Modulation of the cellular redox state by distinct selenocompounds and their effects on cell signaling processes

Dissertation

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Known. Some. Call. Is. Air. Am.
Incoherent? Yes. Entirely without meaning? I´m afraid not.
(Mark Z. Danielewski, House of Leaves)

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List of abbreviations

3´UTR	3´untranslated region
AKT	Protein kinase B
AP-1	Activator protein 1
APM	Antigen processing and presentation machinery
ARE	Antioxidant response element
ATP	Adenosine triphosphate
BSO	Buthionine sulphoximine
CLCA	Calcium activated chloride channel regulator 1
CTL	Cytotoxic T lymphocytes
Cys	Cysteine
DMDSe	Dimethyldiselenide
DMSe	Dimethylselenide
ER	Endoplasmic reticulum
FAS	Fatty acid synthase
FOXO	Forkhead box protein
GCL	Glutamate cysteine ligase
GIT	Gastrointestinal tract
GLRX	Glutaredoxin
GPX	Glutathione peroxidase
GPE1	Glutathione *S*-transferase enhancer 1
GR	Glutathione reductase
GSH	Glutathione
GSK3β	Glycogen synthase kinase-3 beta
GSSeSG	Selenodiglutathione
GSSG	Glutathione disulfide (oxidized glutathione)
GST	Glutathione *S*-transferase
H_2O_2	Hydrogen peroxide
H_2Se, HSe^-	Hydrogen selenide
Hemox	Heme oxygenase
IFNγ	Interferon-gamma
IRE	Insulin response element
JAK	Janus kinase
JNK	c-Jun *N*-terminal kinase
Keap1	Kelch-like ECH-associated protein 1
KO	Knockout

LMP	Low molecular weight protein
$MeSe^-$	Methylselenol
Met	Methionine
MHC	Major histocompatibility complex
MSA	Methylseleninic acid
MSC	Methylselenocysteine
NADPH	Nicotinamide adenine dinucleotide phosphate
NKG2D	Natural killer group member D
NPC	Nutritional Prevention of Cancer
NQO1	NAPH dehydrogenase 1
Nrf2	Nuclear factor erythroid 2-related factor 2
OE	Overexpression
p53	Cellular tumor antigen p53
PIIE	Phase II enzymes
PTEN	Phosphate and tensin homolog
PTP1B	Protein tyrosine phosphatase 1B
ROS	Reactive oxygen species
RSSeSG	Selenotrisulfide
SAM	*S*-Adenosylmethionine
SBP2	Selenium-binding protein 2
Se	Selenium
Sec	Selenocysteine
SECIS	Selenocysteine insertion sequence
SELECT	Selenium and Vitamin E Prevention trial
SeMet	Selenomethionine
STAT	Signal transducer and activator of transcription
T2D	Type II Diabetes
TAP	Transporter-associated with antigen processing
TG	Triglyceride
$TMSe^+$	Trimethylselenonium ion
TRE	12-*O*-tetradecanoylphorpbol-β-acetate response element
TXN	Thioredoxin
TXNRD	Thioredoxin reductase
wnt	Wingless/integrated
WT	Wild-type

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1 Introduction

1.1 It´s essential! The history of selenium

In 1817 the Swedish chemist Jöns Jacob Freiherr von Berzelius (1779 – 1848) discovered the element selenium (Se) in a factory that produced sulfuric acid. With the techniques he had, Berzelius characterized the novel element and found that it exhibits similarities to both, sulfur and tellurium[1]. It took almost one and half centuries until Se became recognized as an element performing biological functions. In 1954 Pinsent et al. reported that trace amounts of Se are required for full activity of formic dehydrogenases in coliform organisms[2]. Only three years later two research groups independently found that Se plays also biological roles in mammals[3,4]. The observation that rats deficient in Se and Vitamin E died from necrotic liver degeneration provided the first evidence of Se essentiality[5]. Furthermore, the authors concluded that Se is involved in oxidation-reduction reactions[5]. Together, these findings promoted the discovery of Se-dependent, species-specific diseases, and injuries of organs, including exudative diathesis in poultry[3,4], mulberry heart disease in pigs[6], white muscle disease in calves and lambs[7,8], and male infertility in various mammalian species[9,10]. In humans, Se deficiency has been associated with Keshan disease, a cardiomyopathy, and Kashin-Beck disease, a chronic osteochondropathy, both found in areas of China where the Se content in soil is rather poor[11,12]. In 1973, the discovery that the cellular glutathione peroxidase 1 (GPX1) depends on Se provided a plausible explanation for Se-mediated protective impact on the above-mentioned diseases[13,14]. Since then, the family of selenoproteins increased significantly. Until today, 25 genes encoding for selenoproteins could be identified[15]. Some of the discovered selenoproteins were extensively investigated and functional classified, whereas others are still with unknown functions (see chapter **1.3.3** and **Table 1**).

