *Tα1-Cre*, have demonstrated that loss of Gpx4 in cerebellar neurons leads to cerebellar hypoplasia and Purkinje cell death [48, 49].

More recently, the first inducible KO for *Gpx4* in a neuronal population was reported using *Thy1-Cre/ERT2,-EYFP* (*SLICK-H*) mice [50]. Thereby, it was shown that the inducible deletion in adult animals leads to neurodegeneration manifested by the loss of motor neurons, thus underlining the importance of Gpx4 in preventing motor neuron degeneration. In an earlier study, the same authors have demonstrated that the inducible full body deletion (*ROSA26-CreER^{T2}*) was accompanied by early lethality and loss of hippocampal neurons [51]. Nonetheless, it is unlikely that the lethality in this model is due to the loss of hippocampal neurons (see below). Other cells or organs that have been shown to be sensitive to Gpx4 deletion are photoreceptor cells [52], skin [53] and cells of the hematopoietic system [54].

Remarkably, none of the phenotypes observed above have critically interrogated the role of Gpx4 in the ferroptotic process. Therefore, an important contribution to the understanding of ferroptosis in vivo was recently provided. Using inducible full body deletion of Gpx4 except in the brain (*ROSA26-CreER^{T2}*), we showed that early mortality of mice is due to acute kidney failure and this process is accompanied by an unrestrained and complex lipid oxidation signature [17]. Importantly, in this report, the first in vivo inhibitor of ferroptosis, liproxstatin-1, was described. This molecule was identified in a phenotypic small molecule screen for compounds able to inhibit cell death of mouse embryonic fibroblasts triggered by genetic deletion of *Gpx4*. Of note, liproxstatin-1 was able to prolong survival of *Gpx4* KO animals by preventing cell death in kidneys, thus providing first evidence that, at least in kidneys, Gpx4 deletion leads to cell death in a ferroptotic manner. Similar findings were also observed in T-cell-specific *Gpx4* KO mice [55], where upon deletion, CD8+ T cells not only failed to maintain homeostatic balance in periphery, but also CD8+ and CD4+ T cells failed to expand and to protect the mice from acute lymphocytic choriomeningitis virus and *Leishmania major* parasite infections. Hence, these observations support an important role of Gpx4 in restraining ferroptosis in immunity.

Another important lesson learned from the studies of *Gpx4* KO animals is that not all tissues respond in a similar manner as they may use ways to compensate for the loss of Gpx4. One of the mechanisms that appears to compensate for the loss of Gpx4 is the content of the antioxidant vitamin E in the diet. This notion was convincingly demonstrated using animals deficient for Gpx4 in the endothelium (*Cdh5(PAC)-CreER^{T2}*) [56]. The endothelial-specific *Gpx4* KO animals developed normally and no marked phenotype or dysfunction in the endothelium was evident upon KO induction by tamoxifen, although aortic explants from endothelium-specific *Gpx4* KO presented a reduced number of endothelial branches in an ex vivo sprouting assay. From these findings, it was concluded that an unknown component was rescuing the loss-of Gpx4 in vivo. In order to challenge this, animals were then subjected to a diet with low levels of vitamin E, which in combination with the loss of Gpx4 led to several clinical pathologies, such as thromboembolic events

culminating in heart failure, renal and splenic micro-infarctions or paraplegia. Therefore, at least in the endothelium, an important interrelationship between Gpx4 and vitamin E must be considered, suggesting to some extent overlapping functions in protecting the endothelium against lipid peroxidation-driven dysfunction [56].

43.4 Concluding Remarks

It has become increasingly evident that cells can die in many ways which are not restricted to apoptosis. The recognition of novel cell death modalities has presented the cell death field with renewed breath, spurring research investigating the mechanisms regulating these new novel forms of cell death. Among these pathways, special attention has been attributed to ferroptosis due to its role in pathophysiological scenarios. Additionally, the recent discovery that this form of cell death is negatively regulated by the selenoprotein Gpx4 has opened new avenues of investigation in order to better understand the importance of Gpx4 and selenium biology in the context of tumor development and cytoprotective strategies. We believe that the integrated study of selenoprotein biology and ferroptosis will provide exciting future insights in the cross-talk between cell death and survival decisions in a myriad of pathophysiological phenomena.

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Chapter 44
Mutations in Humans That Adversely Affect the Selenoprotein Synthesis Pathway

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Abstract Human mutations have been described in three genes implicated in the selenocysteine insertion pathway (*SECISBP2*, *TRU-TCA1-1* and *SEPSECS*), which result in impaired synthesis of multiple selenoproteins. Mutations in these genes result in decreased gene expression and/or generate defective protein or RNA products; however, in all cases the preservation of some residual function is presumed, since selenoprotein expression is not completely abrogated. Patients harbouring *SEPSECS* mutations present with progressive cerebello-cerebral atrophy as the predominant phenotype, whereas this has not been associated with *SECISBP2* and *TRU-TCA1-1* defects. In contrast, patients with mutations in the latter two genes manifest a multisystem disorder with a thyroid hormone biochemical signature secondary to loss of selenoprotein deiodinases, myopathic features due to SEPN1 deficiency and phenotypes attributable to elevated levels of reactive oxygen species as a consequence of lack of antioxidant selenoenzymes. The progressive nature of most reported phenotypes may be explained by cumulative oxidative damage over time, which may also mediate the development of additional pathologies.

Keywords SECISBP2 • Selenocysteine tRNA • Selenoprotein deficiency • SEPSECS

44.1 Introduction

There are 25 known human genes encoding selenoproteins, the synthesis of which requires decoding of an UGA codon as the amino acid (AA) selenocysteine (Sec) instead of a premature stop, which is mediated by a complex Sec-insertion machinery. The synthesis of Sec occurs, uniquely, on its tRNA, encoded by *TRU-TCA1-1*, involving a dedicated pathway, with initial attachment of serine to $tRNA^{[Ser]Sec}$ by seryl-tRNA synthetase, phosphorylation of this residue by phosphoseryl-tRNA

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kinase, subsequent conversion of this phosphoserine to an intermediate by O-phosphoseryl-tRNA:Sec-tRNA synthase (SEPSECS) and eventual acceptance of selenophosphate to generate $tRNA^{[Ser]Sec}$. Delivery and incorporation of Sec into polypeptide chains is achieved via a highly conserved, Sec-insertion machinery, comprising *trans*-acting factors (e.g., Sec insertion sequence binding protein 2 (SECISBP2), Sec tRNA specific eukaryotic elongation factor, $tRNA^{[Ser]Sec}$) interacting with *cis*-acting Sec-insertion sequence (SECIS) elements, usually located in the 3′-UTR of selenoprotein mRNAs. Other factors such as ribosomal protein L30, tRNA selenocysteine 1 associated protein 1 and nucleolin, are also known to regulate the Sec insertion process (Chap. 2 and [1]).

44.2 Human *SECISBP2* Mutations

SECISBP2 is a limiting, obligate factor for selenoprotein synthesis and is also essential for survival as evidenced by the observation that murine *Secisbp2* deletion is embryonic lethal [2, 3]. Human SECISBP2 contains 854 AA (Fig. 44.1) with the amino (N-)-terminal first 400 residues being dispensable for its Sec incorporation function in vitro [4, 5]. The carboxyl (C-)-terminal region (AA399–784) is both essential and sufficient for SECIS binding and Sec incorporation. Two main domains have been identified in the C-terminal region [4–12]: (1) the Sec incorporation domain (SID), dispensable for SECIS binding, but required for Sec incorporation; and (2) the RNA binding domain (RBD). The RBD contains an L7Ae-type RNA interaction motif mediating interaction with the SECIS element and the 28S ribosomal RNA, as well as a bipartite/SID/K-rich region involved in more specific recognition of the SECIS element.

Other functional SECISBP2 domains comprise a lysine-rich nuclear localization signal (NLS), two leucine-rich nuclear export signals (NES) and a redox-sensitive cysteine-rich domain (CRD) involved in cellular localization. Alternative splicing events result in at least five protein isoforms of varying N-terminal sequence [2, 13]. The importance of the splice variants, subcellular location, tissue expression levels

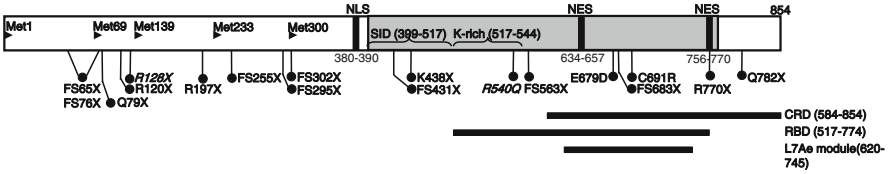

Fig. 44.1 Functional domains of human SECISBP2 with the position of compound heterozygous and homozygous mutations (*italicized*) superimposed. Minimal functional protein (399–784, grey); Sec incorporation domain (399–517, SID); minimal RNA-binding domain (517–774, RBD); Lysine-rich domain (517–544, K-rich); L7Ae homology module (620–745); NLS: 380–390; CRD: 584–854 and two nuclear export signals: NES1 (634–657) and NES2 (756–770). *Arrowheads* denote the location of ATG codons

and redox state for SECISBP2 functions, including prevention of nonsense-mediated selenoprotein RNA decay (NMD), Sec incorporation and hierarchical selenoprotein expression, remain open questions requiring further investigation [14].

To date, nine families with either compound heterozygous or homozygous *SECISBP2* defects have been described [15–21] (Table 44.1). Although patients are from diverse ethnic backgrounds, they share similar clinical phenotypes. With the exception of three missense abnormalities, all *SECISBP2* defects are predicted to prematurely truncate the SECISBP2 protein. Compared to the lethality of murine *Secisbp2* inactivation, the phenotype of *SECISBP2* patients is modest (Table 44.2), suggesting that the mutations result in an altered gene product retaining residual expression or functional activity as has been shown experimentally for the missense mutants (R540Q, E679D and C691R). For most premature stop mutations, it has been suggested that translation initiation occurs from alternative start codons (e.g., Met300) resulting in synthesis of a shorter, but functional C-terminal SECISBP2 protein fragment. Premature stop mutations downstream of Met300 may eliminate synthesis of functional protein completely; however, mutations distal to the RBD, e.g., in the CRD:R770X or Q782X, might generate truncated, but functional proteins, possible affecting RNA binding and nuclear localisation. An intronic mutation IVS8ds+29G>A [17] affecting splicing, only reduces wild type transcript levels by 50 % and other splice site defects may be associated with preserved mRNA levels by a similar mechanism. Alternative mechanisms to explain residual selenoprotein activity have been postulated, e.g., a low-efficiency, SECISBP2-independent, Sec incorporation pathway or incorporation of another AA in the position of Sec, preventing the UGA codon from being read as a nonsense mutation.

Carefully designed studies will be required to elucidate such a mechanism. All patients described to date harbor at least one allele generating partially functional SECISBP2. However, since SECISBP2 is rate-limiting for Sec incorporation, this reduction in protein levels will compromise selenoprotein synthesis. It is unclear whether a correlation between the degree of SECISBP2 deficiency and the severity of clinical phenotype exists, due to the limited number of cases with varying ethnic backgrounds, identified to date. Interestingly, selenoprotein mRNA levels in *SECISBP2* cases do not always follow the canonical rules for NMD, induced by the presence of the in-frame UGA codon, as also described for liver and neuron-specific *Secisbp2* null mice [3, 22]. These findings suggest SECISBP2 deficiency might affect selenoprotein mRNA stability via hitherto uncharacterized mechanisms in addition to susceptibility to NMD, all of which may contribute to hierarchical selenoprotein expression [5, 11].

Abnormal thyroid function tests are a universal finding in all *SECISBP2* cases described hitherto, with typical abnormalities comprising raised circulating thyroxine (T4), low or normal triiodothyronine (T3), elevated reverse T3 (rT3), and normal/high plasma thyroid-stimulating hormone (TSH) levels (Table 44.2). This pattern likely reflects abnormal metabolism of thyroid hormones, due to deficiencies of three, Sec-containing, deiodinase enzymes [23]. Such abnormal thyroid function, together with low plasma Se-levels, reflecting deficiencies of SEPP1 and GPx3, provides a biochemical signature that facilitates identification of putative

Table 44.1 Human selenoprotein synthesis pathway gene defects

Gene	Family	Gene mutation	Protein mutation	Alleles affected	Suggested mechanism	Ref
SECISBP2	A	c.1619G>A	R540Q	Homozygous	SID/bipartite/K-rich region affecting SECIS/ribosome binding	[17]
	B	c.1312A>T	K438X	Compound heterozygous	Premature stop with absence of full length protein	[17]
		c.IVS8ds+29G>A	fs431X			
	C	c.382 C>T	R128X	Homozygous	Premature stop with absence of full length protein/splice variants affected	[16]
	D	c.358 C>T	R120X	Compound heterozygous	Premature stop absence of full length protein/splice variants affected	[48]
		c.2308 C>T	R770X		Premature stop C-terminal end of RNA binding domain missing	
	E	c.668delT	F223fs255X	Compound heterozygous	Premature stop missing full length protein	[21]
		c.IVS7-155, T>A	fs295X+fs302X		exon 6-7 splicing affected resulting in premature stop with absence of full length protein	
	F	c. 2017T>C	C691R	Compound heterozygous	Predicted to affect SECIS/ribosome binding, increased proteasomal degradation	[21]
		1–5 intronic SNP's	fs65X+fs76X		transcripts lack exons 2-3-4 or 3-4 resulting in premature stop with absence of full length protein/splice variants affected	
	G	c.1529_1541dup CCAGCGCCCCACT	M515fs563X	Compound heterozygous	Premature stop missing full length protein	[20]
		c.235 C>T	Q79X		Premature stop missing full length protein	
	H	c.2344 C>T	Q782X	Compound heterozygous	Premature stop missing full length protein	[18]
		c.2045–2048 delAACA	K682fs683X		Premature stop missing full length protein	
	I	c.660 C>T	R197X	Compound heterozygous	Premature stop missing full length protein	[19]
		c.2108G>T or C	E679D		Predicted to affect SECIS and ribosome binding	

tRNAsec	A	c.C65G	–	Homozygous	Results in decreased expression level and affect tRNA modifications	[57]
SEPSECS	A, B	c.1001A>G	Y334C	Homozygous	The mutations are expected to disrupt folding and the catalytic function, as illustrated by In vitro experiments	[63]
	C, D	c.715G>A	A239T	Compound heterozygous		
		c.1001A>G	Y334C			
	E, F, G	c.974>G	T325S	Compound heterozygous	Structural rearrangements in the catalytic pocket would yield an enzyme with reduced catalytic activity, as illustrated by In vitro experiments	[65]
		c.1287>A	Y429X			
	I	c.1001T>C	Y334H	Homozygous	The mutation is expected to disrupt folding and the catalytic function	[68]
	J	c.77delG	R26Profs*42	Compound heterozygous	Premature stop	[66]
		c.356A4G	N119S		Predicted to be pathogenic	
	K	c.356A4G	N119S	Compound heterozygous	Predicted to be pathogenic	[66]
		c.467G4A	R156Q			
	L	c.1A4G	M1V	Compound heterozygous	Removal ATG start, possible alternative ATG or absence of protein	[69]
		c.388+3A4G	FSX		splice site defect leading to premature stop	
	M	c.1466A4T	D489V	Homozygous	Predicted to be pathogenic	[67]
	N	c.1027_1120del	E343Lfs354	ND	Deletion leading to a premature stop	[64]

Table 44.2 Main physiological phenotypes in patients with selenoprotein deficiency

Gene	Family	Age (years) Gender; Ethnicity; phenotype	Effect of treatment	Plasma Se	FT4	T3	rT3	TSH
SECIBP2	A	14 years (M); 7 years (M); 4 years (F); Saudi Arabian; Short stature, DBA; Normal mental development	Se: no effect	↓	↑	↓	↑	↑
	B	6 years (M); Irish/Kenyan; Short stature, DBA	–	↓	↑	↓	↑	N
	C	8 years (M); Ghanaian	Se: no effect	↓	↑	↓	↑	N
		Short stature, DBA	T3: improved growth skeletal maturation					
	D	12 years (F); Brazilian; short stature; kyphoscoliosis; DBA; failure to thrive; impaired mental and motor development; hip girdle weakness; peripheral sensory neuropathy; hypotonia; bilateral sensorineural hearing loss; asymmetric leg length; bilateral clinodactyly; fatty infiltration of muscle; spirometry: reduced expiratory and inspiratory flow; Increased fat mass	Se: increased plasma Se and GPx but not SEPP levels T3	↓	↑	N	↑	↑
	E	35 years (M); British; Developmental delay; bilateral sensorineural hearing loss; vertigo; lumbar spinal rigidity; reduced axial and neck strength; fatty infiltration of muscle; spirometry: reduced vital capacity, nocturnal hypoventilation; Genu valgus; increased fat mass; low insulin and high adiponectin; favourable blood lipid profile; low intrahepatic lipid; Azoopsermia, Raynauds disease, photosensitivity, mild lymphopenia and reduced red cell mass	–	↓	↑	N	↑	↑
	F	2 years (M); British; Failure to thrive, Short Stature, Developmental delay, Bilateral sensorineural hearing loss;	T3: normalised FT3, improved growth, speech and development	↓	↑	↓	↑	N
		Proximal and axial myopathy, lumbar rigidity, fatty infiltration of adductor muscle						
		Increased fat mass, Hypoglycaemia with low fasting insulin; Eosinophilic colitis						

	G	2.6 years (M); Japanese	GH: improved growth	↓	↑	↓	ND	N
		Short Stature; DBA; failure to thrive; delayed motor and intellectual development; IQ 70; rotatory vertigo Bilateral mild conductive hearing loss; fatty infiltration of muscle; increased fat mass; hypoplastic thyroid gland.	GH and T3: normal FT3, low FT4, improved growth and skeletal maturation					
	H	11 years (F); Turkish; Short Stature, Mental and motor retardation, IQ 50	–		↑	↓	↑	N
	I	5 years (F); Argentinian; DBA—Failure to Thrive	–		↑	↓	↑	N
tRNA sec	A	8 years (M); Kuwait/Iraqi; Recurrent episodes of abdominal pain; diarrhoea and/or constipation; fatigue, muscle weakness (proximal), no spinal rigidity; hypermetropia, astigmatism, exophoria	T3: normalised FT3, improved growth	↓	↑	N	↑	N

(continued)

Table 44.2 (continued)

Gene	Family	Age (years) Gender; Ethnicity; phenotype	Effect of treatment	Plasma Se	FT4	T3	rT3	TSH
SEPSECS	A	M/F; Jewish Iraqi; pontocerebellar hypoplasia type 2D (PCH2D): progressive cerebellocerebral atrophy and microcephaly with profound mental retardation; severe spasticity and myoclonic or generalized tonic-clonic seizures	–	ND	ND	ND	ND	ND
	B							
	C	M/F; Iraqi/Moroccan; PCH2D						
	D							
	E	M, M, F, F; Finnish; Age at death (year): 8.5, 4.3, 15. 3, 2.7; PCH2D: irritable from birth; absent head control; severe spasticity (opisthotonus, tremors, myoclonus); epileptic seizures; dysmorphic features; visual impairment; absent psychomotor development; edema of hands, feet and face; moderate degeneration of the liver post-mortem; elevated lactate; abnormal EEG (hypsarrhythmia, slow background activity)	–	ND	N	ND	ND	↑
	F							
	G							
	H							
	I	8 years (M), Arabian, PCH2D; mild mitochondrial myopathy; optic nerve atrophy	Pain relief, supplements	ND	ND	ND	ND	ND
	J	10 years (F), Japanese Late-onset progressive cerebellocerebral atrophy, Developmental delay, Ataxic gait, Dysmetria, Nystagmus, Hypotonia		ND	ND	ND	ND	ND
	K	21 years (F), Japanese Late-onset progressive cerebellocerebral atrophy, congenital microcephaly, Developmental delay, Ataxic gait, Dysmetria, Nystagmus, Hypotonia		ND	ND	ND	ND	ND
	L	PCH2D		ND	ND	ND	ND	ND
	M	Jordan, Syndromic ID/DD		ND	ND	ND	ND	ND
	N	Neurodegenerative disease		ND	ND	ND	ND	ND

DBA delayed bone age, *Se* selenium, *T3* triiodothyronine, *IQ* intelligence quotient, *M* male, *F* female, *N* normal, *ND* not determined

SECISBP2 defect cases. Failure to thrive in infancy and short stature are the commonest presenting symptoms in most *SECISBP2* cases; associated abnormalities include delayed bone age, asymmetric leg length and clinodactyly. The pathogenesis of such growth retardation and delayed bone development is probably multifactorial and has been described in several mouse models (*Dio1* and *2*, *Secisbp2* and *Trsp*) [3, 22, 24–26], with no clear link to a single selenoprotein defect.

Three patients (D, E and F) exhibit sensorineural hearing loss, with the adult male being most severely affected and requiring hearing aids (Proband E), perhaps reflecting progressive, age-related dysfunction. Proband G has conductive hearing deficit, associated with recurrent otitis media. Hearing loss and retarded cochlear development are recognized features in *Dio2* null mice [26]. Alternatively, the hearing loss could reflect damage mediated by elevated cellular reactive oxygen species (ROS) resulting in cumulative damage [27, 28]. Many *SECISBP2* cases exhibit developmental delay (global, speech, motor and intellectual). IQ is variably affected, ranging from normal to severely reduced. However, in those cases in whom intellectual impairment occurs, it cannot be ascribed to deficiency of specific selenoproteins (see neuropathologic phenotype in *SEPSECS* cases). Motor delay in these patients may be a consequence of muscular dystrophy with fatigue and weakness, affecting proximal (leg and hip girdle), neck and axial muscles, a recognized feature in several patients (family D, E and F). This musculoskeletal phenotype in *SECISBP2* cases is highly analogous to that seen in a spectrum of myopathic disorders collectively termed the SEPN1-myopathies, which occur due to defects in *SEPN1*. This group of myopathies varies in clinical severity and age of onset and includes rigid spine muscular dystrophy, desmin-related myopathy with mallory body-like inclusions and congenital fibre type disproportion [29–31].

The adult *SECISBP2* case (Proband E) has a high total body fat mass which is paradoxically associated with favorable metabolic parameters including low fasting insulin, raised adiponectin levels, a favorable lipid profile and negligible intrahepatic lipid levels. Elevated fat mass index and high circulating adiponectin were also recorded in a childhood case (Proband F), who additionally experiences recurrent, symptomatic, hypoglycemia in the presence of low circulating insulin levels, necessitating nocturnal feeds by gastrostomy tube [21]. Increased fat mass was also documented in probands D and F, but was not evaluated in the remaining cases. A substantial body of evidence suggests a link between selenoproteins and systemic insulin sensitivity, although data are sometimes conflicting [32–39]. Proband E is infertile due to maturation arrest in the latter spermatogenic stages, with preservation of early cell types. This is consistent with deficiencies of three selenoproteins (mGPX4, TXNRD3 and SELV), all of which have recognized roles in spermatogenesis [40–42].

Abnormal cutaneous photosensitivity was first documented in Proband E at 13 years of age and confirmed formally by UV skin testing. Abnormal photosensitivity has not been recorded in other *SECISBP2* cases, perhaps because of naturally-occurring pigmentation in non-Caucasian individuals or the absence of formal testing. The photosensitivity cannot be linked to specific selenoprotein deficiencies, but studies have none the less shown that selenoproteins are essential for normal skin development [43].

Proband E has mildly reduced red blood cell and total lymphocyte counts, with normal levels of other cell lineages (e.g., platelets, neutrophils, monocytes), lymphocyte subsets and immunoglobulin levels. His T cell proliferation was impaired, similar to observations in a T cell–specific, *Trsp* null mouse [44]. Peripheral blood mononuclear cells showed significant telomere shortening and, when stimulated, showed markedly enhanced secretion of IL-6 and TNF-α, with diminished IFN-γ production. The telomere shortening was postulated to contribute to reduced red blood cell number and lymphopenia. Intriguingly, shortened telomere length in T cells has also been specifically associated with connective tissue disorders (e.g., lupus, rheumatoid arthritis and scleroderma) [45, 46]; in this context, it was noted that patient E has marked Raynaud's disease, albeit without other clinical or serological features of a connective tissue disorder. Patient F exhibited colitis of unknown etiology, and although there are no known gastrointestinal symptoms in other cases; several human and mouse studies suggest involvement of selenoproteins in colonic function [47]. Since overt immunodeficiency is not a feature of impaired selenoprotein synthesis, haematological and immune cell phenotypes of other *SECISBP2* cases have not been formally evaluated.

Treatment of some *SECISBP2* cases with Se-supplementation augmented serum Se-levels; however, there was no clinical [16, 48] or biochemical (circulating GPX, SEPP1 and thyroid hormone) effect [49]. Treatment of Probands C and F with T3 alone (T4 was not effective in one case) or in conjunction with growth hormone (Proband G) resulted in an improvement in growth, development and bone maturation. More promising results were documented for treatment of Proband G with vitamin E and T3, which resulted in decreased lipid peroxidation products (e.g., 7β-hydroxycholesterol), increased/decreased free T3/T4 levels respectively and increased numbers of circulating white blood cell and neutrophils, all of which reversed after treatment withdrawal [50]. The number of lymphocytes and red blood cells, serum levels of AST, ALT and blood glucose were all within the normal range in Proband G and vitamin E treatment did not cause obvious changes. These results suggest that antioxidant treatment, to counteract effects of elevated cellular ROS, effectively inhibits the generation of lipid peroxidation products, restores T3/T4 levels and affects blood cell numbers [50].

44.3 *TRU-TCA1-1* Mutation

Transfer RNA$^{[Ser]Sec}$ is encoded by *TRU-TCA1-1* and deletion is embryonic lethal in mice [51]. Several unique features distinguish tRNA$^{[Ser]Sec}$ from other tRNAs, e.g., atypical longer acceptor- and D-stems, and only a few modified bases [1]. The two major tRNA$^{[Ser]Sec}$ isoforms contain either 5-methoxycarbonylmethyl-2′-O-methyluridine (mcm^5Um) or its precursor 5-methoxycarbonylmethyluridine (mcm^5U) at position 34, located in the anticodon loop and this may contribute to the stabilization of the codon-anticodon interaction (see Chap. 1 and [52]). The final step in tRNA$^{[Ser]Sec}$ maturation, methylation of mcm^5U, requires other prior base modifications (e.g., pseudouridine (pψ) and 1-methyladenosine (pm1A) at positions

55 and 58, respectively) [52], intact secondary/tertiary structure [53] and aminoacylation of tRNA$^{[Ser]Sec}$ [54]. The relative preponderance of the two isoforms is influenced by systemic Se-status [51, 52, 55] and each subtype has a differing role in selenoprotein synthesis. Synthesis of housekeeping selenoproteins (e.g., TXNRD1 and 3 and GPX4) is reported to be dependent on the mcm^{5}U isoform, whereas production of stress-related selenoproteins (e.g., GPX1, GPX3 and SEPW1), is directed by the mcm^{5}Um species [51, 56]. One patient with a homozygous mutation for a single nucleotide change (C65G) in tRNA$^{[Ser]Sec}$ has been identified to date (Fig. 44.2, Tables 44.1 and 44.2) [57]. The phenotype of the proband is similar to that described in *SECISBP2* patients. However, comparison of selenoprotein expression profiles revealed significant differences, preservation of housekeeping selenoproteins (e.g., TXRNDs and GPX4) compared to *SECISBP2* cases. In contrast, expression of stress-related selenoproteins (e.g., GPX1, GPX3 and SEPW1) was similarly reduced in both contexts. Transfer RNA$^{[Ser]Sec}$ populations in patient-derived primary cells showed preservation of mcm^{5}U levels in the heterozygous parent with some

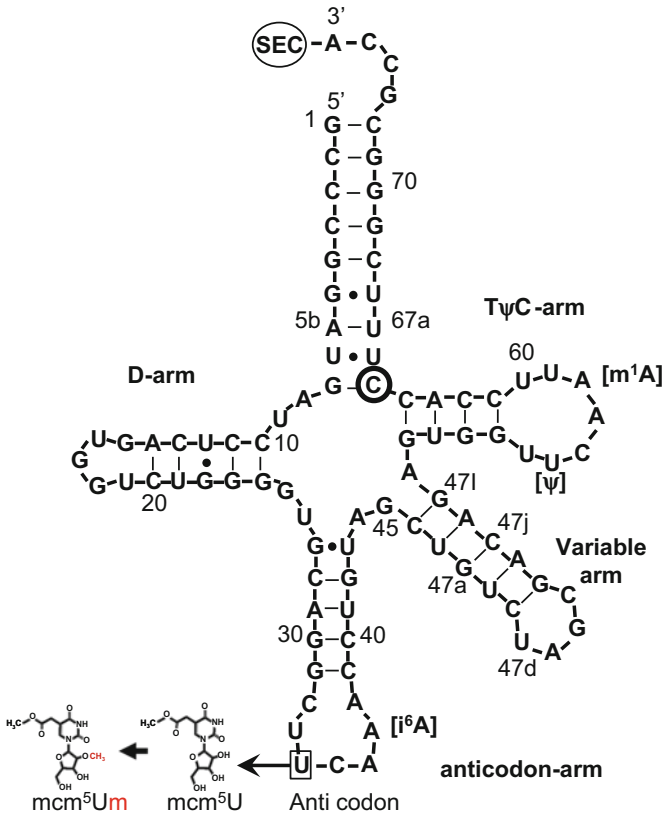

Fig. 44.2 Cloverleaf model of tRNA$^{[Ser]Sec}$ with the mutated base (*ringed*), the uridine (*boxed*) that is modified (tRNA$^{[Ser]Sec}_{mcm^5U}$ or tRNA$^{[Ser]Sec}_{mcm^5Um}$) and other depicted modified bases

reduction of mcm^{5}Um. However, in the proband, disproportionately greater diminution in tRNA$^{[Ser]Sec}$ mcm^{5}Um levels was observed. The C65G tRNA$^{[Ser]Sec}$ mutation also affects the modification i6A at position 37, but there is no indication that aminoacylation, Sec synthesis or binding to SEPSECS were affected. The basis for the reduction of the mutant tRNA$^{[Ser]Sec}$ population is unclear, but possibilities include anomalous transcription, greater mutant transcript turnover or transcript degradation secondary to defective mutant tRNA$^{[Ser]Sec}$ maturation. Preserved selenoprotein levels in heterozygous parents, suggests that moderate reduction in tRNA$^{[Ser]Sec}$ levels is not rate limiting for selenoprotein biosynthesis, as has been documented in other contexts [51]. However, while much lower levels of tRNA$^{[Ser]Sec}$ in the proband are still sufficient for synthesis of some housekeeping selenoproteins, they are insufficient for efficient synthesis of stress-related selenoproteins. Interestingly, synthesis of the essential selenoprotein GPX4 is preserved, although severely impaired, as described in mice lacking the mcm^{5}Um isoform, suggesting that both tRNA$^{[Ser]Sec}$ isoforms are required for its synthesis [reviewed in 1]. In summary, these data suggest that overall decreased tRNA$^{[Ser]Sec}$ levels, with severely reduced tRNA$^{[Ser]Sec}$ mcm^{5}Um isoform levels in the proband, contributed to the specific selenoprotein deficiency pattern.

44.4 *SEPSECS* Mutations

The conversion of Ser-tRNA$^{[Ser]Sec}$ to Sec-tRNA$^{[Ser]Sec}$ is catalyzed by SEPSECS, an enzyme reported to be part of the Sec-insertion complex [58, 59]. The human SEPSECS is also known as soluble liver antigen/liver pancreas (SLA/LP), which represents one of the autoantigens in autoimmune hepatitis [60]. The crystal structures of the archaeal, murine SEPSECS apo-enzymes and the human SEPSECS in complex with tRNA$^{[Ser]Sec}$ have been reported [15, 61, 62]. These provide substantial information on the conversion of Ser-tRNA$^{[Ser]Sec}$ to Sec-tRNA$^{[Ser]Sec}$ and suggest that it forms its own branch in the fold type I family of pyridoxal phosphate dependent enzymes. In the human structure, four SEPSECS subunits form a tetramer binding two tRNA$^{[Ser]Sec}$ molecules through their long acceptor-TΨC arms. Interaction of tRNA$^{[Ser]Sec}$ with the active site of SEPSECS induces a conformational change allowing binding of O-phosphoseryl-tRNA$^{[Ser]Sec}$, but not free phosphoserine, in order for the reaction to occur.

To date, ten families with either compound heterozygous or homozygous *SEPSECS* defects have been described ([63–69] and Table 44.1 and Fig. 44.3). Most *SEPSECS* defects are missense mutations, but within these families two deletions, one predicted splice site variant and one nonsense mutation all leading to a premature stop have been identified. In silico analysis, based on the SEPSECS structures, predicts disruption of protein folding and catalytic activity, confirmed by in vitro assays for T325S mutation showing some residual SEPSECS activity. Compared to the lethality of murine *Trsp* knockout [51], the phenotype of *SEPSECS* cases is modest, suggesting that the altered gene products retain some residual functional activity

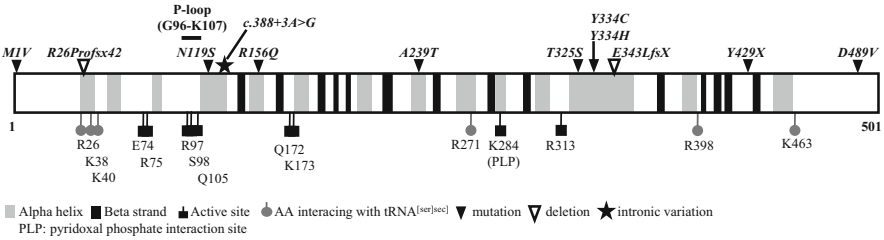

Fig. 44.3 Functional and structural domains of SEPSECS with location of human mutations (*italicized*). The position of the P-loop, alpha helixes, beta strands, AA part of the active site and interacting with the $tRNA^{[Ser]Sec}$ are indicated

in vivo. Although patients are from diverse ethnic backgrounds, they do share similar clinical features (Table 44.2), with the predominant phenotypes comprising progressive cerebellocerebral atrophy, profound mental retardation, severe spasticity and seizures, classified as pontocerebellar hypoplasia type 2D (PCH2D) [63].

Murine and human studies have demonstrated the importance of selenoproteins for normal brain development, maintenance and function [70, 71]. The neuropathologic phenotype is reminiscent of some mitochondrial diseases (e.g., PEHO, PCH6 and PCH2 types, A, B and C, and Alpers syndrome) [72–76], with some (indirect) evidence of mitochondrial dysfunction in brain samples of probands E, F, G and H and mild mitochondrial myopathy in Proband H, although no significant alterations of the mitochondrial respiratory chain complexes were detected. Since mitochondria are a major source of cellular H_2O_2, the lack of antioxidant selenoenzymes may result in ROS-mediated cellular damage, possibly accounting for phenotypes which mimic features seen in mitochondrial diseases.

The thyroid phenotype, a biochemical signature in *SECISBP2* and *TRU-TCA1-1* cases, was not addressed in detail in *SEPSECS* cases. Probands E, F, G and H also presented with a progressively deteriorating hypsarrhythmia EEG signal and microvacuolar fatty degeneration of the liver parenchyma. Interestingly, while selenoprotein expression (GPX1, GPX4 and TXNRD1) was reduced in the brain, expression in fibroblasts and myoblasts was found to be normal in families E, F and G which is difficult to explain, since SEPSECS is thought to be essential for generation of $tRNA^{[Ser]Sec}$. Alternatively pathways for rescue of selenoprotein expression have been suggested despite a paucity of supporting evidence. An important difference between the brain and most other tissues during systemic selenium deficiency, is its efficiency in retaining appropriate Se-concentrations due to preferential cerebral Se transport via SEPP1 and ApoER2 at the cost of other organs [77]. This suggests that preservation of adequate selenoprotein expression is crucial for cerebral development, maintenance and functioning which comes at a cost for other tissues in a Se-deficient environment; and, for unknown reasons, CNS selenoprotein synthesis exhibits greater dependence on SEPSECS than on SECISBP2 or $tRNA^{[Ser]Sec}$.

44.5 Concluding Remarks

With the expression of most members of the selenoproteome being affected, it is not surprising that the phenotype of general selenoprotein deficiency disorder is complex (Table 44.2). Several features of this syndrome, such as abnormal thyroid function, azoospermia, myopathy and low plasma Se, are attributable to deficiencies of particular selenoproteins, DIO1, 2 and 3, SELV, GPX4, TXNRD3, SEPN1, SEPP1 and GPX3, and the associated biochemical signature facilitates identification of putative selenoprotein synthesis defect cases. Deficiency of antioxidant selenoenzymes mediates raised cellular ROS, which may manifest as photosensitivity, age-dependent hearing loss and may contribute to enhanced insulin sensitivity, and phenotypes similar to mitochondrial disorders. Other clinical features, e.g., growth retardation, delayed mental development and motor milestones and neurodegeneration, may have a complex, multifactorial basis, due to abnormalities in multiple selenoprotein pathways.

The possibility of additional phenotypes secondary to oxidative damage, e.g., neoplasia and premature aging, which may emerge with time, cannot be discounted. Consistent with a recessive mode of inheritance, documented in some kindreds, heterozygous individuals are unaffected. The nature of the gene defect and environmental factors such as iodine and Se-status, might contribute to inter-individual differences in phenotypes in patients from different ethnic and geographical backgrounds. However, it is interesting that mutations in *SECISBP2* and *TRU-TCA1-1* lead to a very similar phenotype with a specific thyroid signature and muscle myopathy, while the main phenotype in *SEPSECS* cases is progressive cerebellocerebral atrophy, with the severity of this phenotype having possibly prevented more detailed analysis to ascertain other phenotypes. In *SECISBP2* and *TRU-TCA1-1* cases, there is no obvious neuropathological phenotype, although IQ is variably affected.

Evidence from *SEPSECS* cases and mouse models suggests a progressive neurological phenotype that could present at older age; to date this has not manifested in the adult *SECISBP2* case (Proband E), and it is conceivable that most human *SECISBP2* mutations are not severe enough to be associated with CNS abnormality. In summary, *SECISBP2*, *TRU-TCA1-1* and *SEPSECS* have been shown to be essential elements in the generation of human selenoproteins. While there is a substantial body of information regarding their function, detailed understanding of the Sec-insertion pathway is far from being elucidated. Patterns of defective selenoprotein synthesis in these patients are consonant with our current knowledge of the Sec-insertion pathway. However, the basis for residual selenoprotein expression and different, sometimes progressive, clinical phenotypes in these disorders warrants further investigation. Identification of further cases may also reveal additional phenotypes, linked to deficiencies of selenoproteins of unknown function. In addition to careful evaluation of selenoprotein deficient patients, transgenic mouse models are of great utility, with cellular and biochemical characterisation of tissues not accessible in human patients providing mechanistic insights.

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Chapter 45
Alteration of Selenoprotein Expression During Stress and in Aging

Zahia Touat-Hamici*, Yona Legrain*, Jordan Sonet, Anne-Laure Bulteau, and Laurent Chavatte

Abstract Selenium (Se) is an essential trace element implicated in many facets of human health and disease. Most of its beneficial effects are attributed to its presence as selenocysteine in a small, but vital group of proteins, namely the selenoproteins. They are implicated in antioxidant defense, redox homeostasis, redox signaling and possibly other cellular processes. The selenoproteome is primarily controlled by Se bioavailability that induces prioritization of protein biosynthesis, when this trace element is deficient. The hierarchical regulation of the selenoproteome by other exogenous stimuli, cellular stressors or pathophysiological conditions is poorly understood. Understanding biological causes of aging also remains challenging, although several theories and concepts have emerged in the past decades. Characterization of biomarkers of aging is controversial even with the impressive amount of 'omic' analyses performed in many living organisms. Accumulation of age-related damage, including oxidative-induced cellular damage, and the decreasing efficiency in elimination and repair systems have been extensively reported,

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being either a cause or consequence of the aging phenomenon. In this regard, and given the role of Se in redox biology of organisms, studying regulation of the selenoproteome in response to oxidative stress and aging is essential. This chapter reviews the current knowledge in this area.

Keywords Cellular senescence • Gene regulation • Oxidative stress • Selenocysteine • Selenoproteome • Translation control • UGA recoding

45.1 Introduction

Integrity and homeostasis of organisms are constantly challenged at the molecular level by reactive oxygen and nitrogen species (RONS) that are either generated endogenously during cellular metabolism, mostly by mitochondria, or originate from exogenous sources including drugs, xenobiotics, metals and radiation. RONS alter a wide variety of macromolecules, including proteins, lipids, sugars, DNAs and RNAs. Depending on the severity of oxidative damage, newly oxidized molecules are repaired by various cellular machineries or are eliminated by dedicated degradation systems. When RONS production is overwhelming or when repair/degradation systems are impaired, oxidative stress occurs. It follows that oxidized molecules accumulate and hamper biological functions, leading to pathological conditions and eventually to death. Accumulation of RONS-induced cellular damage is associated with cancer, neurological disorders, atherosclerosis, inflammation, and aging. Given the essential role of several selenoproteins in antioxidant defense, redox homeostasis and signaling, RONS may link the biological functions of selenium (Se) and selenoprotein in mammals with these pathologies.

45.2 Aging, Lifespan and Senescence

45.2.1 Definitions

45.2.1.1 Aging

Aging is a slow, complex, and multifactorial process, resulting in a gradual and irreversible decline of various functions of the body, making organisms more vulnerable and more likely to die. Several theories and concepts tried to comprehend the biological basis of aging, with the most recognized being the free radical/damage and evolution theories of aging.

45.2.1.2 Longevity

Longevity of a species is defined as the time between birth and death, and also referred to as lifespan. Maximum longevity refers to the maximum lifespan achieved by individuals of a species, while mean longevity is a statistics reference for life

expectancy of a defined organism or individual based on different genetic background or risk factors.

45.2.1.3 Cellular Senescence

Cellular senescence (or replicative senescence) reflects the fact that diploid cells have a finite number of divisions, also referred to as Hayflick limits [1]. Cellular senescence is characterized by an irreversible cell cycle arrest, telomere shortening, accumulation of oxidative damage and several senescence-associated markers, including ß-galactosidase (SABG) and heterochromatin foci (SAHF). Since several aspects of organismal aging are recapitulated in senescent cells, they offer a commonly used cellular model to study the aging phenomenon. Indeed, the number of senescent cells increases with age and senescent-associated phenotypes are considered to be predictive features of age-related phenotypes.

45.2.1.4 Rejuvenation

Rejuvenation refers to a possibility of reversing the aging process, *via* removal of age-related damage and replacement of aged cells by novel ones. Rejuvenation can therefore lead to an increase in lifespan, although this has not been experimentally demonstrated.

45.2.1.5 Reactive Oxygen Species (ROS)

Cellular ROS are generated endogenously as side-products of mitochondrial oxidative phosphorylation, and also arise from exogenous sources. ROS consist of radical and non-radical oxygen species formed by partial reduction of molecular oxygen. They include superoxide anion ($O_2^{\bullet}$), hydrogen peroxide (H_2O_2), and hydroxyl radical ($HO^{\bullet}$). ROS are also essential molecules in physiological processes such as cell signaling, proliferation, tumor suppression, and the immune system.

45.2.1.6 Reactive Nitrogen Species (RNS)

Cellular RNS are generated mostly from nitric oxide (NO) reactions with other radical species, NO being produced by nitric oxide synthase (NOS). While NO is an essential signaling component for vascular functions, RNS such as peroxynitrite, $ONOO^-$, are highly reactive with proteins, predominantly tyrosine residues, rendering those targets compromised with deteriorated function, comparable to carbonyls in proteins. In addition, peroxynitrite can also react with proteins containing transition metals and oxidize cysteine (Cys) residues, thereafter impairing their integrity.

45.2.1.7 Oxidative and Nitrosative Stresses

Oxidative and nitrosative stresses result from overproduction of RONS relative to their elimination. Conversely, excessive use of antioxidants reduces the levels of signaling RONS and therefore creates reductive stress.

45.2.1.8 Age-Related Diseases

Age-related diseases concern chronic diseases that arise with aging, such as cancer, diabetes and neurodegenerative diseases. When occurring in young individuals, these diseases have often a strong genetic component.

45.2.1.9 Healthy Aging

Healthy aging, which is also known as successful aging, is the maintenance of health and optimal physiological, psychological and social functions in older people.

45.2.2 Free Radical Theory of Aging

The free radical theory of aging, initially proposed by Harman in the 1950s [2], proposes that accumulation of oxidative damage is the main driving force in the aging process. Many components of cells (proteins, lipids and nucleic acids) are susceptible to react with ROS and RNS to give rise to a wide variety of oxidized biomolecules. Some of these modifications could be critical for optimal performance of a given cell, tissue or organism. Proteins are targets of reactive species leading to peptide backbone cleavage, cross-linking and alteration of the side chain of virtually all amino acids, several of them being more vulnerable, such as Cys, methionine, lysine, tyrosine, histidine and tryptophan. Although somewhat controversial, the detection of these oxidatively modified molecules is widely used as a marker of oxidative stress and aging [3, 4].

Interestingly, Se has the property of being highly resistant to irreversible oxidation in comparison with sulfur, suggesting that selenoproteins could be more resistant to inactivation by oxidation than Cys counterparts. This has been validated in a thioredoxin reductase (TrxR) model where the mitochondrial Sec-containing TrxR was far more resistant to a wide variety of oxidants (including H_2O_2, hydroxyl radical, peroxynitrite, hypochlorous acid, hypobromous acid, and hypothiocyanous acid) than the Cys-ortholog in *Drosophila melanogaster* [5].

45.2.3 Damage Theory of Aging

The free radical theory of aging, although followed by a large number of scientists, is challenged by the lack of causality between oxidative damage and aging in certain models. Recently, a more global picture of damage theory of aging is emerging.

Enzymatic and metabolic reactions are far from perfect in real life, and this inherent imperfectness or heterogeneity leads to the generation of damage in every biological reaction, from molecular to cellular levels. Cells cannot deal with every damage, since the variety of damage will always be higher than the number of repair or elimination systems. In addition, this damage could escape natural selection pressures when such damage is low in abundance, or only slightly deleterious. The variety of damage is far too numerous to deal with, and its accumulation manifests as aging. The removal of the damage only occurs by dilution (cell division), or when cells are renewed from stem cells. Somehow, the imperfectness theory of aging extends the free radical theory, not only to oxidatively related damage, but also to all kinds of damage, caused by endogenous reactions and by exogenous hazards or xenobiotics [6].

45.2.4 Evolutionary Theory of Aging

Natural selection of individuals is a phenomenon that ensures survival and adaptation of the species to its environment and predators. Mutations impairing older organisms have a greater chance of escaping removal from the population by natural selection. From these observations, two key evolutionary theories have emerged: the mutation-accumulation theory and the antagonistic pleiotropy or trade-off theory [7]. The mutation-accumulation theory stipulates that mutations with deleterious effects at a later age will undergo less selection pressure than mutations with early age onset. These mutations will appear in accordance with the endogenous mutational rate of the species, the selection being less efficient for the older population. Therefore, mutations with deleterious effect later in life will accumulate and lead to the aging phenotype. The concept of antagonistic pleiotropy predicts that under a natural environment, if a mutation presents a selective advantage to escape predators or diseases, or to adapt to the environment in early age, it will be incorporated in the population even though it is detrimental later in life. In both theories, rates of aging will evolve as a function of mutational rates and external hazards: lifestyle, environment or predators. When applied, the force of natural selection will improve survival and fertility capabilities that benefit early in life to the detriment of aging rates as soon as it ensures the species preservation.

45.3 Selenoprotein Synthesis and Regulation

45.3.1 Mechanism and Factors for Sec Insertion

Sec is referred to as the 21st proteogenic residue since it was the first addition to the genetic code deciphered in 1960s. Sec is inserted during translation of selenoprotein mRNAs when the ribosome encounters a UGA codon, which is generally used as a stop codon. This UGA-Sec recoding event implies an intricate machinery for Sec

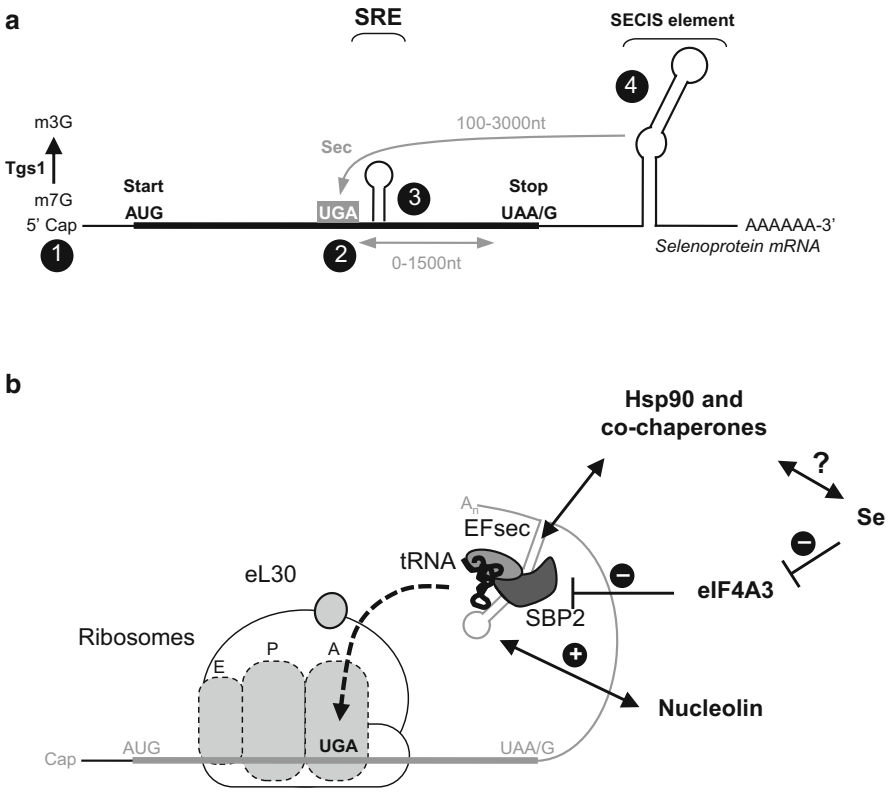

Fig. 45.1 Translational control mechanism for selenoprotein expression in eukaryotes. (**a**) Schematic representation of a selenoprotein mRNA with its different *cis*-acting regulatory features: (*1*) modification of the 5′Cap; (*2*) context and position of the UGA codon; (*3*) the presence of a hairpin structure (SRE) downstream of UGA codon; and (*4*) the nature of the SECIS element. (**b**) *Trans*-acting factors influencing the translation efficiency of UGA recoding. Levels of eIF4A3 are reduced upon Se supplementation

insertion centered around two RNA molecules: a dedicated Sec-tRNA, designated Sec-tRNA$^{[Ser]Sec}$ (see also Chap. 1), and the Sec insertion sequence (SECIS) element present in the 3′UTR of selenoprotein mRNAs; and their interacting protein partners ([8–15] and see also Chap. 2).

The SECIS element is a stem-loop-stem-loop structure with poor sequence conservation in eukaryotes (Fig. 45.1). Different SECIS binding proteins have been characterized to date, that include SECIS binding protein-2 (SBP2 or SECISBP2), SBP2-like (SBP2L), ribosomal protein eL30, nucleolin and eIF4A3 [9]. Among these proteins, SBP2 is a limiting factor and thought to dictate Sec insertion efficiency and therefore control selenoprotein levels.

The other central RNA component of Sec insertion machinery is Sec-tRNA$^{[Ser]Sec}$, which is the only tRNA governing by itself the expression of an entire family of

proteins, the selenoproteome (see [16] and Chap. 1). The Sec-tRNA$^{[Ser]Sec}$ is distinct from other cellular tRNAs regarding its size, aminoacylation, transcription, modification and delivery to the ribosome. Detection of Sec-tRNA$^{[Ser]Sec}$ gene in a genome often defines the presence of selenoproteins in a species. The aminoacylation of Sec-tRNA$^{[Ser]Sec}$ is particularly unique since it requires four enzymes rather than one for classical tRNAs. In addition, due to its unusual structure, a dedicated elongation factor, EFsec, is required to transport and faithfully deliver the Sec-tRNA$^{[Ser]Sec}$ to the ribosomal A site in presence of an UGA-Sec codon (Chap. 2).

45.3.2 Translational Control of Selenoproteins

Selenoprotein biosynthesis is an intricate process that is mostly controlled at the translational stage, with the recoding of UGA as Sec, also referred to as Sec insertion efficiency, being the rate limiting stage. A hierarchical response has been described in response to Se status that maintains essential (or housekeeping) selenoproteins at the expense of other proteins, which are considered stress-related. Although well described, the precise mechanism for the prioritized use of Se remains elusive, mostly because of the multiple layers of regulation. As initially observed by polysome profiling for *Gpx4* mRNA [17] and extended by RNA-Seq and ribosome profiling to other mRNAs, the recoding of UGA is a limiting step that induces a pause of the ribosome [18]. When detectable, this pause is alleviated by Se supplementation demonstrating that recoding of UGA is a critical regulatory step for selenoprotein expression.

Pause of ribosome at the UGA-Sec codon can be modulated by different features inherent to selenoprotein mRNA, including the upstream and downstream UGA codon context, the presence of a downstream RNA structure, and the nature of the SECIS element ([9] and see also Chap. 2 and Fig. 45.1). The influence of codon context on Sec insertion efficiency has been demonstrated by different groups [19, 20], but did not generate conclusive rules for predicting recoding activity. Additionally, the distance between the UGA and the SECIS element, initially thought to be important, does not strongly affect Sec insertion efficiency. On the other hand, other *cis*-acting RNA structures than the SECIS element have also been detected in different mRNAs, including *SelN*, *SelK*, *SelO*, *SelH*, *SelT*, *SelS* and selenophosphate synthetase 2 (*Sps2*) [9, 21]. The so-called Sec redefinition elements (SRE) are hairpin structures located downstream of UGA codon, which improve stop codon readthrough efficiency by steric hindrance. Finally, the nature of the SECIS element has also been found to modulate Sec insertion efficiency [22]. In addition, the response to Se variation also depends on the nature of the SECIS element, making it an important determinant for selenoprotein hierarchy in response to various stimuli [23].

An additional feature of several selenoprotein mRNAs has been recently noticed and concerns the 5′end, which can be hypermethylated by trimethylguanosine synthase 1 (Tgs1) enzyme [24]. Indeed, Tgs1 already catalyzes the transfer of two

methyl groups to the m^7G cap to form a 2,2,7-trimethylguanosine cap of snRNAs, snoRNAs, telomerase RNA TLC1 and several selenoprotein mRNAs, including *SelX*, *Gpx1*, *Gpx4*, *SelM* and *SelW*. The precise role of the trimethylated cap on mRNAs is unclear, but since this modification does not impair association to polysome fractions and does not occur equally in selenoprotein mRNAs, it may participate to translational control of selenoproteome hierarchy.

Several *trans*-acting factors that bind SECIS selectively regulate Sec insertion. Nucleolin and eIF4A3 have been characterized for their selective SECIS binding properties and influence on UGA-Sec recoding efficiency. While nucleolin appears to be a positive regulator of Sec insertion [25], eIF4A3 acts as a negative regulator by preventing the SECIS binding of SBP2, supposedly by steric hindrance [26]. Interestingly, eIF4A3 seems to be inversely regulated by Se levels to regulate a cohort of non-essential selenoproteins.

45.3.3 Transcriptional Control of Selenoproteins

Similar to other genes, selenoprotein expression is controlled at the level of transcription. A 2009 review summarized the published studies on transcriptional regulation of mammalian selenoprotein genes, followed by *in silico* analysis on predicted regulatory elements [27]. NFkB transcription factor, which is one of the cellular sensors responding to oxidative stress, has been reported to regulate several selenoprotein genes, including *Gpx4*, *SelS* and *Dio2*. NFkB is ubiquitous and involved in cellular responses to several stimuli or stressors, including cytokines, free radicals, UV irradiation, oxidized LDL, and bacterial or viral antigens. NFkB binding sites are predicted *in silico* in all 25 selenoprotein genes [27], but awaits further investigation for a comprehensive picture of selenoproteome regulation by NFkB.

Metal response elements (MREs) are short *cis*-acting sequences that are bound by metal responsive transcription factor 1 (MTF-1). Upon heavy metal exposure or cellular stress, MTF-1 translocates to the nucleus, where it regulates expression of metallothioneins and selenoproteins, including SelH, TrxR2 and SelW [28]. Interestingly, MREs are also predicted in almost all human selenoprotein genomic regions surrounding transcription start sites [27].

Antioxidant responsive elements (AREs) are *cis*-acting sequences that are bound by transcription factor NF-E2-related factor 2 (Nrf2). Nrf2 targets a multitude of genes involved in stress response, antioxidant activity, anti-inflammatory response, DNA repair, molecular chaperones, proteasome systems, and two selenoproteins, Gpx2 and TrxR1. Activation of this pathway is induced in certain cell lines during severe Se deficiency or complete loss of selenoproteins obtained by $tRNA^{[Ser]Sec}$ gene inactivation [29]. Whether these NF-kB binding sites, MREs or AREs are present in genes involved in Sec insertion machinery remains to be investigated to grasp a better picture of transcriptional control of selenoprotein expression in response to cellular stressors or xenobiotics.

45.4 Interplay Between Se, Selenoproteins, Oxidative Stress and Aging

45.4.1 Oxidative Stress

Selenoproteins are enzymes mostly implicated in antioxidant defense, redox homeostasis and signaling, in which Sec is often located in the catalytic site. Well-characterized members are Gpxs, thioredoxin reductases (TrxRs), methionine sulfoxide reductases (Msrs), iodothyronine deiodinases (Dios), and several endoplasmic reticulum (ER) selenoproteins. In addition, Sps2 is involved in selenoprotein biosynthesis, and is therefore a Se sensor that regulates its own expression. For about a third of the selenoproteome a redox function is only predicted by homology. Gpxs act as a major ROS defense by detoxifying hydrogen and lipid peroxides in mitochondria (Gpx4), cytoplasm (Gpx1, Gpx2), nucleus (Gpx4), plasma membrane (Gpx4), epithelium (Gpx2, Gpx6) and serum (Gpx3). TrxRs are involved in thiol homeostasis by reducing thioredoxin and various other substrates. They are also distributed in different cellular compartments, i.e., TrxR1 in the nucleus/cytosol, TrxR2 in mitochondria, and TrxR3 (or TGR) in testis. Finally, the ER selenoproteins (Sel15, SelS, SelK, SelN, SelM, and SelT) have important functions in protein folding and ER stress response. To date, the precise function of selenoproteins localized in the nucleus (SelH, Gpx4 and TrxR1) remains unclear. Animal models with selenoprotein gene inactivation have not been performed systematically, making difficult the classification between housekeeping (or essential) and stress-related (non-essential) members [30]. The requirement of selenoproteins for cell protection against oxidative stress and maintenance of redox homeostasis is evidenced by human genetic inherited diseases and animal/cellular models, where recoding factors are targeted. As proof, mutations in SBP2, causing a global defect of the selenoproteome, lead to a severe and complex phenotype, including increased cellular ROS and susceptibility to ultraviolet radiation-induced oxidative damage in skin [31–33].

In regard to their activity in redox reactions, it has long been hypothesized that the selenoprotein expression could be regulated by oxidative stress or stress-related processes such as replicative senescence or aging. However, only limited work has been performed to this aim. An initial report has shown a selective downregulation selenoproteins in response to H_2O_2 treatment (from 100 up to 1000 μM) [34]. Concomitantly, a transient relocation of SBP2 in the nucleus is observed. Further studies show that this regulation of selenoproteins by oxidative stress is highly dependent on Se status [35]. Interestingly, while a selective upregulation of selenoprotein activity (Gpx and TrxR) and expression (Gpx1, SelK, Sps2, Gpx4, TrxR1, SelS and see Fig. 45.2) is observed after H_2O_2 treatment upon Se deficiency, very little change is noted in optimal conditions. Furthermore, Se depleted cells are much more sensitive to H_2O_2 induced oxidative stress as revealed by different markers such as levels of oxidized proteins, and levels of ROS in mitochondria. It appears from selenoprotein mRNA analyses and reporter constructs measuring UGA

recoding efficiency that most of this regulation occurs at the translational levels. A nucleocytoplasmic shuttling of recoding factors has been further detected for SBP2, eL30, and, to a lesser extent, EFsec [35]. Interestingly, SBP2 contains a redox sensitive Cys-rich domain that overlaps with its RNA binding domain and nuclear export signal [34]. It is tempting to speculate that SBP2 could sense cellular stressors to selectively regulate the selenoproteome. Additionally, selenoprotein messenger ribonucleoprotein (mRNP) assembly, which is controlled by the Hsp90 chaperone machinery (Hsp90, R2TP, and Nufip) in the nucleus [37], could modulate mRNP export to the cytoplasm and therefore translation efficiency.

Another control for selective UGA recoding efficiency resides in the wobble position modification (U34) of Sec-tRNA$^{[Ser]Sec}$. Indeed, the tRNA methyltransferase ALKBH8 that regulates the level of methylated U34 is induced in response to ROS ([38] and also see Fig. 45.2). Additionally *Alkbh8*$^{-/-}$ embryonic fibroblasts (MEFs) display elevated ROS levels, increase in DNA and lipid damage and in hallmarks of cellular stress. This chapter demonstrates the requirement of ALKBH8 for selective upregulation of Gpxs (Gpx1, 3 and 6) and TrxRs (TrxR1 and 2) in response to H_2O_2 treatments of MEFs.

45.4.2 Replicative Senescence

Only few studies investigated a link between Se, age, and longevity in humans. One study conducted in Italy on healthy subjects showed a decrease in serum Se and in Se-dependent Gpx activity with age [39]. Interestingly, the heterogeneity in Se levels in elderly was so noticeable that it was studied as a predictor of longevity [40, 41]. It appeared that low Se levels led to a weakening of the antioxidant defense and decrease in longevity, although the molecular mechanisms and selenoproteins involved remain to be elucidated. At the cellular level, two studies reported the extension of the replicative life span of cultured cells with Se supplementation [36, 42]. In the most recent study, Se supplementation significantly impacts the level of senescence markers, including signaling molecules (p16, p21, p53), telomere shortening, SABG and SAHF. In this cellular model, and as shown in Fig. 45.2, several selenoproteins are dramatically regulated in response to replicative senescence. It is proposed that this modulation is linked to both a transcriptional and a translational control of selenoprotein mRNA, possibly *via* stress related transcription factors (NFkB, Nrf2, MTF-1) and relocation of SBP2 in the nucleus [36], respectively.

45.5 Concluding Remarks

The selenoproteome is an important component of antioxidant defense against cellular and exogenous stressors. The repertoire of regulatory mechanisms responsible for selenoprotein hierarchy is getting more and more complex. It appears that the

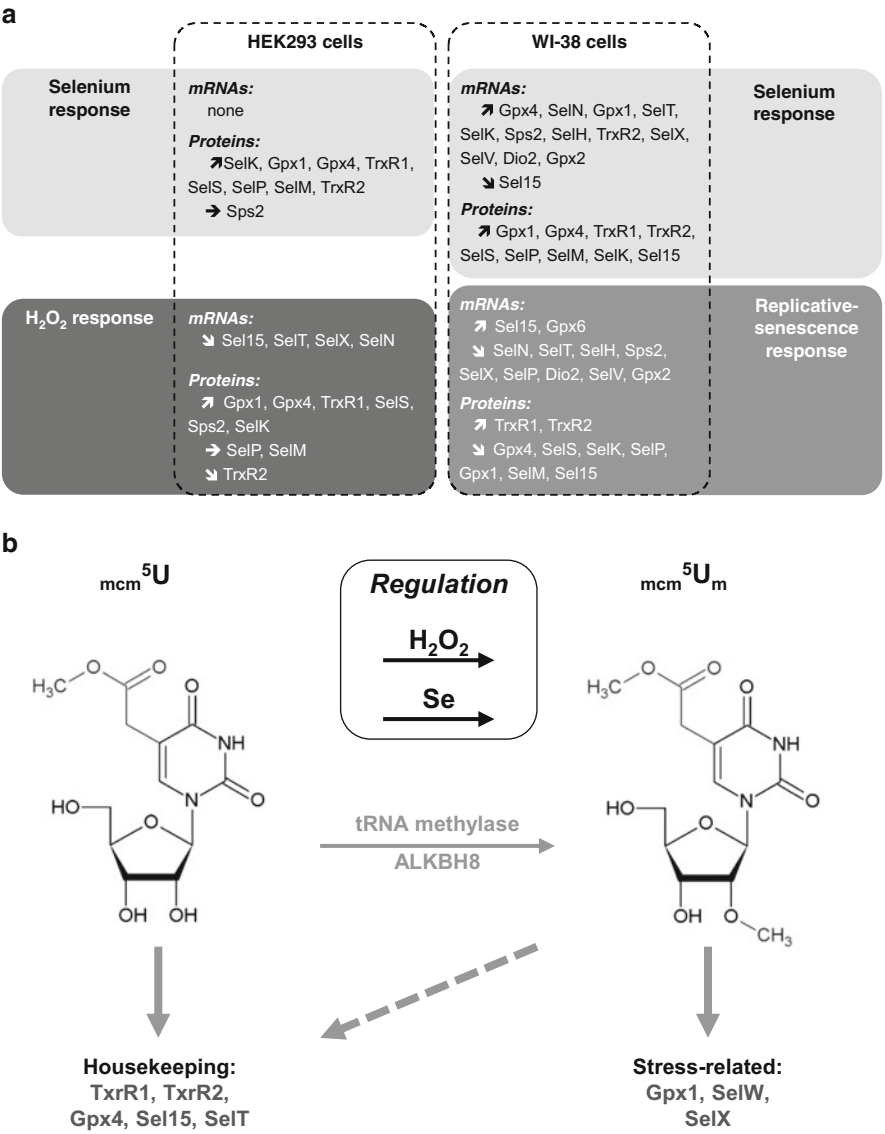

Fig. 45.2 Selenoprotein targets of oxidative stress and aging. (**a**) Schematic of selenoprotein regulation in response to Se (*light grey*) and oxidative stress (H_2O_2) or stress-related process (replicative senescence), in *darker grey*. Experiments were performed in immortalized HEK293 cells [35] and in primary cells WI-38 [36]. (**b**) Regulation of tRNA modification at position U34 between $_{mcm}{}^5U$ and $_{mcm}{}^5U_m$ in response to Se levels and H_2O_2 treatments. The two isoforms of $tRNA^{[Ser]Sec}$ are selectively used for biosynthesis of housekeeping or stress-related selenoproteins, respectively

stage of recoding of UGA as Sec constitutes a checkpoint for translational regulation, which is influenced by many factors, including the SECIS binding proteins (levels, modification and localization), the tRNA (aminoacylation and modifications), and the mRNA itself (5′Cap and SECIS). In certain cases, a transcriptional control also occurs in response to specific stimuli. Like other defense and repair systems, selenoproteins could also be targeted by oxidative stress and stress-related processes. The functional and regulatory characterization of the selenoproteome is a prerequisite to grasp its importance in aging.

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Part V
Biological Models for Elucidating the Role of Selenium and Selenoproteins in Biology and Medicine

A number of biological models have been developed that have provided novel and unique ways to examine selenium and selenoproteins. The chapters in this section discuss such models and how they have been used to further elucidate the many mechanisms of selenium and selenoproteins in health.

Chapter 46
Selenocysteine tRNA[Ser]Sec Mouse Models for Elucidating Roles of Selenoproteins in Health and Development

Bradley A. Carlson

Abstract Selenium (Se), an essential element in the diet of mammals, has been known for many years to play major roles in numerous health benefits and in development. More recently, Se-containing proteins, designated selenoproteins, have been shown to be highly significant components in carrying out these health benefits and also to have essential roles in mammalian development. Se is incorporated into protein as the Se-containing amino acid, selenocysteine (Sec). Selenoprotein synthesis is dependent on Sec tRNA for the insertion of Sec, the 21st amino acid in the genetic code, into protein, providing us with a tool to modulate the expression of selenoproteins by modulating the expression of Sec tRNA. Taking advantage of this dependency, we made various mutants of the Sec tRNA gene that have been used to generate transgenic, conditional knockout, transgenic/knockout and transgenic/conditional knockout mouse models to elucidate many roles of this protein class in health and development.

Keywords Housekeeping selenoproteins • Mouse models • Selenocysteine tRNA • Selenocysteine tRNA modifications • Selenocysteine tRNA mutants • Stress-related selenoproteins

46.1 Introduction

Selenium (Se) was first reported to be an essential element in the diet of mammals by Schwartz and Foltz in 1958, when they found that this element prevented liver necrosis in rats [1]. Since this seminal discovery of a prominent role of Se in mammalian health, many more health benefits of Se have been reported, including roles in preventing cancer, heart disease and other cardiovascular and muscle disorders,

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inhibiting viral expression and delaying the onset of AIDS in HIV-positive patients, enhancing male reproduction and immune function, and roles in mammalian development [2–4]. At the turn of this century, a dispute in the Se field existed whether small molecular weight selenocompounds or selenoproteins were responsible for the many health benefits attributed to Se (see [2, 5–9] and references therein). However, very little was known at that time about selenoproteins, their metabolic roles or the number of selenoproteins encoded in the genomes of mammals. Thus, much additional knowledge about selenoproteins was necessary to assess their metabolic roles.

Two major approaches were undertaken in the early 2000s to elucidate the roles of selenoproteins in health and development. One approach involved specifically designed computational bioinformatics techniques to initially identify the selenoproteome in mammals for subsequent characterization of the roles of individual selenoproteins [10]. This study demonstrated the presence of 25 selenoprotein genes in humans and 24 in rodents, which, upon expression, generated many more selenoproteins, due to alternative transcription initiation and alternative splicing, as well as termination at the second and third UGA codons in Selenoprotein P (*Sepp1*) mRNA [11].

The second approach involved the generation of mouse models expressing selenocysteine (Sec) tRNA (designated $tRNA^{[Ser]Sec}$) mutants for modulating the expression of selenoproteins to assess the function of this protein class in health and development. This latter approach is the subject of this chapter.

46.2 Transfer $RNA^{[Ser]Sec}$ Mouse Models to Elucidate Selenoprotein Function

One of the unique features of selenoproteins is that selenoprotein synthesis is dependent on a single tRNA, $tRNA^{[Ser]Sec}$ (see Chap. 1). Furthermore, the primary and tertiary structures of $tRNA^{[Ser]Sec}$ are critical to the efficiency of this tRNA in expressing selenoproteins [12] and, unlike most other tRNAs, the $tRNA^{[Ser]Sec}$ gene (*Trsp*) is encoded by a single copy gene [13]. *Trsp* can therefore be targeted for deletion in a specific organ or tissue by conditional knockout, removing the expression of all selenoproteins in the targeted tissue. Thus, the expression of this selenoprotein class can be tempered by introducing a mutant *Trsp* transgene into the genome or by generating a mouse line, wherein the mice lack this gene, but the *Trsp* deficient mice are complemented with a mutant transgene. Mouse models using these approaches were prepared to perturb the synthesis of selenoproteins in order to provide a means of elucidating the roles of these Se-containing proteins and providing further insight into whether small molecular weight selenocompounds or selenoproteins have the more significant role in health and development.

46.2.1 *Mouse Models Involving only* Trsp *Transgenes*

Transfer RNA transgenes were first introduced into the genome of mice in 2001 to provide mouse models for better understanding the role of tRNA[Ser]Sec [13, 14]. The initial mouse models contained wild type *Trsp*, specified *Trsp*^{t}, as a transgene, or either one of two mutant transgenes. One of the mutant transgenes, specified *Trsp*^{tG37}, wherein the adenine at position 37 (A37) was changed to guanine (G37), prevented the formation of *N*6-isopentenyladenosine (i^6A) in the mature tRNA [13, 14]. The second mutant transgene, specified *Trsp*^{tA34}, wherein thymine at position 34, which is the wobble codon within the anticodon, was changed to adenine, prevented the formation of 5-methoxycarbonylmethyluridine (mcm^5U) in the mature gene [15].

Mice carrying multiple copies of *Trsp*^{t} overexpressed tRNA[Ser]Sec, but this did not lead to enhanced expression of selenoproteins in various tissues and organs that were examined [13, 14]. These observations suggested that tRNA[Ser]Sec was normally expressed in sufficient levels so that it was not limiting in selenoprotein synthesis. In *Trsp*^{tG37} mice carrying from a few to as many as 40 mutant transgenes, the amounts of stress-related selenoproteins, e.g., GPX1, GPX3, SELR, and SEPW1, decreased in a selenoprotein and organ-specific manner. However, the levels of housekeeping selenoproteins, e.g., TXNRD1 and TXNRD2, were largely unaffected. In liver and testis, GPX1 was most affected, while TXNRD1 and TXNRD2 were least affected. The decrease in stress-related protein expression was proportional to the increase in mutant tRNA[Ser]Sec levels. Interestingly, the amounts of most selenoprotein mRNAs were unaltered in *Trsp*^{tG37} mice, demonstrating that the flaw in selenoprotein synthesis involving the mutant tRNA[Ser]Sec must be at the level of translation [13, 14]. Transfer RNA[Ser]Sec exists in two isoforms, one further methylated at the 2′-*O* position of mcm^5U at position 34, designated mcm^5Um tRNA[Ser]Sec (see Chap. 1). Interestingly, the methylase responsible for mcm^5Um synthesis on tRNA[Ser]Sec appears to have a strict requirement for intact Sec-tRNA[Ser]Sec without any alterations in its primary or tertiary structure [12, 16].

The above studies, as well as additional studies, involving *Trsp*^{tG37} mice provided strong evidence that the mcm^5Um isoform was responsible for synthesizing stress-related selenoproteins, while housekeeping selenoproteins, e.g., TXNRD1 and TXNRD2, were synthesized by the non-Um34 isoform, mcm^5U [15–18]. Other essential selenoproteins, e.g., GPX4 and SEPP1, appeared to be synthesized by both isoforms.

The physical phenotypes of *Trsp*^{tG37} mice encoding as many as 40 mutant transgenes appeared to be normal [13, 14], although *Trsp*^{tA34} mice could not encode more than 12 mutant transgenic copies [15]. An adenine (A) located in the anticodon wobble position of *Trsp*^{tA34} is converted to inosine (I) in all tRNAs encoding A at this location that in turn read codons ending in uracil (U), cytosine (C) and A. Transfer $RNA^{[Ser]Sec}_{ICA}$ would therefore decode the Cys codons, UGU and UGC, as well as the Sec codon, UGA [15], which likely explains why these mice, which

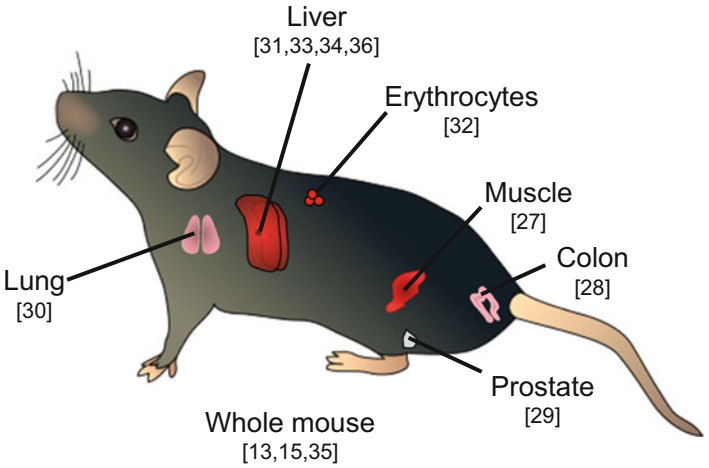

Fig. 46.1 *Trsp* transgenic mouse models. Each mouse model shown in the figure encodes a mutant *Trsp* in all tissues and organs. Tissues analyzed in studies are depicted in the figure. Descriptions of each mouse model and the major findings of each study are found in Table 46.1

would be expected to insert Sec in place of Cys, cannot, as noted above, encode more than 12 mutant transgenes. Mouse models encoding either $Trsp^{tG37}$ or $Trsp^{tA34}$ were generated to assess the effect of either mutant tRNA on stress-related selenoprotein expression and on health and/or development in specific tissues as shown in Fig. 46.1. The effects of these transgenes on selenoprotein expression and the consequences are summarized in Table 46.1.

46.2.2 Mouse Models Involving Trsp Knockout and Trsp Conditional Knockout Mice

The loss of *Trsp*, designated $Trsp^{\Delta}$, is lethal in early embryogenesis [19]. In order to assess the role of selenoproteins in health and development, we therefore constructed a conditional knockout of *Trsp*, designated $Trsp^{C\Delta}$, employing *loxP-Cre* technology to target *Trsp* loss in several specific organs and tissues [20]. In the initial study, the removal of *Trsp* was targeted in mouse mammary epithelium during lactation when the mammary gland consists of about 90 % epithelial cells [20]. Selenoprotein expression was reduced substantially, but not completely, most likely due to contamination from other cell types. No phenotypic changes were observed in the mammary gland with respect to milk generation or the transfer of milk to pups, suggesting that altered selenoprotein synthesis does not play a role in these processes. This early study clearly demonstrated that the selenoprotein population could be targeted for removal by deleting *Trsp* and provided the foundation for

Table 46.1 Transgenic *Trsp* mouse models elucidating the roles of stress-related selenoproteins in health and development[a]

Model description	Major findings[b]
Trsp^{t}	First transgenic mouse encoding tRNA transgenes. Little or no variation in selenoprotein expression in *Trsp*^{t} mice, showing that tRNA[Ser]Sec levels are not limiting in selenoprotein synthesis. Stress-related selenoprotein expression decreased in a protein and tissue specific manner in *Trsp*^{tG37}mice providing an important tool for elucidating the role of this selenoprotein subclass in health and development [13].
Trsp^{tG37}	
Trsp^{tG37}	Enhanced skeletal muscle adaptation after exercise enhanced growth that was blocked by inhibition of the target of rapamycin (mTOR) pathway. Muscles of *Trsp*^{tG37} mice exhibited increased site-specific phosphorylation on both Akt and p70 ribosomal S6 kinase before ablation [27].
Exercise regimen	
Trsp^{tG37}	Enhanced azoxymethane-induced aberrant crypt (a preneoplastic lesion for colon cancer) formation. First study suggesting that stress-related selenoproteins reduce colon cancer incidence [28].
Colon targeted by azoxymethane exposure	
Trsp^{tG37}/*Tag*	Accelerated development of lesions associated with prostate cancer progression ([C3(1)/Tag] is a prostate cancer driver gene). First study showing stress-related selenoproteins have a role in preventing prostate cancer [29].
Trsp^{tA34}	Stress-related selenoprotein expression decreased in a protein and tissue specific manner. A total of only 12 copies of *Trsp*^{tA34} was tolerated, likely due to mistranslation [15].
Trsp^{tG37}	Only minor changes observed in the immune system of *Trsp*^{tG37} mice and lung pathology was similar to control mice, suggesting that stress-related selenoproteins have a limited role in protecting mice from influenza viral infection [30].
Lung targeted by exposure to influenza virus	
Trsp^{tG37}	*Trsp*^{tG37} and *Trsp*^{tA34} mice were compared to *Trsp*$^{c\Delta}$ hepatocyte-specific knockout mice. First study to demonstrate that housekeeping selenoproteins are essential in lipoprotein biosynthesis and metabolism [31].
Trsp^{tA34}	
Trsp^{tG37}	Higher incidence of micronuclei formation in erythrocytes following exposure to X-rays, indicating a role of stress-related selenoproteins in protecting DNA from damage [32].
X-ray exposure	
Trsp^{tG37}	Mice maintained on Se-deficient, adequate and supplemented diets. Increased incidence of liver tumors in *Trsp*^{tG37} mice on Se adequate diets, whereas Se-deficient and supplemented levels protected against tumor formation. Se-deficient *Trsp*^{tG37} mice manifested a neurological phenotype [33].
Varying dietary Se levels	
Liver targeted by diethylnitrosamine (DEN) exposure	
Trsp^{tG37}	Mice maintained on Se-deficient, adequate and supplemented diets or diets with a non-metabolized selenium compound, triphenylselenonium chloride (TPSC). Widespread pyogranuloma formation, severe neurological phenotype associated with early morbidity and mortality in *Trsp*^{tG37}and *Trsp*^{tG37}/*TGFα* mice on Se-deficient or TPSC diets. Liver tumors significantly enhanced in *TGFα* (liver cancer driver gene) mice irrespective of selenium or selenoprotein status [34].
Trsp^{tG37}/*TGFα*	
Varying dietary Se levels or TPSC	

(continued)

Table 46.1 (continued)

Model description	Major findings[b]
Trsp^{rG37}	Reduced synthesis of stress-related selenoproteins promoted glucose intolerance and led to a diabetes-like phenotype [35].
Trsp^{rG37} Varying dietary Se levels	Mice maintained on Se-deficient, adequate and supplemented diets. Translational mechanisms controlling selenoprotein synthesis in liver examined by ribosome profiling. Under Se-deficiency conditions, housekeeping selenoproteins were preferentially synthesized. Increased ribosome density occurs at or upstream of the UGA codon. Overall determining factor of selenoprotein translation proficiency is the effectiveness of Sec insertion and not the initiation of translation [36].

[a]Table was adapted from [37]
[b]The studies shown in this column describe the major findings observed in genetically altered mice relative to the corresponding control mice in the study

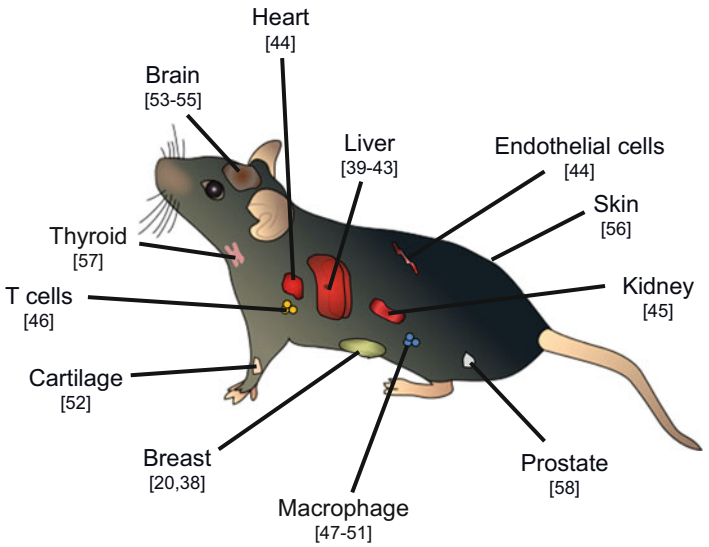

Fig. 46.2 *Trsp* conditional knockout mouse models. Each of these mouse models used *loxP-Cre* technology to target the removal of *Trsp* in a specific tissue or organ. The *Cre* promoter, the targeted organ or tissue and the major findings of each study are found in Table 46.2

examining the role of selenoproteins in health and development. Many *Trsp*$^{C\Delta}$ mouse models have subsequently been generated to examine the effects of selenoprotein loss on a variety of tissues and organs. The targeted sites in mice thus far examined are illustrated in Fig. 46.2, and the effects of selenoprotein loss on the targeted tissue or organ are summarized in Table 46.2.

Table 46.2 Conditional *Trsp*$^{\Delta}$ mouse models used to determine the roles of selenoproteins in health and development[a]

Targeted organ or tissue	Cre promoter	Major findings[b]
Mammary gland	*MMTV-Cre*; *Wap-Cre*	First *Trsp*$^{c\Delta}$ mouse, providing an important tool for elucidating the role of selenoproteins in health and development [20]. *MMTV-Cre* mice treated with 7,12-dimethylbenz[a]anthracene (DMBA) had significantly more tumors, suggesting that selenoproteins protect against carcinogen-induced mammary cancer [38].
Liver	*Alb-Cre*	Mice died between 1 and 3 months of age due to severe hepatocellular degeneration, demonstrating that selenoproteins have a role in proper liver function [39]. Sepp1 and Gpx3 were reduced in serum and kidney, supporting a Se transport role for liver-derived Sepp1 [40]. Loss of *Trsp* in liver was compensated for by an enhanced expression of phase II response genes [41]. Mice used as controls to monitor Se pools in kidney due to reduction of Gpx3 imported from liver [42]. In hepatocytes, *Secisbp2* gene inactivation shown to be less detrimental than *Trsp* inactivation [43].
Endothelial cells	*TieTek2-Cre*	Embryonic lethal. 14.5 dpc embryos were smaller, more fragile, had poorly developed vascular system, underdeveloped limbs, tails and heads, demonstrating that selenoproteins have a role in endothelial cell development and function [44].
Heart and skeletal muscle	*MCK-Cre*	Mice died from acute myocardial failure 12 days after birth, demonstrating that selenoproteins play a role in preventing heart disease [44].
Kidney	*NPHS2-Cre*	No increase in oxidative stress or nephropathy found in podocyte selenoprotein-deficient mice [45].
T cells	*LCK-Cre*	Reduction of mature T cells and a defect in T cell-dependent antibody responses. Antioxidant hyperproduction and suppression of T cell proliferation in response to T cell receptor stimulation. Selenoproteins have a role in immune function [46].
Macrophage	*LysM-Cre*	Elevated oxidative stress and induction of cytoprotective antioxidant and detoxification genes. Accumulation of ROS levels and impaired invasiveness. Altered expression of several extracellular matrix and fibrosis-associated genes. Selenoproteins have a role in immune function [47]. Selenoproteins are essential for the balance of pro- and anti-inflammatory oxylipids during inflammation [48]. Selenoproteins protect mice from DSS-induced colitis by alleviating inflammation [49]. Selenoproteins play a role in the epigenetic modulation of pro-inflammatory genes [50]. When infected with *N. brasiliensis*, Se supplemented knockout mice showed a complete abrogation in M2 marker expression with a significant increase in intestinal worms and fecal eggs [51].

(continued)

Table 46.2 (continued)

Targeted organ or tissue	Cre promoter	Major findings[b]
Osteo-chondroprogenitor	*Col2a1-Cre*	Mice had post-natal growth retardation, chondrodyplasia, chondronecrosis and delayed skeletal ossification characteristic of Kashin-Beck disease. First mouse model for Kashin-Beck disease [52].
Neurons	*Tα1- Cre*; *CamK-Cre*	Enhanced neuronal excitation followed by neurodegeneration of hippocampus. Cerebellar hypoplasia associated with degeneration of Purkinje and granule cells. Cerebellar interneurons were essentially absent. Selenoproteins have a role in neuronal function [53]. Striatal interneuron density was reduced in mice with impaired selenoprotein expression [54]. Selenoproteins are required in post-mitotic neurons of the developing cerebellum [55].
Skin	*K14-Cre*	Runt phenotype, premature death, alopecia with flaky and fragile skin, epidermal hyperplasia with disturbed hair cycle and an early regression of hair follicles. Selenoproteins have a role in skin and hair follicle development [56].
Thyroid	*Pax8-Cre*; *Tg-Cre^{ER}*	Mice lacking selenoproteins in thyrocytes showed increased oxidative stress in thyroid. Gross morphology remained intact for at least 6 months. Thyroid hormone levels remained normal in knockout mice; thyrotropin levels moderately elevated [57].
Prostate	*PB-Cre4*	Mice developed PIN-like lesions and microinvasive carcinoma by 24 weeks that was associated with loss of basement membrane and increased cell cycle and apoptotic activity [58].

[a]Table was adapted from [37]
[b]The studies shown in this column describe the major findings observed in genetically altered mice relative to the corresponding control mice in the study

46.2.3 Trsp$^{\Delta}$ and Trsp$^{c\Delta}$ Mice Complemented with Transgenes

Both *Trsp*$^{\Delta}$ and *Trsp*$^{c\Delta}$ mice have been complemented with *Trsp* transgenes. Interestingly, *Trsp*$^{\Delta}$ mice can be rescued with *Trsp^{tG37}*, and the resulting mice appear phenotypically normal, with the exception of reduced fertility in males and an apparent reduced litter size in females. These mice expressed stress-related selenoproteins in very low levels [17, 18], demonstrating that this subclass of selenoproteins, whose expression are very susceptible to Se status, are not essential to life of the animal. *Trsp*/*Trsp^{tG37}* males were found to generate sperm with abnormal morphology that most certainly attributed to their reduced fertility. *Trsp*$^{\Delta}$ mice were rescued with *Trsp^{t}*, and as expected, the selenoprotein expression in these mice was virtually identical to that found in wild type, control mice [17, 18]. *Trsp*$^{\Delta}$ mice

apparently cannot be rescued with *Trsp*^{tA34}, most likely because the resulting tRNA[Ser]Sec generated from this tRNA contains I in the wobble position of the anticodon which translates UGU and UGC codons in addition to UGA (see [17, 18] and above).

Trsp$^{c\Delta}$ mice that were targeted for loss of selenoproteins in liver and complemented with either *Trsp*^{t}, *Trsp*^{tG37} or *Trsp*^{tA34} were also generated [15]. As expected, selenoprotein expression was fully restored in hepatocyte-specific *Trsp*$^{c\Delta}$ mice by introducing *Trsp*^{t} and was partially restored by introducing *Trsp*^{tG37} or *Trsp*^{tA34}. Because both *Trsp*^{tG37} and *Trsp*^{tA34} transgenes produced tRNAs[Ser]Sec lacking Um34, this observation provided further evidence that the 2′-*O*-modification on tRNA[Ser]Sec is critical for the synthesis of stress-related selenoproteins. Only low copy numbers of the *Trsp*^{tA34} transgene were tolerated without endogenous *Trsp*, whereas both low and high copy numbers of *Trsp*^{tG37} restored housekeeping selenoprotein expression, irrespective of the presence of endogenous *Trsp* [15]. Interestingly, ^{75}Se-labeling of liver *Trsp*$^{c\Delta}$ mice carrying *Trsp*^{tA34} showed that only selenoproteins incorporated the label. Whether non-Sec-containing proteins also incorporated Sec in place of Cys at levels below detection, ascribing to the consequences of Sec-tRNA[Ser]Sec$_{ICA}$, at least in part, is not known. However, the major impact of Sec-tRNA[Ser]Sec$_{ICA}$ on protein synthesis is most certainly due to the misreading of this mutant tRNA by replacing Cys with Sec within the limits of Sec-tRNA[Ser]Sec boundaries for utilization as a result of the Sec insertion sequence (SECIS) element in selenoproteins [21, 22]. Such an observation would suggest that tRNA[Ser]Sec$_{ICA}$ did not randomly insert Sec in place of Cys in protein, but only at Cys/Sec codons in selenoproteins, and within the boundaries set by the UGA/Sec-tRNA[Ser]Sec/SECIS element [21, 22]. However, what was not resolved in this study was whether Ser-tRNA[Ser]Sec, which is known to suppress UGA stop codons [23, 24], can decode UGU and UGC randomly in proteins.

Trsp$^{\Delta}$ mice were also crossed with mice carrying a transgene with a deletion in the activator element (AE) region of *Trsp* yielding a *Trsp*$^{\Delta}$ mouse that was dependent on the *Trsp*$^{tAE-}$ transgene for expression of its tRNA[Ser]Sec [25]. The AE region is necessary for the binding of Sec tRNA gene transcription activating factor (STAF). Interestingly, this mouse, designated *Trsp*$^{\Delta}$/*Trsp*$^{tAE-}$, expressed tRNA[Ser]Sec in varying amounts in different organs and tissues. The level of the mcm^{5}Um-containing isoform was dramatically reduced in all tissues and organs examined compared to the corresponding controls, and selenoprotein synthesis was affected, wherein tRNA[Ser]Sec levels were most severely reduced [25]. Interestingly, *Trsp*$^{\Delta}$/*Trsp*$^{tAE-}$ mice manifested a neurological phenotype similar to mice lacking the *Sepp1* gene (see [25] and references therein). These data suggest that STAF controls selenoprotein synthesis by increasing *Trsp* transcription in an organ/tissue-specific manner and by regulating Sec tRNA[Ser]Sec modification. The mechanism of how the tRNA[Ser]Sec population in *Trsp*$^{\Delta}$/*Trsp*$^{tAE-}$ mice was affected in this manner has not yet been resolved.

The mouse models described above and the tissues analyzed are depicted in Fig. 46.3. The consequences of these mouse models on health and/or development are summarized in Table 46.3.

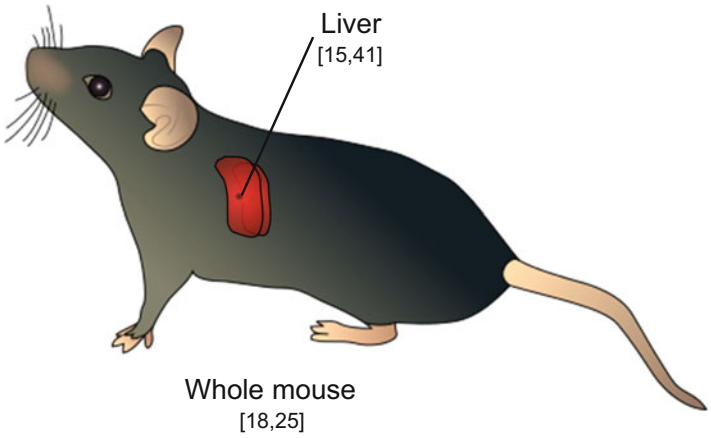

Fig. 46.3 Standard *Trsp* knockout/transgenic and conditional *Trsp* knockout/mutant transgenic mouse models. Descriptions of each mouse model, the targeted organ or tissue and the major findings of each study are found in Table 46.3, and are discussed in the text

Table 46.3 Transgenic/knockout *Trsp* mouse models assessing the roles of stress-related selenoproteins in health and development[a]

Model description	Major findings[b]
$Trsp^{\Delta}/Trsp^{t}$	Mice synthesize stress-related selenoproteins very poorly, demonstrating that proper base modification in the anticodon is essential in stress-related selenoprotein expression as discussed in the text. Males had abnormal sperm and reduced fertility and females showed reduced litter size [18].
$Trsp^{\Delta}/Trsp^{tG37}$	
$Trsp^{\Delta}/Trsp^{tAE-}$	Mice expressed $tRNA^{[Ser]Sec}$ in varying amounts in different organs and tissues. The level of the mcm^5Um isoform was always less in all tissues and organs examined in the $Trsp^{\Delta}/Trsp^{tAE-}$ mouse. Mice developed a neurological phenotype similar to mice without the *Sepp1* gene and a reduced life span [25].
$Trsp^{c\Delta}/Trsp^{t}$	The replacement of selenoprotein synthesis in hepatocytes of mice carrying mutant *Trsp* transgenes, wherein housekeeping, but not stress-related selenoproteins were expressed (see text for details), led to normal expression of phase II response genes, providing strong evidence of a functional link between housekeeping selenoproteins and phase II enzymes [15, 41].
$Trsp^{c\Delta}/Trsp^{tG37}$	
$Trsp^{c\Delta}/Trsp^{tA34}$	
Alb-Cre used to target liver	

[a]Data in the table were taken from [37]
[b]The studies shown in this column describe the major findings observed in genetically altered mice relative to the corresponding control mice in the study

46.3 Concluding Remarks

Mouse models involving manipulation of the expression of selenoproteins have provided a powerful tool in elucidating the role of selenium-containing proteins in health and development as shown in Tables 46.1, 46.2, and 46.3. In addition, mouse

models, which target removal of a single selenoprotein, have provided a wealth of information regarding health, development and the function of specific selenoproteins (see Chap. 47).

Those models which exploit changes in Sec tRNA expression, even though they have been used widely to modulate selenoprotein expression in a number of specific cell types, organs and tissues (see Figs. 46.1, 46.2, and 46.3 and Tables 46.1, 46.2, and 46.3), can be used to target other tissues and organs. Furthermore, new combinations of other mutant transgenic *Trsp* and *Trsp* knockout mice, as well as subjecting mice encoding current *Trsp* models to environmental challenges, will aid in further illuminating the roles of selenoproteins in specific health issues. More specifically, the mutant tRNA[Ser]Sec recently reported in humans ([26] and Chap. 44) would be a prime candidate to explore the consequences of this tRNA on selenoprotein synthesis, and also the consequences of mutant tRNA[Ser]Sec with A at the third position of the anticodon (discussed in Sect. 46.2.3 above) could be assessed.

The importance of Se in many aspects of health has been known for many years. However, in recent years, mouse models involving the loss or alteration of selenoprotein expression by the *Trsp* mouse models described herein, or of individual selenoprotein genes (Chap. 47 and references therein), have clearly demonstrated that selenoproteins play critical roles in these health benefits. Although the role of small molecular weight selenocompounds cannot be excluded, these studies, along with numerous in vitro studies characterizing individual selenoproteins, have shifted the focus of how Se exerts its effects on health and development onto selenoproteins as the likely key components in providing these benefits. However, as noted in the Preface of this book, small molecular weight selenocompounds will likely again come into focus as major players in selenium research.

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Chapter 47
Mouse Models that Target Individual Selenoproteins

Marcus Conrad and Ulrich Schweizer

Abstract Selenium deficiency in humans or livestock and impaired selenoprotein synthesis in patients due to mutations in relevant genes have revealed the importance of selenoproteins for healthy life. Twenty-four genes for selenoproteins are encoded in the murine genome, and most of them have been genetically inactivated. We summarize the results from those studies that have provided a series of intriguing mechanistic details about the importance of selenoprotein-dependent processes for proper neuronal, retinal, cardiac, endothelial, and renal function as well as the contribution of selenoprotein activity to the endocrine, immune and gastrointestinal systems, and reproduction. Mouse models were particularly useful for the study of organs that are not easily accessible for biopsy in humans. While four selenoproteins have proven to be crucial for murine embryo and tissue development, others confer more specific functions particularly in response to stress.

Keywords Conditional knockout • Deiodinase • Glutathione peroxidase • Mouse models • SECIS binding protein 2 • Selenoprotein P • Selenoproteins • Transgenics • Thioredoxin reductase • Trsp

47.1 Introduction

The early recognition that selenium (Se) is an essential trace element in humans has spurred researchers for many decades to better understand the molecular mechanisms of Se in human health. Once considered a toxic element because of its detrimental effects observed in livestock at high concentrations [1], it is now

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well-established that Se is an essential trace element and functions through selenoproteins, which are encoded by 25 genes in humans and 24 in mice [2]. Accordingly, conditions of severe Se deficiency have been linked to human diseases, such as Keshan disease, a Se deficiency disease once endemic in China and characterized by congestive cardiomyopathy [3], and Kashin–Beck disease, a multi-factorial, chronic and endemic osteochondropathy with highest incidence rates in China and other areas in East Asia [4]. Familial mutations in SECIS binding protein 2 (*SECISBP2*) affect many selenoproteins at a time, but patients seek medical help because of endocrine problems [5], although more severe cases show that deficient selenoprotein expression affects many more organ systems [6]. Interestingly, mutations in selenocysteine synthase (*SEPSECS*) severely affect the nervous system (see Chaps. 36 and 44). This chapter is a synopsis of all mouse models generated thus far involving selenoprotein genes or their biosynthesis factors (Table 47.1) and discusses briefly their key phenotypes. This, of course, will entail discussing selenoproteins summarized in detail in other chapters which are referred to.

47.2 Studies On Mice With Mutations Interfering with General Selenoprotein Synthesis

Early studies with mice deficient in the selenocysteine (Sec)-specific tRNA gene, *Trsp* (covered in Chap. 46 and references therein), implied that at least one or several selenoproteins confer a vitally important role for early embryogenesis as $Trsp^{-/-}$ embyros fail to develop beyond embryonic day (E) 6.5 [7]. In fact, mice lacking mitochondrial and cytosolic thioredoxin reductase (Txnrd2 and Txnrd1, respectively) and glutathione peroxidase 4 (Gpx4) die during early or mid-gestation [8], as do mice deficient for selenoprotein T [9].

Likewise, inactivating mutations in *Secisbp2* are embryonic lethal and resemble *Txnrd1*- or *Gpx4*-deficient embryos [10]. When *Trsp* or *Secisbp2* were conditionally inactivated in liver or neurons, it became apparent that deficiency of *Trsp* was more severe than of *Secisbp2* [10–12]. In *Secisbp2*-deficient tissues, selenoprotein expression was affected in a gene-specific manner underlining the concept of hierarchical selenoprotein expression [13].

Inactivation of the major Se carrier protein, selenoprotein P (Sepp1), affects selenoprotein expression in the whole body [14–18], and is treated in detail in Chap. 22. Recently, selenocysteine lyase (Scly) has been genetically inactivated reducing overall selenoprotein expression including Gpx1 [19]. What is remarkable is that $Scly^{-/-}$ mice suffer from metabolic syndrome and hyperinsulinemia in contrast to mice lacking Gpx1 only (see below). Two different exon deletions in the presumed selenoprotein biosynthetic factor SECp43 (*Trnau1ap*; *Trspap1*) have been made in mice without any detectable effect on selenoprotein expression in liver, brain, and other organs [20].

Table 47.1 Global knockout (KO) and conditional knockout (CKO) mouse models for individual selenoproteins and those involved in synthesis and Sec incorporation

Gene	Year	Approach	Phenotype	Ref.
Trsp	1996	KO	Embryonic lethality at E6.5	[7]
Trsp	2003	CKO (mammary epithelium and many other cell types)	Altered expression of selenoproteins and tumor suppressor genes, BRCA1 and p53; selenoprotein biosynthesis abrogated in targeted cells	[112]
Secispb2	2014	KO, CKO (liver, neurons)	KO embryonic lethality E7.5; reduced selenoproteins in liver and neurons; reduced numbers of interneurons	[10, 11]
Sepp1	2003	KO and transgene	Sepp is central to Se homeostasis and expression is needed in brain	[14, 15, 18]
Sepp1	2012	CKO (liver)	Plasma Sepp is made in liver and transports Se to target tissues	[16]
Scly	2012	KO	Fully viable, metabolic syndrome	[113]
Trspap1, *Trnau1ap*		KO, CKO (liver and neurons)	Deletion of exons 3+4 without effect on selenoproteins or survival; deletion of exons 7+8 embryonic lethal, but no effect on selenoproteins in liver CKO	[20]
Gpx1	1997	KO	Fully viable, stress sensitive in many models	[24-36]
Gpx2	2001	KO	Fully viable	[37]
Gpx3	2011	KO	Larger brain infarcts upon stroke	[43]
Gpx4	2003	KO	Embryonic lethality at E7.5	[47, 114, 115]
Gpx4	2008	CKO (neurons)	Seizures, ataxia and neuronal loss in hippocampus	[48]
mGpx4	2009	KO	Male infertility	[56]
nGpx4	2005	KO	Delay in sperm chromatin condensation	[59]
Txnrd2	2004	KO/CKO (heart)	Embryonic lethality at E13.5 and cardiac failure	[62]
Txnrd1	2005	KO/CKO (heart)	Embryonic lethality at ~E8.5	[68]
Txnrd1	2007	KO (CKO)	Embryonic lethal at E7.5 - E8.5	[69]
Dio2	2001	KO	Pituitary resistance to thyroid hormone; development of cochlea	[81]
Dio2	2013	CKO (pituitary, astrocytes, muscle)	Pituitary resistance to thyroid hormone, role of tanycytes in hypothalamic control of thyroid hormones	[86]
Dio1	2006	KO	Higher T4 and rT3 levels, loss of iodine through feces	[87]
Dio3	2006	KO	Central hypothyroidism, development of cochlea, testis	[90–95]

(continued)

Table 47.1 (continued)

Gene	Year	Approach	Phenotype	Ref.
Dio3	2014	CKO	Dio3 needed for muscle regeneration, skin carcinogenesis	[96]
Sep15	2011	KO	Cataract formation	[97]
Selk	2011	KO	Impaired control of Ca^{2+} fluxes in immune cells	[99]
Selm	2013	KO	Obesity without cognitive deficits	[102]
Sepn1	2011	KO/CKO	Fully viable, muscle defects	[116, 117]
Sepr	2011	KO	Increased signs of oxidative stress	[107, 108]
Selt	2013	CKO (β cells, neurons)	Impaired insulin production/secretion, increased activity, cell death in brain	[9, 111]

47.3 Glutathione Peroxidases

Eight Gpx are known in mammals [21], of which Gpx1-4 and Gpx6 are selenoproteins, whereas the others are Cys-containing analogues instead (Gpx6 is the sole exception and a selenoprotein in man). Gpx1 was the first selenoprotein to be discovered [22, 23] and targeted in mice, which revealed its dispensability for "normal" life [24]. A requirement for Gpx1 can be uncovered only upon various chemical and physical challenges and in certain disease models like ischemia/reperfusion injury (reviewed in [21, 25]). Among others, Gpx1 protects against ischemia/reperfusion injury in male myocardium [26] and in brain [27], diquat-induced oxidative stress and lethality [28], noise-induced hearing loss [29], viral-induced myocarditis [30], 1-methyl-4-phenyl-1,2,5,6-tetrahydropyridine (MPTP)-triggered striatal neurotoxicity [31], γ-irradiation damage in crypts of jejunum [32], impaired endothelium-dependent vasodilator function [33], UV-induced squamous cell carcinoma formation [34], and doxorubicin-induced cardiotoxicity [35]. A shift in paradigm of Gpx1 research occurred when it was discovered that Gpx1 is involved in insulin signaling [36]. Cells and tissues from *Gpx1*$^{-/-}$ mice present increased insulin sensitivity, which is mediated through increased phosphatase and tensin homolog (PTEN) oxidation and elevated phosphoinositide 3-kinase (PI3K)/Akt signaling, thus rendering *Gpx1*$^{-/-}$ mice more resistant to high fat diet-induced obesity [36]. The underlying mechanism is that some of the protein tyrosine phosphatases are known to be highly sensitive to oxidation leading to reversible oxidation and transient inactivation and to a net increase in receptor tyrosine kinase (RTK) signaling including the insulin receptor. Hence, besides its classical role as an H_2O_2 scavenger, Gpx1 is directly involved in insulin sensitivity, and thus diabetic complications (see Chap. 49).

Gpx2 was the second family member to be inactivated in mice [37]. Similar to Gpx1, loss of Gpx2 is compatible with life, but *Gpx1*$^{-/-}$/*Gpx2*$^{-/-}$ mice develop colitis

depending on their gut flora. Most studies with *Gpx2*$^{-/-}$ mice have centered on the gastro-intestinal tract. For instance, increased apoptosis rates in colon, particularly under Se-deficiency, could be detected in *Gpx2*$^{-/-}$ mice [38]. Its role in colon inflammation and tumorigenesis in colon, however, appears to be more complex and strongly depends on the chemical inducer and Se status of mice (see [39, 40] and also Chap. 38). One function not related to the gastro-intestinal tract is that *Gpx2*$^{-/-}$ mice are more sensitive to ovalbumin-induced allergic airway inflammation [41].

Gpx3 is a plasma protein and specifically binds to basement membranes of renal cortical tubules [42]. Loss of Gpx3 augments thromboembolic events via potentiation of platelet activation, possibly by reduced NO availability [43]. Specifically, these mice show reduced bleeding times, increased platelet activation, endothelial dysfunction leading to ADP infusion-induced platelet aggregation in the pulmonary vasculature and larger brain infarcts in a model of cerebral ischemia/reperfusion. Interestingly, polymorphisms in the promoter region of *GPX3* have been suggested as risk factor for ischemic stroke among children and young adults [44]. Besides in kidney and in the vasculature, Gpx3 expression can also be detected on the basolateral surface in colon [45]. Here, Gpx3 acts as a tumor suppressor in the murine model of azoxymethane/dextran sodium sulfate (AOM/DSS) inflammatory carcinogenesis [45]. Increased intratumoral proliferation, likely driven by augmented total β-catenin levels, nuclear localization and Wnt signaling, increased M2 macrophage infiltration and oxidative DNA damage are some of the findings in *Gpx3*$^{-/-}$ animals subjected to AOM and DSS.

Gpx4 is the only Se-dependent enzyme that can efficiently detoxify peroxides in lipid bilayers and lipoprotein particles and that can use electrons from thiol sources other than GSH [46]. *Gpx4*$^{-/-}$ embryos die as early as E7.5, i.e., after gastrulation [47]. The first conditional knockout of *Gpx4* was reported in 2008 demonstrating that Gpx4 controls a hitherto unrecognized, caspase-independent cell death pathway, and that it prevents newborn mice from ataxia, seizures, and neurodegeneration in the hippocampus [48]. In recent years, a series of tissue-specific deletion approaches and additional knockout models have been published (as reviewed in [49] and Chap. 43), thus further broadening our understanding of lipid peroxidation in cell death and possibly RTK signaling [50]. Interest in Gpx4 research has gained momentum in 2014, when Gpx4 was discovered as the key upstream regulator of a novel regulated necrotic pathway, called ferroptosis [51, 52]. Due to this recent discovery and the importance of Gpx4 in the regulation of this highly pervasive and novel form of non-apoptotic cell death in some cancer entities and pathological settings, including ischemia/reperfusion injury scenarios in liver and kidney (Chap. 43 is dedicated to this topic). Mutations in *GPX4* have been associated with a rare genetic disorder called Spondylometaphyseal Dysplasia of the Sedaghatian type [53].

Beyond the importance of the cytosolic (short) form of Gpx4 for many somatic cells and tissues, a series of studies unambiguously demonstrated that Gpx4 is also the main target of testicular Se essential for male fertility. Severe experimental Se deficiency in rodents is linked to male infertility [54], and, in 1999, Gpx4 was found in an enzymatically inactive form as the main structural protein of the so-called

keratin-like mitochondrial capsule surrounding the mid-piece of mature sperm [55]. In fact, targeted knockout of the mitochondrial (long) form of Gpx4 (mGpx4) alone is associated with male infertility, and in epididymal spermatozoa phenocopies most of the effects observed under severe Se deficiency including bent and kinked mid-piece, extrusion of outer dense fibers, and consequently a strongly impaired motility and progressivity of isolated sperm [56]. Mice transgenic for the cytosolic form of Gpx4 are able to rescue embryonic lethality of knockout of the entire Gpx4 gene, while male mice are infertile due to lack of mGpx4 [57], confirming that cytosolic Gpx4 is the only form essential to prevent ferroptotic cell death, whereas mGpx4 is dispensable for survival of mice but indispensable for male fertility. Another form of Gpx4 is the nuclear from (previously called "sperm-nuclei specific Gpx", snGpx) of Gpx4 (nGpx4) that is expressed dependent on its own promoter located in the first exon of the *Gpx4* gene and predominantly abundant in late spermatids [58]. While the knockout of nGpx4 is fully compatible with life and, unexpectedly, for male fertility [59], knockout spermatozoa display defects in sperm chromatin condensation and decondensation upon sperm maturation and oocyte fertilization, respectively, due to its lacking protamine thiol peroxidase function [59, 60]. The role of Gpx4 in fertility is further expanded on in Chap. 18.

47.4 Thioredoxin Reductases

Three distinct genes encoding cytosolic thioredoxin reductase (Txnrd1), mitochondrial thioredoxin reductase (Txnrd2) and thioredoxin-glutathione reductase (TGR or Txnrd3) are present in mammals (see [61] and Chap. 16). Insights into the (patho) physiological functions of Txnrd1 and 2 have come from a number of conditional knockout approaches [8]. Txnrd2 was the first *Txnrd* gene to be inactivated in mice showing that Txnrd2 is essential for proper fetal hematopoiesis and proper heart development by maintaining mitochondrial redox balance [62]. Heart-specific (inducible) Txnrd2 deletion further demonstrated that Txnrd2 is not only crucial for cardiac function [62], but also for tissue protection in an experimental model of cardiac infarction [63], and for preserving mitochondrial integrity and energy metabolism in the ageing heart [64]. Patients carrying missense mutations in *TXNRD2* (Ala59Thr and Gly375Arg) suffer from dilated cardiomyopathy, corroborating the significance of TXNRD2 also for the human heart [65]. Besides heart, loss of Txnrd2 in transformed fibroblasts leads to altered mitochondrial redox balance, that was recently reported to impair tumor growth and tumor-related angiogenesis by stabilizing prolyl hydroxylase 2 and abrogating Hif-1α target genes [66]. Interestingly, a nonsense mutation in *TXNRD2* causes familial glucocorticoid deficiency with a variable phenotype, but cardiac failure in some patients [67].

Systemic knockout of Txnrd1 causes embryonic death, although earlier than that of Txnrd2 (between E7.5 and E9.0) due to general proliferation deficits (except the developing heart) and massive overall developmental retardation [68, 69]. This is

compatible with the idea that Txnrd1 keeps thioredoxin 1 (Txn1) in its reduced state, which provides electrons to ribonucleotide reductase and is thus important for cell cycle progression. While dispensable for cardiac development and function [63, 68], knockout of Txnrd1, but not Txnrd2, in the neural precursors causes profound cerebellar hypoplasia [70]. Notably, a null mutation in mitochondrial thioredoxin (*TXN2*) was recently reported to cause neurodegeneration with cerebellar atrophy and epilepsy among other severe neuropathological defects in a 16-year-old adolescent [71], indicating that TXNRD1 may be responsible for keeping TXN2 in its reduced state at least in the cerebellum. Besides brain, Txnrd1 appears to be playing more specific roles in liver, although Txnrd1 deletion does not impact hepatic proliferation during development and regeneration [72]. By contrast, hepatocyte-specific deletion of Txnrd1 dramatically increases diethylnitrosamine-induced hepatocarcinogenesis [73], whereas knockout livers are by far more protected against acetaminophen (APAP)-induced hepatotoxicity [74]. Increased APAP-resistance appears to be the consequence of upregulation of nuclear factor erythroid 2-related factor 2 (NRF2) target genes and in particular those involved in GSH biosynthesis and GSH utilization [74]. Moreover, deletion of Txnrd1 in liver is also linked to a metabolic switch from lipogenesis to increased glycogen storage [75], although the precise mechanisms still need to be explored. Interestingly, dietary methionine supplementation can bypass combined Txnrd1 and glutathione reductase (GR) deficiency [76], indicating that cysteine provided by the transsulfuration pathway fuels GSH synthesis via an NADPH-independent pathway. Unlike Txnrd1 and 2, a knockout model for Txnrd3 (TGR) has not been reported for this enzyme, which was initially described to be mainly expressed in sperm cells and to functionally interact with Gpx4 [77].

47.5 Deiodinases

Deiodinases (Dio) comprise a family of three enzymes capable of eliminating iodide from thyroid hormones (TH) and their metabolites [78]. Depending on substrate and product, Dio can locally activate or inactivate THs, e.g., Dio2 activates TH signaling (see Chap. 41). Accordingly, *Dio2*$^{-/-}$ mice show pituitary resistance to thyroxine, impaired cochlear development, and deafness as well as a defect in thermogenesis [79–81]. Conditional gene targeting continues to reveal novel functions of Dio2 in tissue regeneration beyond regulating the thyroid hormone axis [82–86]. Two functions have been proposed for Dio1: activation of TH and degradation of THs and their metabolites and recycling of iodine from inactivated hormones. Gene targeting of Dio1 clearly supports the second hypothesis [87, 88]. Double knockout of both activating deiodinases leads to a surprisingly mild phenotype in *Dio1*$^{-/-}$/*Dio2*$^{-/-}$ mice [89]. Inactivation of THs is important in proliferating tissues or needed to delay TH-dependent differentiation. *Dio3* gene targeting thus leads to dysregulation of the TH axis, and developmental defects in cochlea, retina, cerebellum, endocrine

pancreas, and testis [90–95]. Conditional gene targeting of *Dio3* revealed further roles in muscle regeneration and carcinogenesis [96]. Patients with mutations in *SECISBP2* have phenotypes compatible with deficiency of both *DIO1* and *DIO2* [5].

47.6 Other Selenoproteins

The physiological role of 15 kDa selenoprotein (Sep15) is difficult to grasp at present. It has been inactivated in mice and the most specific phenotype was cataract formation, while ER-stress was not induced, although Sep15 was proposed to be involved in regulation of protein folding [97]. Moreover, loss of Sep15 rather protected from chemically-induced aberrant crypt formation in colon [98]. Sep15 is further discussed in Chap. 19.

SelK is a small endoplasmic reticulum (ER) transmembrane protein predominantly expressed in immune cells. While loss of SelK does not impinge on organismal growth, fertility and normal immune function, it appears to confer more specific functions in immune cell activation by controlling Ca^{2+} fluxes in a variety of immune cells [99]. Consequently, Ca^{2+}-dependent functions such as T cell proliferation, migration of T cells and neutrophils and Fcγ receptor-mediated oxidative burst in macrophages as well as viral clearance are impaired in knockout animals. In macrophages, SelK was found to be crucial for palmitoylation of CD36, which is known to occur at the ER membrane, thus stabilizing and directing CD36 to lipid rafts [100]. This impairment was found to cause reduced foam cell formation and thus decreased atherosclerotic lesions in a model of high fat diet-induced atherosclerosis. Palmitoylation of inositol 1,4,5-triphosphate receptor (IP3R) and IP3R-dependent Ca^{2+} flux also requires SelK, further establishing the specific role of SelK in this cellular process [101]. The many roles of SelK are covered in more detail in Chap. 20.

SelM is an ER-resident, thioredoxin-like protein with preferential expression in the brain. Although knockout animals appear to be fully viable and brain morphology, anxiety, locomotion, motor coordination and learning are indistinguishable between wild type and knockout littermates [102], *Selm*$^{-/-}$ mice display increased weight gain, more white adipose tissue deposits and reduced leptin sensitivity in the arcuate nucleus of the hypothalamus. SelM is further discussed in Chap. 21.

Mutations in the selenoprotein N gene (*SEPN1*) lead to a disease now called SEPN1-related myopathy with a variable clinical presentation. From the perspective of selenoproteins, *SEPN1* was the first selenoprotein gene recognized to specifically be linked to a disease in humans [103]. In addition, *SEPN1* is linked to the first disease-causing mutations in SECIS elements [104]. Moreover, the first disease-causing mutations in the selenocysteine redefinition element were found in *SEPN1* [105, 106]. The biology of Sepn1 and the phenotypes of *Sepn1*$^{-/-}$ mice are covered in Chap. 40.

Methionine sulfoxide reductase B1 (MsrB1, Selenoprotein R, Sepr) is one of three isoforms of the MsrB family, and with its other family members specifically

reduces the R-enantiomer of methionine-sulfoxide (MsrA reduces the S-enantiomer of methionine-sulfoxide). *Sepr*$^{-/-}$ mice are fully viable [107, 108], although they show clear signs of increased oxidative stress in liver and kidney manifested by a shift towards more oxidized GSH, higher levels of malondialdehyde, protein carbonyls and protein methionine-sulfoxide, coinciding with reduced levels of free and protein thiols [107]. Reversible methionine oxidation and reduction by Msrs could represent a novel posttranslational protein modification as demonstrated for signal-dependent actin assembly [109]. This issue is discussed in depth in Chap. 24.

SelT, a Txn-like protein, was first functionally described being readily induced by the neuropeptide pituitary adenylate cyclase-activating polypeptide (PACAP) and implicated in increases of intracellular Ca^{2+} concentrations during cell differentiation of PC12 cells [110]. Conditional knockout of SelT in pancreatic β-cells is associated with higher glucose:insulin ratio in response to glucose treatment of starved mice [111]. SelT deficiency does not alter insulin sensitivity indicating impaired insulin production/secretion in SelT-deficient mice. Besides the endocrine system, SelT contributes to normal brain development. A transient reduction in the overall size of different brain regions including neocortex, hippocampus and cerebellum along with persistent hyperactivity was noticed recently in brain-specific *Selt* knockout animals [9]. The functions of SelT and phenotypes of *Selt*-deficient mice are further presented in Chap. 23.

To date, no mouse models have been reported for six selenoproteins, SelH, SelI, SelO, SelS, SelV and Txnrd3 as well as three biosynthetic factors Pstk, Sephs2 and Sepsecs. Whether Sephs1 is involved in selenoprotein biosynthesis is not clear at present.

47.7 Concluding Remarks

When the first edition of this book was published, the first genetic models for selenoproteins were just being reported and no human genetic condition associated with selenoprotein-deficiency was known. Now, almost all selenoproteins have been inactivated in mice (often conditionally in several cell types), mutations have been discovered in selenoprotein genes associated with disease, and two syndromes caused by general selenoprotein biosynthesis defects have been identified. By and large, genetic models in mice and inborn errors of selenoprotein metabolism in patients account for what has been reported as symptoms associated with Se deficiency. These findings support the notion that Se exerts its physiological functions in mammals via selenoproteins, but does not exclude the possibility that pharmacological effects can as well be mediated via small molecular-weight Se species. Now that most of the cast is known, the scientific community can focus on the roles that selenoproteins play in physiology and pathophysiology.

Acknowledgements We have tried to avoid too much overlap with other Chapters of this book and thus apologize to those, whose work could not be cited here for space restrictions. This work

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Chapter 48
The Naked Mole Rat and Selenium

Alexei V. Lobanov and Vadim N. Gladyshev

Abstract The naked mole rat, *Heterocephalus glaber*, has emerged as a popular model organism for studying aging, hypoxia tolerance and cancer resistance. The sequencing of its genome and transcriptome allowed researchers to get insights into the associated molecular mechanisms, pathways and gene networks. The analysis of trace element content of tissue samples of the naked mole rat revealed a low level of selenium in kidney and liver. This was also consistent with the observed negative correlation between selenium levels in these organs and longevity across mammals. A low selenocysteine content in selenoprotein P and a premature stop codon in glutathione peroxidase 1 account in part for the low selenium utilization by the naked mole rat. As such, this organism emerges as a useful model to study selenium metabolism in mammals.

Keywords Glutathione peroxidase • Model organism • Naked mole rat • Selenium • Selenoproteins

48.1 Introduction

The naked mole rat, *Heterocephalus glaber*, the only currently classified species in the genus *Heterocephalus* [1], is a small mouse-sized rodent (3–4 in. long, 30–35 g of weight) native to East Africa. This is the only known mammal that does not maintain stable body temperature and one of only two (the other one being Damaraland mole rat, *Fukomys damarensis*) that exhibits eusociality [2].

Naked mole rats live underground in complex systems of burrows, in colonies ranging in size from 20 to 300 members in a eusocial structure similar to insects, such as ants and bees [3]. Their diet consists primarily of very large tubers, but they have also been known to eat their own feces, which supposedly serves as a means of distributing hormones from the queen [4]. Adaptations to underground lifestyle include an almost complete absence of hair (hence the name "naked"), poor vision

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(their eyes are quite small), an ability to move backward as fast as forward, lack of pain sensitivity in its skin and very low metabolic and respiratory rates.

However, the most remarkable characteristics of naked mole rats are their resistance to cancer and their longevity. Being the longest-lived rodent currently known, their lifespan exceeds 30 years. Moreover, naked mole rats show negligible senescence, and there is no observed age-related increase in mortality [5]. Because of these features, naked mole rats emerged as an increasingly popular model organism for aging- and cancer-related studies.

48.2 Features of a Model Organism

Several unique features of naked mole rats discussed above make this animal an ideal model for research on longevity, cancer and disease resistance. These features include:

48.2.1 Thermoregulation

Because of their subterranean habitat, where both heat and gas exchanges are impeded [6, 7], the naked mole rat does not regulate its body temperature like other mammals. It is poikilothermic, and its body temperature depends on ambient temperature [8]. To get warm, naked mole rats can huddle together or go closer to the surface; and to cool down, they can retreat to the deeper, cooler areas.

The lower resting metabolic rates and lower body temperature than those of above-ground dwelling mammals help to overcome some of the problems of living below ground [9, 10]. Hypothermia in naked mole rats affects metabolic rate, but its influence on brain hypoxia tolerance is currently unknown.

48.2.2 Resistance to Cancer

Surprisingly, for such a long-lived organism, cancer is extremely rare in naked mole rats [5]. Currently, there are two proposed mechanisms that may play a role in averting cancer: i) contact inhibition mediated by p16; and ii) production of extremely high-molecular-mass hyaluronan.

In humans and mice, contact inhibition is mediated by $p27^{Kip1}$ only, but in naked mole rats both $p16^{Ink4a}$ and $p27^{Kip1}$ may play a role [11]. The analysis of the transcript of tumor suppressor $p16^{Ink4a}$ revealed a low sequence similarity in the last exon (compared to other mammals); also, while ankyrin repeat residues important for CDK binding were intact, the resulting protein was predicted to be shorter (because of two early stop codons in the second exon), so its function may be impacted [12].

Also, it was found that naked mole rat fibroblasts secrete extremely high-molecular-mass hyaluronan (more than five times larger than that in mouse), which

accumulates abundantly in naked mole rat tissues [13]. It was suggested that naked mole rats have evolved a higher concentration of high-molecular-mass hyaluronan in their skin to provide elasticity needed for underground life, and later this trait was co-opted to provide cancer resistance and longevity. In addition, a study of ribosomes showed that production of proteins in naked mole rats is extremely error-free [14], which may also help to prevent cancer.

48.2.3 Pain Insensitivity

While naked mole rats react normally to the mechanical pain such as pinching and prodding, these animals are insensitive to acid or capsaicin [15]. Analyses showed that the lack of pain sensitivity is caused by the absence of substance P neurotransmitter, and injection of substance P restored pain signaling [16]. The genome analysis revealed that the gene *TAC1* encoding substance P is intact; however, there is a deletion in the core promoter region, suggesting that this neurotransmitter is functional, but may be under unique regulation [12]. It was hypothesized that pain insensitivity represents an adaptation to the high levels of carbon dioxide which might cause acid build up in naked mole rat tissues. Because of the uniqueness of the pain biology of the naked mole rat, it can serve as a valuable outlier for studies of pain mechanisms in mammals.

48.2.4 Longevity

The maximum longevity of the naked mole rat is exceptional for a rodent of its size (Fig. 48.1). Currently, it holds the record for the longest living rodent [17]. The exact reason for its longevity is not known, but most likely it is linked to the ability to substantially reduce its metabolic rate, thus minimizing the damage generated as a consequence of metabolic activity [18].

Also, it is possible that other factors, such as eusociality, play important and perhaps primary roles in increased longevity. It was shown that eusociality in insects is highly correlated with longevity, and preliminary analyses suggested that it may have influenced the evolution of longevity [19].

48.2.5 Eusociality

Naked mole rats live in colonies, with a behavioral division of labor: maintenance activities are carried out by smaller individuals, “workers”, while defensive roles are fulfilled by larger colony members, “soldiers” [20]. Most colonies contain one to three breeding males and only one breeding female, the so called “queen” [2, 21]. While the other colony members of both sexes are reproductively suppressed, they are not sterile,

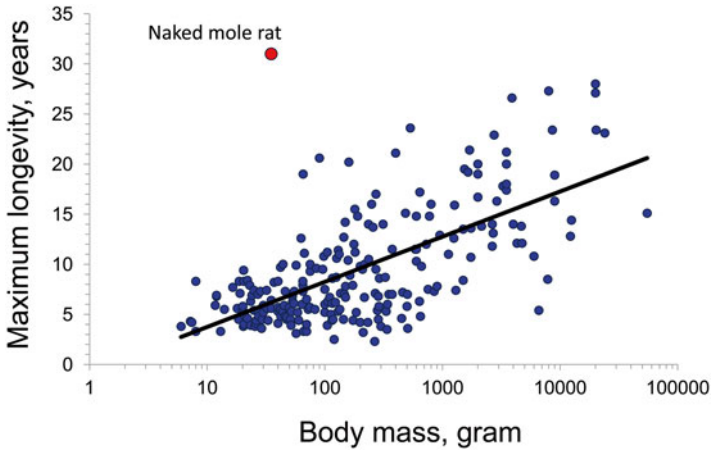

Fig. 48.1 Naked mole rats live longer than other rodents of similar size. Body mass vs Maximum longevity plot for rodents is shown. Each *circle* represents one species. Longevity data and weights for all available *Rodentia* species (229 total) were obtained from the AnAge database. The naked mole rat, a clear outlier, is labeled and shown by a larger *red circle*

and when the queen dies, another female takes her place [22]. Queens are larger than other colony members and may reach 80 g, twice the size of regular "worker" animals. Interestingly, a newly established queen stretches the space between the vertebrae in her backbone to become longer, but the skull width remains the same [23]. The reproductive males also appear to be bigger in size than other colony members.

48.2.6 Hypoxia Tolerance

As a part of adaptation to the limited availability of oxygen within the tunnels of their typical habitat, naked mole rats have very low respiration and metabolic rates for an animal of such size. Their lungs are small and the blood has a very strong affinity for oxygen, increasing the efficiency of oxygen uptake [24]. In addition to the chronic environmental hypoxia, naked mole rats also experience acute hypoxia when performing tunnel excavation.

Because mammalian brains have extremely high levels of aerobic metabolism, they typically suffer irreversible damage after brief periods of oxygen deprivation; however, the brain tissue from naked mole rats is remarkably resistant to hypoxia [25]. It was suggested that brain tolerance to hypoxia may result from slowed or arrested brain development.

Sequence analysis of hypoxia-inducible factor 1α (HIF1α) in naked mole rats revealed a unique amino acid change located in the VHL-binding domain, consistent with relaxation of ubiquitin-dependent degradation of HIF1α and with adaptation to low oxygen conditions [12]. Gene expression changes in several naked mole rat tissues subjected to 8 % oxygen for 1 week also revealed many changes associated with energy metabolism [12].

48.3 Genome and Transcriptome

Following initial gene-based studies [26], our laboratory sequenced the naked mole rat genome and transcriptome in 2011 [12], which received much attention from the research community. Most of the genome (93 %) shared synteny with human, mouse or rat genomes, and it was found that functional categories for olfactory receptor activity, visual perception, spermatogenesis, and RING domain were enriched for pseudogenes.

These studies were followed with additional high-throughput approaches [27–30]. In particular, we sequenced the genome of the Damaraland mole rat, which helped us to narrow down the unique features of the naked mole rat. For example, the naked mole rat uncoupling protein 1 (UCP1) exhibited alterations in the sequences that correspond to a motif regulated by nucleotides and fatty acids, whereas this motif is intact in the Damaraland mole rat. Since UCP1 is responsible for non-shivering thermogenesis, this protein is an attractive target for studies on thermoregulation in naked mole rats. We also found that the naked mole rat is a natural melatonin system knockout as both its melatonin receptors are pseudogenes, whereas one of these receptors is intact in the Damaraland mole rat.

48.4 Ionome and Selenoproteome

An earlier curious observation was that naked mole rat tissues, in particular liver and kidney, are selenium-deficient [26]. However, initial analyses of the selenoproteome of the naked mole rat revealed a standard set of selenoprotein genes. Since selenium is present in mammalian organs, primarily in the form of selenoproteins, it was important to address this discrepancy. Indeed, further analyses revealed low selenoprotein gene expression levels. Moreover, we observed low selenocysteine content of selenoprotein P. Only seven selenocysteine (Sec) residues were present in this naked mole rat protein, compared to 10 in mouse and human selenoprotein P and 14–16 in pig, dog, bear and bat proteins.

Can low selenium utilization in the naked mole rat contribute to its exceptional longevity? To address this issue, we analyzed the levels of 18 chemical elements, i.e., the ionome, of liver, kidney, brain and heart across 26 species of mammals, including the naked mole rat. In both liver and kidney, selenium levels were negatively correlated with longevity, whereas no other element showed this pattern [31]. This is consistent with the idea that while selenoproteins serve important and often essential functions, selenium is also a toxic element. This toxicity may be especially pronounced in long-lived animals, such as naked mole rats. Such species may need to strike a balance between the beneficial functions of selenium, e.g., redox catalysis by selenoenzymes, and detrimental properties of this element. Interestingly, the overall pattern of selenium levels across mammalian organs was most similar to those of arsenic and cadmium, both of which are toxic elements [31]. Thus, the interplay between favorable and unfavorable properties, with a narrow margin between the two, is a distinctive feature of the trace element selenium.

48.5 Defect in Glutathione Peroxidase 1

Selenium deficiency in the naked mole rat prompted us to further examine functions dependent on this trace element. Thus, total methionine sulfoxide reductase activity was measured in a laboratory mouse strain and the naked mole rat, and no significant difference was found. As this activity is represented by MsrA and three MsrBs, only one of which is a selenoprotein, the methionine sulfoxide reduction function was not compromised. In contrast, the activity of glutathione peroxidase (GPX) in the liver and kidney of the naked mole rat was found to be an order of magnitude lower than that in mouse tissues (Fig. 48.2).

A similar distribution of selenium was observed in *Gpx1* knockout mice, thus confirming that the reduced utilization of selenium by the naked mole rat is caused at least in part by GPX1 deficiency. Further examination of the *Gpx1* gene revealed a premature stop codon in its C-terminal region. Thus, GPX function is indeed compromised in the naked mole rat.

48.6 Concluding Remarks

The naked mole rat represents a promising model for studies on aging, cancer resistance, hypoxia, thermoregulation, pain sensitivity and eusociality. Unexpectedly, in the last few years, this organism also emerged as a natural model of selenium and selenoprotein deficiency. How this selenium status is linked to various traits of this animal is currently unknown, but at least in the case of aging, we observed a clear link between low selenium status and longevity. It would be important to further

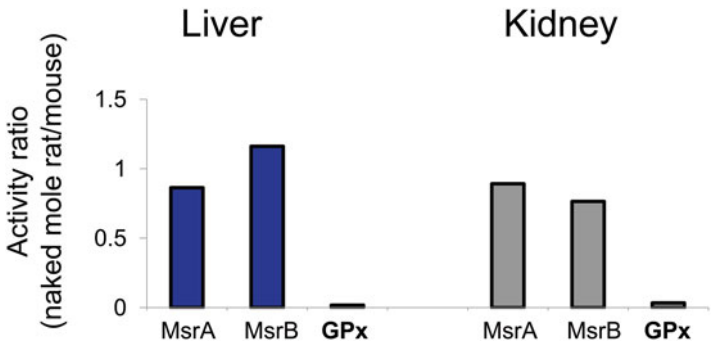

Fig. 48.2 Compromised glutathione peroxidase function in naked mole rats. The figure compares mice and naked mole rats with regard to methionine sulfoxide reductase and glutathione peroxidase activities. Both in liver (*left*) and kidney (*right*), GPx activity was much lower in the naked mole rat, whereas methionine sulfoxide reductase A (MsrA) and methionine sulfoxide reductase B (MsrB) activities were similar

investigate if selenium deficiency also contributes to other traits observed in this animal. We suggest that the naked mole rat may be used as a model to help to uncover new functions of selenium-dependent pathways in mammals.

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Chapter 49
Glutathione Peroxidase 1: Models for Diabetes and Obesity

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Abstract Glutathione peroxidase 1 (GPX1), the most abundant selenoprotein, was initially recognized as only a major antioxidant enzyme against oxidative stress. Strikingly, overexpression of *Gpx1* in mice induces type 2 diabetes-like phenotypes and obesity, whereas knockout of the gene leads to metabolic disorders similar to type 1 diabetes. This chapter elaborates the physiological characteristics, molecular mechanisms, and human health relevance of these maladies resulting from alterations of *Gpx1* expression levels.

Keywords Diabetes • Glutathione peroxidase • Insulin resistance • Mouse model • Obesity

49.1 Introduction

Glutathione peroxidases (GPX) are selenium-containing enzymes that degrade hydrogen and lipid peroxides. The first member of the enzyme family (GPX1) was initially found by Gordon Mills to protect hemoglobin from oxidation in erythrocytes [1]. Later, GPX1 was identified as the first selenoenzyme in mammals [2], containing one molecule of selenium per subunit [3]. The chemical moiety of selenium in GPX1, as in other selenoproteins, is selenocysteine [4] that requires a special selenocysteyl-tRNA and a special codon (UGA) for its biosynthesis through a co-translational mechanism [5]. There is a direct link between GPX1 enzyme activity and protein concentration [6]. GPX1 is often called classic, cytosolic, or cellular GPX [EC 1.11.1.9], and can metabolize a wide range of peroxides, using glutathione as the reducing agent. GPX1 is a tetramer composed of four identical subunits [7]. There are four other selenium-containing GPX enzymes. Specifically, GPX2 [EC 1.11.1.9] is called gastrointestinal GPX, and is also a tetramer with substrate specificity similar to that of GPX1 [8]. GPX3 [EC 1.11.1.9] is called plasma GPX. As an extracellular glycoprotein [9], GPX3 is also a tetramer, with a molecular weight of 23–25 kDa for each subunit [10]. GPX4 [EC 1.11.1.12] is a monomer that

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can catalyze the degradation of phospholipid hydroperoxides [11] in contrast to the other GPX enzymes [12]. GPX6 is a selenoprotein in humans with cysteine-containing homologs in rats and mice [13]. *GPX1* genomic localization was assigned to human chromosome 3 [14], and in situ hybridization localized the gene to 3p13-q12 [15]. Later work reassigned *GPX1* to 3q11-q13 [16], and finally to 3p21.3 [17]. Mouse *Gpx1* was mapped to chromosome 9, in a region of conserved homology with human chromosome 3 [18].

49.2 *Gpx1* Transgenic Mouse Model

Overproduction of GPX1 leads to a thermosensitive phenotype, due to a decreased concentration of heat shock protein (HSP). Notably, serum level of HSP70 was significantly higher in type 2 diabetics and it positively correlated with the duration of disease [19], while restoration of HSP70 deficiencies improves glucose tolerance in diabetic monkeys [20, 21]. Even though the overproduction of GPX1 did not alter GPX3 or GPX4 activities or selenium concentrations in various tissues [22], it conferred protection against post-myocardial infarction remodeling and heart failure in mice [23]. Intriguingly, insulin resistance in healthy pregnant women is associated with an increased erythrocyte GPX1 activity in late gestation [24]. More strikingly, *Gpx1* overexpressing (OE) mice developed type 2 diabetes mellitus (T2DM)-like phenotypes including insulin resistance, hyperglycemia, hyperinsulinemia, and obesity at an age of 6 months [25]. Insulin resistance in the *Gpx1* OE mice was associated with decreased phosphorylation of insulin receptor and protein kinase B (Akt at Ser473 and Thr308) in liver and soleus muscle [25]. This model revealed a novel role of GPX1 in the transduction of insulin signaling via regulating the transient inhibitions of protein phosphatases that have cysteines in the active sites that can be oxidized by intracellular hydroperoxides [26, 27] Dietary selenium deficiency can partially rescue the type 2 diabetes-like phenotype in male mice [28]. The mechanisms were related to reversing gene expression of key factors involved in insulin synthesis and secretion (*Beta2*, *Cfos*, *Foxa2*, *Pregluc*, *Ins1*, *p53*, and *Sur1*) to wild type levels. This in turn down-regulated hepatic gene expression of two rate-limiting enzymes for lipogenesis (acetyl-coenzyme A carboxylase, *Acc1*) and glycolysis (glucokinase 1, *Gk1*), and lowered activities of hepatic glucokinase and muscle phosphoenolpyruvate carboxykinase (PEPCK) [28, 29].

Dietary restriction can prevent or reverse all type 2 diabetes-like phenotypes in OE mice except for hyperinsulinemia [30]. Thus, hyperinsulinemia is considered a primary effect of the global metabolic phenotype by GPX1 overexpression [30], and is attributed to elevated beta cell mass, insulin synthesis, and insulin secretion. The molecular mechanism was mediated by an up-regulation of pancreatic and duodenal homeobox 1 (PDX1), a key transcriptional factor in beta cell differentiation, growth, and survival; and, in addition, insulin synthesis and secretion, and a down-regulation of uncoupling protein 2 (UCP2), a mitochondrial protein that suppresses insulin secretion. Furthermore, GPX1 overexpression enhanced acetylation of H3 and H4

histones at the proximal *Pdx1* promoter in islets, illustrating another novel role of this enzyme in insulin physiology via epigenetic regulation of *Pdx1* expression in pancreatic islets [30]. GPX1 overexpression also protected the *Pdx1* promoter from H_2O_2-induced histone deacetylation.

Prolonged hyperinsulinemia in *Gpx1* OE mice may be the driving force for the development of their overt metabolic phenotypes. This implied that hyperinsulinemia preceded the onset of insulin resistance. In classic T2DM, however, insulin resistance often induced a compensatory insulin production and/or secretion, resulting in a temporary hyperinsulinemia followed by fading insulin release, and occurrence of hyperglycemia and complications. Furthermore, the T2DM-like phenotypes in *Gpx1* OE mice also concurred with elevated triglyceride, cholesterol, and non-esterified fatty acids in liver, along with obesity in adulthood [25, 28].

Dietary selenium repletion can elevate *Gpx1* translational efficiency by 20-fold [31], whereas dietary selenium deficiency reduced the *Gpx1* mRNA abundance by 20-fold [32]. This helped explain why dietary selenium deficiency partially rescued *Gpx1* OE phenotypes [28, 29]. In contrast, several studies in rats [33], pigs [34, 35], and mice [36] demonstrated diabetogenic potential of supranutrition of dietary selenium. Prolonged feeding of female rats with a high selenium diet (3 mg/kg diet) from breeding to lactation induced gestational diabetes in the dams and insulin resistance in their offspring [33]. Feeding pigs 0.5 mg selenium/kg led to alterations in molecular targets related to energy metabolism in skeletal muscles and visceral adipose tissue [34]. Feeding pigs 3 mg selenium/kg from weaning produced hyperinsulinemia and decreased hepatic Akt protein [35]. These diabetogenic effects of dietary selenium excess were associated with elevated production of GPX1 and certain other selenoproteins as well. However, a high fat diet-induced obesity in pigs and up-regulated expression of 12 selenoprotein genes in six tissues and down-regulated 13 selenoprotein genes in seven tissues, but exerted no effect on *Gpx1* gene expression in any tissue [37].

49.3 *Gpx1* Knockout Model

Three different *Gpx1*$^{-/-}$ mouse models have been reported [38–40]. Mice lacking *Gpx1* were apparently healthy and fertile, without increased sensitivity to hyperoxia. Their tissues exhibited neither a retarded rate in consuming extracellular hydrogen peroxide nor an increased content of protein carbonyl groups and lipid peroxidation compared with those of the corresponding wild type mice [38]. However, *Gpx1*$^{-/-}$ mice were drastically more susceptible to paraquat-mediated lethality and hepatotoxicity than wild type mice, while this herbicide showed a transcriptional regulation of *Gpx1* in normal cells [41, 42]. High doses of dietary vitamin E did not compensate or help the protection afforded by GPX1 in *Gpx1*$^{-/-}$ mice [43]. Growth retardation was seen by one group in *Gpx1*$^{-/-}$ mice, presumably due to impaired mitochondrial energy production as an outcome of increased oxidative stress [40].

While overexpression of GPX1 in mice induced T2DM-like phenotypes, the knockout of *Gpx1* caused metabolic changes similar to T1DM. Compared with wild type mice, *Gpx1*$^{-/-}$ mice have lower pancreatic β-cell mass, hypoinsulinemia, mild hyperglycemia, and impaired ATP production and glucose-stimulated insulin secretion in islets [44]. The molecular mechanism was found to be associated with decreased PDX1 and elevated UCP2 in pancreas [44]. The comparative impacts and mechanisms of GPX1 overproduction and deficiency on insulin physiology and glucose metabolism are summarized in Fig. 49.1.

In fact, knockout of superoxide dismutase-1 (*Sod1*) or double knockout of *Sod1* and *Gpx1* produced the same phenotypes as those of *Gpx1*$^{-/-}$ mice, but to a much more severe extent [44]. Knockout of *Sod1* down-regulated the fork head box A2/Pdx1 pathway in a superoxide-dependent fashion at epigenetic, mRNA, and protein levels. *Sod1*$^{-/-}$ mice showed more apparent pancreatitis than *Gpx1*$^{-/-}$ mice, whereas

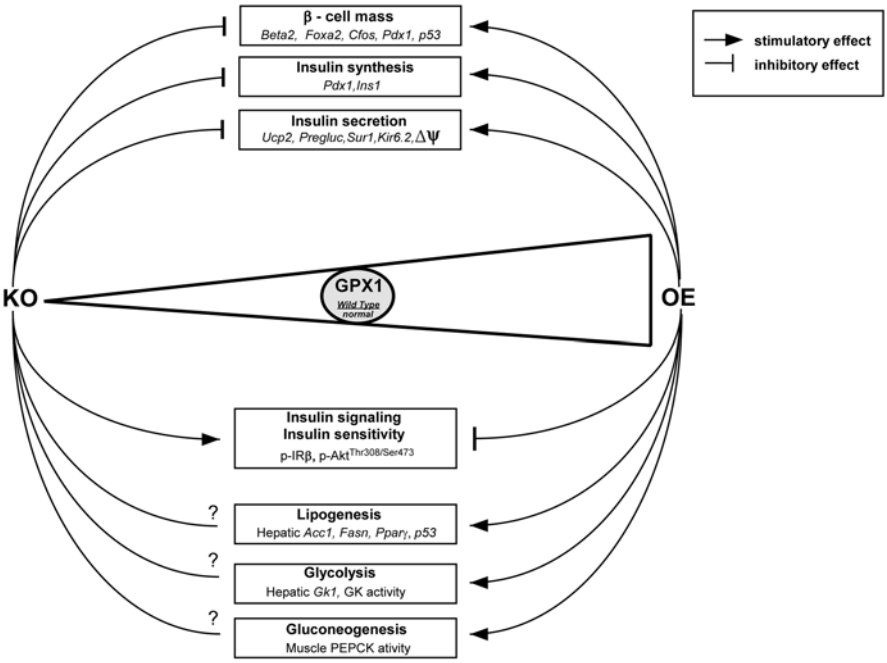

Fig. 49.1 Role of GPX1 in insulin physiology and glucose metabolism. Overexpression of Gpx1 (OE) induces hypertrophy of beta cells, hyperinsulinemia, hyper secretion of insulin, hyperglycemia, hyperlipidemia, insulin resistance, and obesity. In contrast, knockout of Gpx1 leads to hypotrophy of β-cells, hypoinsulinemia, hypo secretion of insulin, and elevated insulin sensitivity. *Acc1* acetyl-coenzyme A carboxylase 1, *Beta2* transcription factor Beta 2, *Cat* catalase, *C-fos* transcription factor C-fos, *Fasn* fatty acid synthase, *Foxa2* forkhead box protein A2, *Gk1* glucokinase, *Ins1* insulin 1, *Kir6*.2 inward-rectifier potassium ion channel, *p53* tumor protein 53, *PEPCK* phospho-enolpyruvate carboxykinase, *Pdx1* pancreatic and duodenal homeobox 1, *p-IRb* phosphorylated insulin receptor subunit b, *p-AktThr308*/*Ser473* phosphorylated protein kinase B, *Pparγ* peroxisome proliferator-activated receptor gamma, *Pregluc* preglucagon, *Sur1* sulfonylurea receptor 1, *Ucp2* uncoupling protein 2, *Δψ* mitochondrial membrane potential

the latter were more susceptible to pancreatitis and amylase release induced by cerulean-injection than the former [44, 45]. Furthermore, *Sod1*$^{-/-}$ mice displayed decreased pyruvate tolerance and liver glycogen storage, but elevated hepatic triglycerides and non-esterified fatty acids [46]. Mechanistically, *Sod1*$^{-/-}$ mice had decreased hepatic activities of PEPCK, total protein phosphatase, and protein phosphatase 2A (PP2A), but increased hepatic GK activity compared to those of wild type controls. Meanwhile, these mice also have elevated hepatic protein levels of sterol regulatory element binding proteins 1 and 2, p53, mitogen activated protein kinase, total and phosphorylated AMP-activated protein kinase α1 protein, protein tyrosine phosphatase 1B, and protein phosphatase 2B compared with wild type controls. It is very intriguing that knockout of *Gpx1* exerts no evident effect on glycolysis, gluconeogenesis, or lipogenesis.

The attenuated glucose-stimulated insulin secretion in *Gpx1*$^{-/-}$ mouse islets can be improved by the synthetic GPX mimic ebselen [47]. This rescue resulted from a coordinated transcriptional regulation of four key glucose-stimulated insulin secretion regulators and was mediated by the peroxisome proliferator-activated receptor γ co-activator 1α (PGC-1α)-mediated signaling pathways. Although ebselen also improved glucose-stimulated insulin secretion in the *Sod1*$^{-/-}$ mouse or the *Sod1* and *Gpx1* double knockout mouse islets, the SOD mimic copper diisopropylsalicylate exerted such effects only in the *Sod1*$^{-/-}$ mouse islets and suppressed gene expression of the PGC-1α pathway.

Knockout of *Gpx1*, contrary to overexpression of *Gpx1*, improved insulin sensitivity [48]. This is because intracellular reactive oxygen species (ROS) are important for sensitizing insulin signaling [48, 49]. Overproduction of GPX1 accelerated cellular H_2O_2 quenching, alleviated peroxides-mediated inhibition of tyrosine phosphatase activity, and subsequently attenuated phosphorylation of insulin receptor and downstream signal proteins [25]. In contrast, improved insulin sensitivity in *Gpx1*$^{-/-}$ mice was associated with elevated intracellular hydroperoxide and phosphorylation of p53 and p38-AMP-activated protein kinase in islets [48] and enhanced phosphatidylinositol-3-kinase (PI3K)/Akt signaling and glucose uptake in muscle [48, 50]. Meanwhile, *Gpx1*$^{-/-}$ mice were protected from insulin resistance associated with the high fat diet-induced obesity by a mechanism correlated with enhanced oxidation of the PI3K antagonist phosphatase and tensin homolog, PTEN [50].

49.4 Human Health Relevance of GPX1-Related Metabolic Disorders

Pancreatic islets of Langerhans account for approximately 1–2 % of total pancreatic mass and have five types of hormone-producing cells: α-cells (glucagon), β-cells (insulin), δ-cells (somatostatin), PP-cells (pancreatic polypeptide), and ε-cells (ghrelin). Because pancreatic islets have naturally low expression of antioxidant enzymes, all these hormone-secreting cells, and in particular β-cells, were perceived to be very vulnerable targets of oxidative stress [51–54]. This observation helped

explain why pro-oxidant streptozotocin and alloxan were very potent diabetogenic agents [55, 56] and why the GPX mimic ebselen rescued glucose stimulated insulin secretion in isolated *Gpx1*$^{-/-}$ islets and mice [60], and was effective for treating diabetes-related atherosclerosis and nephropathy in the diabetic apolipoprotein E(*ApoE*)/*Gpx1* double knockout mouse model [57]. Although overproduction of GPX1 induced T2DM-like phenotypes in mice without diabetic or obese-prone genetic background [25], β-cell-specific overexpression of *Gpx1* in diabetic and obese (*ob*/*ob*) mice actually reversed hyperglycemia and improved β-cell volume and granulation [58]. Furthermore, elevated β-cell mass and insulin synthesis in pancreatic islets of *Gpx1* OE mice implied a unique trophic effect of the enzyme, because no similar changes were produced by overexpressing catalase [59, 60], metallothionein [59, 61], or SOD enzymes [62, 63]. This feature may be applied to treat T2DM as the β-cell mass loss is recognized as a pivotal factor in pathogenesis of T2DM [64, 65].

High plasma or serum selenium status has been reported to be associated with an increased risk of hyperglycemia, hyperlipidemia, or T2DM [66]. The increased risk may be related to abnormal plasma selenoprotein P and/or tissue GPX1 and other selenoproteins [67]. Meanwhile, *GPX1* polymorphisms were implicated in human obesity. While the *GPX1* Pro198Leu variant contributed to the metabolic syndrome in Japanese men [68], the *GPX1* Pro200Leu was linked to morbid obesity in central Mexican women [69]. Gene polymorphisms of *GPX1*, *SOD1*, and *SOD2* were found to be associated with T2DM in the Indian population [70]. A recent study has shown the rescue by overexpression of *Gpx1* of the selective inactivation of β-cell transcription factors involved in insulin synthesis, *Mafa*, *Nkx6.1*, and *Pdx1* in the diabetic (*db*/*db*) T2DM mouse model. This type of rescue is relevant as the same impaired expression pattern was found in islets of human T2DM patients [71]. Rayman and Stranges [72] have reviewed a complex correlation between systemic selenium status and T2DM risk, and suggested a "U-shaped" curve for such correlation. This implied possible harm to occur outside a physiological range. Certainly, the human responses were in accordance with metabolic effects or phenotypes associated with *Gpx1* overexpression or knockout in mice and dietary selenium supranutrition or deficiency in several species discussed above [25, 50, 73, 74].

49.5 Concluding Remarks

Overall, this chapter reveals the physiological characteristics, biochemical pathways, and human health relevance of dual roles of GPX1 in insulin physiology and development of diabetes and obesity. The developments of T2DM-like phenotypes and obesity in OE *Gpx1* mice and T1DM-like phenotypes in the *Gpx1*$^{-/-}$ mice seem to be reciprocal, which reflects the importance for maintaining a balance between antioxidant enzyme activity and ROS at cellular, tissue, and body levels. Excessive ROS accumulation, due to GPX1-deficiency, inhibits gene expression or protein production of key transcriptional factors like PDX1, leading to lowered islet β-cell

mass, insulin synthesis, and insulin secretion. However, the physiological level of ROS is essential for controlling protein phosphatase activity in insulin signaling. Overly diminishing intracellular ROS by *Gpx1* overexpression desensitizes insulin signaling. Along with the chronic hyperinsulinemia resultant from dysregulated islet β-cell mass, insulin synthesis, and insulin secretion, this desensitization leads to insulin resistance in *Gpx1* OE mice. Because GPX1 is emerging as a novel, key regulator of insulin physiology and energy metabolism, effects of *GPX1* polymorphisms and dietary selenium on its activity or function should be fully considered at physiological and pathological conditions.

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Chapter 50
Selenium in Livestock and Other Domestic Animals

Peter F. Surai and Vladimir I. Fisinin

Abstract Selenium (Se) is an essential element of poultry and livestock nutrition, and its dietary supplementation in commercial trace-mineral premixes is key in preventing deficiency. The efficiency of Se assimilation from the diet depends on the form used in the diet. In comparison to sodium selenite, selenomethionine is more effectively transferred to chicken eggs and developing embryos, as well as via placenta to mammalian embryos and is used by newly born animals. Recent developments in selenoprotein studies and the new roles of Se in antioxidant defenses and cell signaling have opened new areas of Se research, which have aided our understanding of the application of this element to the livestock industry.

Keywords Chickens • Pigs • Ruminants • Selenium • Selenomethionine • Sows

50.1 Introduction

Selenium (Se) is an essential element of livestock nutrition. Severe Se deficiency had been associated with the development of various disorders, which is no longer the case in modern commercial poultry industry and other areas of animal production. However, decreased productive and reproductive performance due to suboptimal levels of dietary Se is still observed in livestock. The introduction of modern genetics to the livestock industry has substantially improved the growth rates of animals. However, a major downside of such improvements in performance is that the

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livestock are often highly sensitive to various stresses. Therefore, in modern animal production for human consumption, there is an important movement to prevent Se deficiency by meeting the exact Se requirement for optimizing performance. In particular, the discovery and characterization of a range of new selenoproteins, as well as a better understanding of the relationships between different antioxidants, are providing new insights in this area. For the last 30 years, a mandatory approach to regulate Se supplementation in commercial feed has been used in the animal industry worldwide. To meet Se requirements, this industry has relied completely on supplemental Se delivered with trace-mineral premixes (usually at 0.2–0.3 mg/kg diet), while the Se levels in the feed ingredients have not been taken into account [1].

50.2 Se in Poultry Nutrition

The Se requirements of poultry have been shown to be quite low, ranging from 0.06 mg/kg diet for laying hens to as high as 0.2 mg/kg diet for turkeys and ducks [1, 2]. However, it is generally agreed upon that Se requirements may be increased substantially by the stress conditions of commercial poultry production. It should be noted that the benefits of dietary Se supplementation for chickens, as described below, likely have the same relevance to the commercial production for turkeys, geese, ducks, quail, and other avian species. Poultry production technology is based on usage of adult parent birds (breeders) used for meat and egg type chicken reproduction and producing incubation eggs; commercial layers (adult egg type birds, producing table eggs for human consumption); broiler chickens (meat type chickens, usually growing up to 6 weeks) and rearing chickens (from hatch to a reproductive age).

50.2.1 Breeders

Egg production and egg quality are two major parameters that determine the profitability of Se supplementation for breeders. Therefore, the most important issue is to transfer adequate amounts of Se to the fertilized, developing egg. Se supplementation enhances Se levels in egg albumen and yolk as well as affecting eggshell quality. More importantly, it helps the developing embryo withstand the oxidative stress that accompanies hatching [3], as high temperature and humidity with increased availability of oxygen at the time of hatching make this period especially stressful in terms of redox balance. However, producing eggs with optimal concentrations of Se appears to be a difficult task. Poultry feed ingredients in many parts of the world are quite low in Se, and traditional dietary supplementation with sodium selenite or selenate has not been effective [1, 4, 5], because, as only recently determined, supplemental inorganic Se is not accumulated within the egg. The main form of Se in egg yolks and egg whites is selenomethionine (SeMet), which is not synthesized in

chickens or other animals. Therefore, development and commercialization of various sources of organic Se, mainly in the form of SeMet, solved the problem of Se transfer to the egg, thereby providing the developing embryo with optimal Se concentrations associated with high productive and reproductive performance for breeders [2]. Our analysis of published research and commercial data indicated that 0.2–0.3 mg/kg diet supplemental Se in an organic form is the recommended level for poultry breeders to use.

50.2.2 Layers

For layers, Se is important for three main reasons. That is, this element is essential i) to maintain layer health and productive ability [1, 3], ii) to foster optimal internal egg yolk, egg white and external egg shell quality [1], since it is likely that the beneficial Se effects on bone structure and metabolism in laying hens [6] would also be of considerable interest to the egg-producing industry, and iii) to potentially provide an important source of Se for human consumption [7], and thus production of healthy foods through Se-enriched eggs [8].

50.2.3 Broilers

In broilers, improving immunocompetence often improves stress resistance. Immunocompetence is the most important parameter for guaranteeing the performance of broilers because of their high rates of microbial and viral infections and busy vaccination schedules [9].

Due to the drastic increase in growth rates of chickens that may exceed 60 g/day, the animals are very sensitive to various stresses, and antioxidant defenses become the driving force of supporting chicken health and development [1–3]. Therefore, the need for optimal Se status and tissue-specific expression of a range of selenoproteins makes Se a very important element in broiler nutrition. Under conditions of Se deficiency, a decrease in mRNA levels of 23 selenoproteins in the thymus, spleen and bursa of Fabricius of broiler chickens has been observed [10].

Meat quality, including its appearance, drip loss, juiciness and off-taste due to lipid peroxidation and/or protein oxidation during storage, makes antioxidant defenses, and Se in particular, major contributors to consider [11]. It seems likely that a selenoprotein such as methionine-R-sulfoxide reductase B (MsrB), which is responsible for the prevention of protein oxidation, is of great importance in relation to meat quality. Furthermore, Se deficiency has been shown to mainly influence expression of antioxidative selenoproteins in chicken muscles, including glutathione peroxidase (GPx), thioredoxin reductase, selenoproteins W, K and H, etc. [12]. Finally, when using organic Se sources in chicken diets, it is possible to substantially increase Se content in chicken meat to such extent that 100 g of meat would deliver 50 % of the recommended daily amount of Se for humans [8] based on US RDA.

50.3 Se in Swine Nutrition

There are three main applications of Se in pig and other swine nutrition: Se in swine breeders (boars and sows), Se in newborn piglets, and Se for finishing pigs. The Se requirement in swine depends on many environmental and other conditions and is generally considered to be 0.15–0.30 mg/kg feed. It seems likely that reproducing sows and boars are especially sensitive to Se deficiency, and meeting their Se requirements is an important challenge for pig nutritionists [1].

50.3.1 Se for Swine Breeders: Boars and Sows

Se plays an important role in boar nutrition by participating in selenoprotein synthesis. The Se-dependent enzyme, GPx, is the most studied selenoprotein in swine production, because these selenoproteins are important for antioxidant system regulation in the animal. The roles of other selenoproteins in boar semen production and the maintenance of semen quality remain to be investigated. Mammalian spermatozoa are unique in structure and chemical composition, and are characterized by high proportions of polyunsaturated fatty acids (PUFAs) in the phospholipid fractions of their membranes [1].

The feature of these highly specialized cells reflects the specific needs of their membranes for high levels of fluidity and flexibility, which are necessary for sperm motility and fusion with the egg. This functional advantage conferred by PUFAs, however, is associated with disadvantages in terms of the susceptibility of sperm to free radical attack and lipid peroxidation [1, 3]. Therefore, antioxidant protection is a vital element of maintaining sperm membrane integrity, motility and fertilizing ability. In this respect, dietary Se through its role in antioxidant systems is an important modulator of semen quality.

Boar spermatozoa were thought to be poorly adapted to counteract the toxic effects of induced reactive oxygen species (ROS) because of comparatively low levels of superoxide dismutase (SOD) and GPx in seminal plasma. In fact, GPx activity in boar seminal plasma was initially shown to be very low, but later studies showed that GPx was present in boar seminal plasma (for review see [13]). Indeed, the Se-dependent form of GPx in boar plasma comprised between 80.7 and 90.8 % of total GPx enzymatic activity. GPx4 levels in boar semen were shown to be affected by Se status, and organic Se in the boar diet positively affected GPx4 gene expression in boar testes. The primary focus thus far has been on GPx1 as an important biomarker for Se status and potentially for antioxidant defenses [13]. However, current evidence suggests that other selenoproteins could be even more important than GPx for Se antioxidant action and that optimal levels achieved in tissues may depend upon the form of Se being ingested.

The effects of dietary Se on boar reproduction depend on the basic level and the chemical form of Se in the diet. When Se levels in the diet were found to be comparatively low (0.06–0.07 mg/kg diet), characteristic detrimental changes in testes structure were observed (for review see [13]).

Since Se levels in European feedstuffs are substantially lower than in the USA [1], one could expect a better response in terms of productive and reproductive performance to dietary Se supplementation in European commercially raised swines. Since 1998, the recommended Se requirement worldwide for swine is 0.15–0.30 mg/kg diet, but in commercial conditions, this likely still may be suboptimal depending on the levels of stress to which the animals are exposed. Deviation from the optimal quantities of dietary Se, either above or below these quantities, may cause multiple abnormalities of spermatozoa and affect their motility and fertilizing ability [14]. Indeed, the Se requirements for pigs depends on their living conditions, as well as levels for other antioxidants (vitamin E) and pro-oxidants (unsaturated fat) in the diet. It has been suggested that boars in big scale commercial, European farms and other areas with low Se dietary levels and high environmental challenges would require increased antioxidant defenses [1]. They would also most likely benefit from additional dietary Se supplementation in an organic form [13].

A great body of evidence indicates that the antioxidant-prooxidant balance is an important regulator of mammalian reproductive functions, including ovarian follicular development, ovulation, fertilization, luteal steroidogenesis, endometrium receptivity and shedding, embryonic development, implantation and early placental growth and development (for review see [15]). In fact, gestation is considered a source of constant oxidative stress for the dam. It seems likely that at the time of gestation, sows experience decreased antioxidant protection. A decline in serum vitamin E levels has been observed from about 80–90 days after insemination through farrowing, and the plasma concentration of this vitamin was substantially lower at the end of gestation than at the beginning (for review see [15]). It seems likely there is a risk that increased oxidative damage in sows during late gestation may negatively affect the growth and health of fetuses as well as postpartum growth of piglets [16]. Furthermore, the sow's ascorbic acid status and GPx activity may be low during this period of extensive stress. Therefore, oxidative stress in gestating sows is a risk factor of decreased sow productive and reproductive performance that could detrimentally affect progeny.

50.3.2 Se in Newborn Piglets

Newborn animals are at high risk of oxidative stress and are very susceptible to free radical oxidative damage. Several lines of evidence support this assumption, including their exposure to high oxygen concentrations, reduced antioxidant defense and free iron that enhances the Fenton reaction leading to production of highly toxic hydroxyl radicals. Indeed, newborn piglets suffer seriously from birth oxidative stress because of their not fully functional antioxidant systems [1]. It seems likely, due to limited placental transfer, piglets are born with low vitamin E content, including low levels in liver, brain, heart and kidney (for review see [15]). Moreover, the fetal piglet looses the ability to synthesize vitamin C as gestation advances. The neonatal pig does not synthesize vitamin C during the first week of life and is

completely dependent on its supply via placental transfer and in colostrum and milk. Therefore, oxidative stress is the main concern in pregnant sows and newborn piglets. Improvement of antioxidant defenses of sows and piglets at those critical periods is of great importance to their health, productiveness and reproductive performance. Since vitamin E and ascorbic acid status of newly born piglets is comparatively low, the role of selenoproteins in antioxidant defenses and redox status maintenance is crucial. In fact, SeMet supplementation of the maternal diet significantly improves, not only GPx activity in the liver, kidney, pancreas, muscle and serum, but also positively affects other important elements of the antioxidant system of the piglets. Indeed, there was a significantly improved total antioxidant activity in the liver, kidney, pancreas, muscle and serum as well as SOD (pancreas, muscle, and serum) and glutathione (GSH; kidney, pancreas, muscle, and serum) in experimental piglets [17]. This was associated with decreased malondialdehyde (MDA) content in liver, kidney, and pancreas of the piglets. Effects of dietary Se on a variety of indexes of antioxidant protection shown above, including SOD activity and GSH content in various pig tissues, reflect important interactions inside the antioxidant network. Transcription factors such as nuclear factor (erythroid-derived 2)-like 2 (NFE2L2, Nrf2) and NF-κB may mediate these types of interactions. In fact, mammalian selenoprotein thioredoxin reductase 1 has recently been shown to be a potent regulator of Nrf2 activation [18], and Se is found to downregulate NF-κB with decreasing synthesis of pro-inflammatory cytokines in cell culture [19].

50.3.3 Se for Growing Pigs

Similar to growing chickens, young piglets need optimal Se levels for antioxidant defenses related to immunocompetence and the various stresses associated with commercial pig production [16]. Furthermore, like chicken meat, pork can be enriched with Se and be an important source of Se for human consumption [8]. Moreover, improving the Se content of muscle before slaughter could guarantee enhanced meat stability through the reduction of lipid and protein oxidation.

50.4 Se in Ruminant Nutrition

Taking into account various stresses of commercial dairy, beef and sheep production, it is important to consider the antioxidant-prooxidant balance in the feed, digestive tract and tissues of these animals [1, 3, 20]. As mentioned previously, Se as an integral part of a range of selenoproteins plays an important role in regulating various physiological functions, including immunity, reproduction and early postnatal viability. The Se requirement for lactating and dry cows was set in 2001 at 0.3 mg/kg diet. In 2000, the beef cattle requirement for all ages was set at 0.1 mg/kg diet, and the maximum tolerable concentration was set at 2 mg/kg diet. In 1985,

the sheep Se requirement was set at 0.1–0.2 mg/kg diet, with 2 mg/kg diet as the maximal tolerable level. It is important to note that Se deficiency in dairy cows persists globally independent of the current practice of dietary Se supplementation. In fact, Se deficiency alone or in combination with vitamin E deficiency relates to the development of various diseases in ruminants, including nutritional muscular dystrophy, retained placenta, metritis, increased somatic cell counts, reduced growth rates, reduced reproduction rates, immunosuppression and increased susceptibility to various diseases [1]. Therefore, Se deficiency in ruminants remains a global problem in European, Asian and American countries, including the USA, resulting in decreased immunocompetence and productive and reproductive performance of animals. Indeed, suboptimal blood-Se concentrations in ruminants fed Se supplements have recently been observed in the USA [21], reflecting low Se bioavailability from sodium selenite. Therefore, it seems reasonable to suggest that by addressing Se requirements, it will be possible to deal with Se-related diseases/health problems in dairy, beef and sheep production.

Se nutrition of ruminants has created some specific challenges for the dairy and beef industries. In particular, in many places in the world, Se levels in feed ingredients are not adequate to meet the high Se demands of growing, reproducing and lactating animals. The main problem with Se assimilation from the diet is associated with rumen conditions. It is well established that absorption of Se from main feed additives (sodium selenite) is much lower in ruminants than in monogastric animals [1]. For example, absorption of orally administered radio-labeled selenite was 85 % in swine, 86 % in rats and only 34 % in sheep [22]. Because rumen reducing conditions and microorganisms affect Se metabolism, it is possible that sodium selenite may be converted into insoluble forms such as elemental Se or selenides. Furthermore, rumen bacteria can use Se for the synthesis of various compounds, and this process depends on many factors including chemical form of Se. However, Se bioavailability from microbe mass seems to be low. Recently, the incorporation of Se into microbial mass was shown to be greater for SeMet than inorganic Se supplements. Additionally, formation of non-bioavailable, elemental Se was significantly less for ruminant microbes incubated with SeMet compared with inorganic Se sources [23]. Furthermore, as in monogastric animals, Se is poorly transferred via placenta in ruminants (especially when sodium selenite is used as a feed additive), and newly born animals are usually Se deficient [1].

50.5 Se in Nutrition of Other Animals

50.5.1 Horses

The antioxidant-prooxidant balance in horses plays an important role in their health maintenance. Optimal levels of Se in equine diets are thought to increase horses' adaptability to high intensity exercises. Low intakes of Se have been suggested to damage the development of horse embryos and fetuses as well as the viability of the

newborn foals, because of muscle diseases [1]. Similar to the maternal effect of Se in pigs and ruminants, antioxidant transfer via placenta in horses is restricted, and increased Se delivery via colostrum and milk is of major nutritional and physiological importance [1]. Parenteral supplementation trials with mares at late gestation indicate that only limited amounts of Se cross the placental barrier when sodium selenite is used. Therefore, parenteral organic Se supplementation of mares during gestation and lactation as well as supplementation of foals beginning at birth are essential procedures to increase blood Se levels in foals to optimal levels. In the long term, optimal Se and vitamin E supplementation are thought to improve the performance of racing horses as well as keeping them healthy during exercise and periods of recovery and rest. As with other animal species, optimal forms of Se supplementation are key to maintaining optimal Se status of horses.

50.5.2 Se in Domestic Cats

Dietary antioxidants play a crucial role in the health of companion animals and are a major protective mechanism for animals exposed to elevated levels of toxins and free radicals. Kitten Se requirement was recently estimated to be 0.15 mg Se/kg diet [1] and it is recommended that commercial diets for cats contain 0.5 mg Se/kg feed. In many cases, the effects of antioxidants on companion animals are very similar to those described for humans, since they share common environments, stressors and in some cases diseases [1]. Therefore, dietary supplementation with various antioxidant compounds, including Se in an organic form, is an effective strategy to prevent the damaging effects of free radicals on companion animals.

50.6 Organic Se Concept in Animal Nutrition

A great deal of studies over the last two decades have suggested that the dietary form of Se is a major determinant of its efficiency in meeting Se requirement. Indeed, there are two major Se sources for animals, namely inorganic Se (mainly selenite or selenate) and organic Se in the form of SeMet (mainly as Se-Yeast or SeMet preparations). In the forages, grains and oilseed meals that comprise the main part of the chicken and pig diets, Se is found in organic forms, mainly as SeMet [1]. However, the major commercial Se supplements in use for the last 50 years are selenite and selenate, which are inorganic forms of Se. The limitations of using inorganic Se in animal nutrition are well known: high toxicity, interactions with other minerals, low efficiency of transfer to milk, meat and eggs, and inability for Se reserves to be built and maintained in the body [1–3, 13, 15]. Furthermore, the prooxidant effect of the selenite ion is a great disadvantage. Thus, the use of sodium selenite in animal diets has been questioned [1–3, 13, 15, 16], and an organic form of Se (SeMet) may have important advantages that can be exploited commercially.

In general, organic Se has two main advantages over inorganic Se in animal nutrition. Firstly, organic Se allows animals to build Se reserves in tissues, mainly muscles, in the form of SeMet, which can be used in stress conditions to improve antioxidant defenses [1–3, 13, 15, 16]. Secondly, organic Se in the form of SeMet provides an effective transport of Se from dam to fetus and to the newly born animal via placenta, colostrum and milk [1, 3, 13, 15, 16]. The same is true for poultry production, where organic Se is more effectively transferred from feed to the egg and further to the developing embryo and newly-hatched chick. For example, compared with inorganic Se, organic sources of Se appear to better meet the needs of the modern, hyper-prolific sow for the reasons listed in Table 50.1. It seems as if the organic Se concept has found its way into the animal industry.

50.7 Organic Se Sources: a Re-evaluation

The chemical and physical properties of Se and sulphur are very similar, reflecting similar outer-valence-shell electronic configurations and atomic sizes [1]. Therefore, plants don't appear to distinguish between these two elements when synthesizing amino acids. As a result, they can synthesize SeMet instead of Met when sulphur is replaced by Se in the soil or in a growth medium. This biological feature was the basis for the development of the commercial technology of organic Se production from yeast. Subsequently, various commercial forms of Se-yeast found their way to the marketplace and proved to be effective sources of Se for poultry and animal production (for review see [1–3, 13, 15, 16, 24, 25]). In addition, the advantages and disadvantages of Se-yeast usage in poultry, boar and sow diets have been recently evaluated [2, 13, 15] and can be summarized as follows:

- Se-yeast contains SeMet as a major Se compound, comprising 50–70 % of total Se. Recently, a considerable amount of selenocysteine (Sec) was found in proteins of the yeast proteome despite the absence of the UGA codon, and 10–15 % of Se present in Se-enriched yeast was established to be in the form of Sec. However, dietary Sec, like sodium selenite, is not effective in increasing Se tissue concentration in monogastric animals.
- SeMet proportions in Se-yeast are variable and with current technology it is difficult to guarantee the exact percentage of SeMet in the product.
- There are analytical difficulties in precisely determining SeMet concentrations in yeast-based products, and only a few labs worldwide can do such complex analysis.

Another option for improving the Se status of poultry and farm animals would be to use pure SeMet as a dietary supplement [26]. Some publications have demonstrated the beneficial effects of organic Se in the form of SeMet in pig and poultry diets (for review [2, 13, 15]). The main problem with purified SeMet is its stability, since it is easily oxidized. For example, SeMet was the major compound identified in Se-yeast, while SeMet selenoxide was its main degradation product formed after medium and long-term sample storage, respectively. Therefore, in commercial feed

Table 50.1 Advances of organic Se in sows' nutrition [15]

Parameter	Effect of organic *vs*. inorganic Se
Sow	
Se status and antioxidant status of sows	Improved
Se concentration in sow's whole blood	Improved
Se levels in the liver, loin and pancreas of the sows	Improved
Se concentration in sow's serum	Improved
Se content in colostrum and milk	Improved
Antioxidant status of colostrum and milk	Improved
Uterine transfer of Se	Improved
Development of pig embryos	Enhanced
Newly-born piglets	
Se content in loin and liver in the newborn piglets	Improved
Se content in heart in the newborn piglets	Improved
Se concentration in serum in the newborn piglets	Improved
Se in fetal body, fetal liver and maternal liver	Improved
Se levels in the liver and loin of the neonate pigs	Improved
Se levels in the liver and loin of the neonate pigs	Improved
Se transfer via placenta and Se amount in a whole newly born piglet	Improved
Weaned piglets	
Se concentration in the whole blood of piglets at the age of 3 and 14 days	Improved
Se concentration in piglet loin at weaning	Improved
Se concentration in piglet whole blood at weaning	Improved
Se retention in body and liver of progeny piglets during 21-day period	Improved
Se content in serum, liver, kidney, pancreas, muscle, thymus and thyroid gland in weaned pigs at day 28	Improved
GPx activity in serum, liver, kidney, pancreas and muscle in weaned pigs at day 28	Improved
Total antioxidant activity (AOA) in liver, kidney, pancreas, muscle and serum in weaned pigs at day 28	Improved
SOD activity in pancreas, muscle and serum in weaned pigs at day 28	Improved
MDA levels in liver, kidney and pancreas in weaned pigs at day 28	Decreased
Triiodothyronine (T3) concentration in the piglet serum at day 28	Increased
Thyroxine (T4) concentration in the piglet serum at day 28	Decreased
Protease, amylase, and lipase activities in the pancreatic tissue of offspring piglets at day 28	Increased
Weaning litter weight and average weight of progeny piglets	Increased
Daily weight gain of piglet from birth to weaning	Increased
Toxicity for pigs	Less toxic
Se excretion	Decreased

production, heat treatment, transportation and storage, SeMet stability could be a limiting factor of its effective use.

Recently, a new stable organic Se-containing compound, hydroxy-selenomethionine (OH-SeMet), has been developed. Various experiments with broiler chickens, laying

hens and piglets have compared the effect of OH-SeMet with selenite and Se-yeast [27–30]. The results clearly demonstrated that OH-SeMet fed in the same dosage as Se-yeast (normalized to Se content) significantly improved Se status of growing chicks and laying hens, with improved Se transfer to eggs. The same was shown for pigs: regardless of the Se level, the Se deposition in muscle was significantly greater in pigs supplemented with OH-SeMet than those supplemented with Se-yeast. A slope ratio assay revealed that the relative bioavailability of Se from OH-SeMet for plasma, liver and muscle was 170 %, 141 % and 162 %, respectively, in comparison to Se-yeast. More interestingly, this new source of organic Se was able to enhance the Sec content of tissues (muscles, eggs, milk), thereby supporting enhanced levels of selenoproteins and antioxidant potential [27].

50.8 Concluding Remarks

Se is an essential element of animal/poultry nutrition, and its dietary supplementation in optimal form and amount is key for maintaining animal health as well as productive and reproductive performance. The organic form of Se (mainly SeMet) in the animal/poultry diet has been demonstrated to have a range of advantages in comparison to traditional sodium selenite. It should also be mentioned that in the last decade, there has been significant progress in characterizing selenoproteins and in understanding their physiological functions [31]. Substantial progress has recently been made in selenoprotein studies of poultry and pig tissues. In particular, the mRNA levels of 25 selenoprotein genes in birds, pigs and cows have been identified [32]. Furthermore, 25 selenoprotein genes were studied in broiler chicken tissues [33], and, via selenoprotein expression, Se was found to be involved in regulation of cell growth, apoptosis and modifying the action of cell signalling systems and transcription factors. Enhancing Sec *de novo* synthesis in muscle tissues helped to improve meat stability during storage through reduced lipid peroxidation and protein oxidation [34]. Therefore, adequate dietary Se supply is a crucial factor in many physiological processes. The maternal effect of Se in poultry and animal nutrition [2, 15] deserves more attention. The epigenetic effects of Se [35] and their possible implications for animal health also await further investigation.

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