Of all the benefits attributed to Se one received massive attention: Its potential anti-cancer properties. Epidemiological studies in the 1960s revealed higher human cancer death rates in Se-deficient regions of the United States[16]. Similarly, in 1985 Clark et al. reported geographical data on the Se content of food crops by U.S. counties showing an inverse correlation of forage plant Se levels with overall cancer motility[17]. These findings were supported by the first randomized controlled trial analyzing the effects of Se supplementation in patients suffering from skin carcinomas. Here, supplemental Se reduced the incidence of and mortality from carcinomas of several sites[18]. This study set off an extensive interest in researching the fascinating element Se and its impact on human health (**Figure 1**).

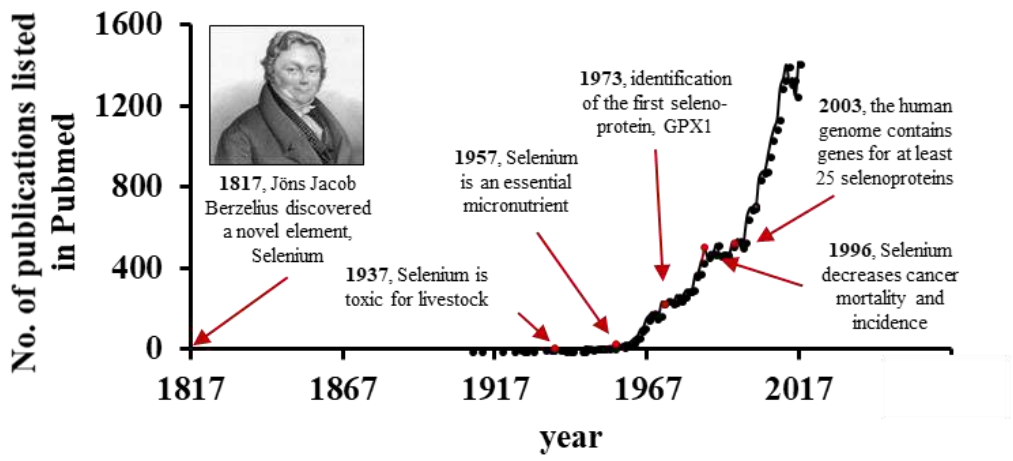

Figure 1: Selenium – from toxicity to essentiality.
While at the beginning of the 20th century Se was primarily considered as a toxin, the picture changed through the discovery of its essential character. Finally, its potential anti-carcinogenic properties led to a huge interest in researching Se (Source of Berzelius image: Wikipedia)

Until now the debate on Se and its association with cancer is ongoing. Meanwhile, observational studies and randomized controlled trials suggest conflicting/controversial results in humans[19]. Furthermore, studies showing negative effects of a high Se status, in particular, the enhanced risk to develop metabolic disorders[20], cannot be ignored. Besides a large number of studies focusing on the connection between Se and human health/diseases, the knowledge of the underlying mechanisms are scarce and still need to be clarified.

1.2 The metabolism of selenium – different forms, different ways

Se exists in different forms each with special properties. Both, organic and inorganic Se compounds play roles in human nutrition. The structures of Se compounds that were of particular interest for this thesis are represented in (**Figure 2**).

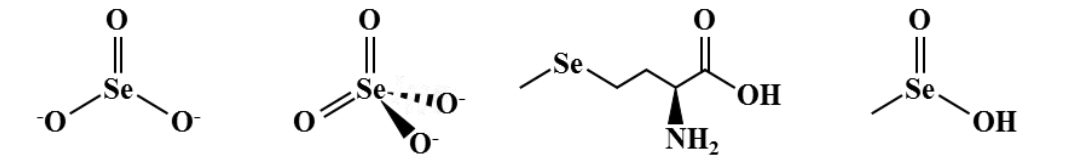

Figure 2: Chemical structures of Se compounds used in this thesis.

The organic derivatives selenocysteine (Sec), selenomethionine (SeMet) and methylselenocysteine (MSC) are found in food products of animal and plant origins, whereas sodium selenate and sodium selenite are inorganic and often used in supplements. Apart from these natural occurring Se compounds, a number of synthetics exist; such as methylseleninic acid (MSA) amongst others.

Intestinal absorption of Se and thus its bioavailability, as well as the intermediary metabolism of Se, are very distinct from one another and highly dependent on the respective Se compound (**Figure 3**). This is important to note as the biological activities of Se are mediated by intermediary formed metabolites[21]. Hydrogen selenide (H_2Se; HSe^- at physiological pH) is the metabolite formed by all Se compounds. HSe^- is directly generated from selenite via thiol-dependent reduction processes but could also be released from Sec or formed through demethylation of methylselenol. Furthermore, HSe^- is the key metabolite necessary for selenoprotein biosynthesis (see chapter: **1.3.1**).

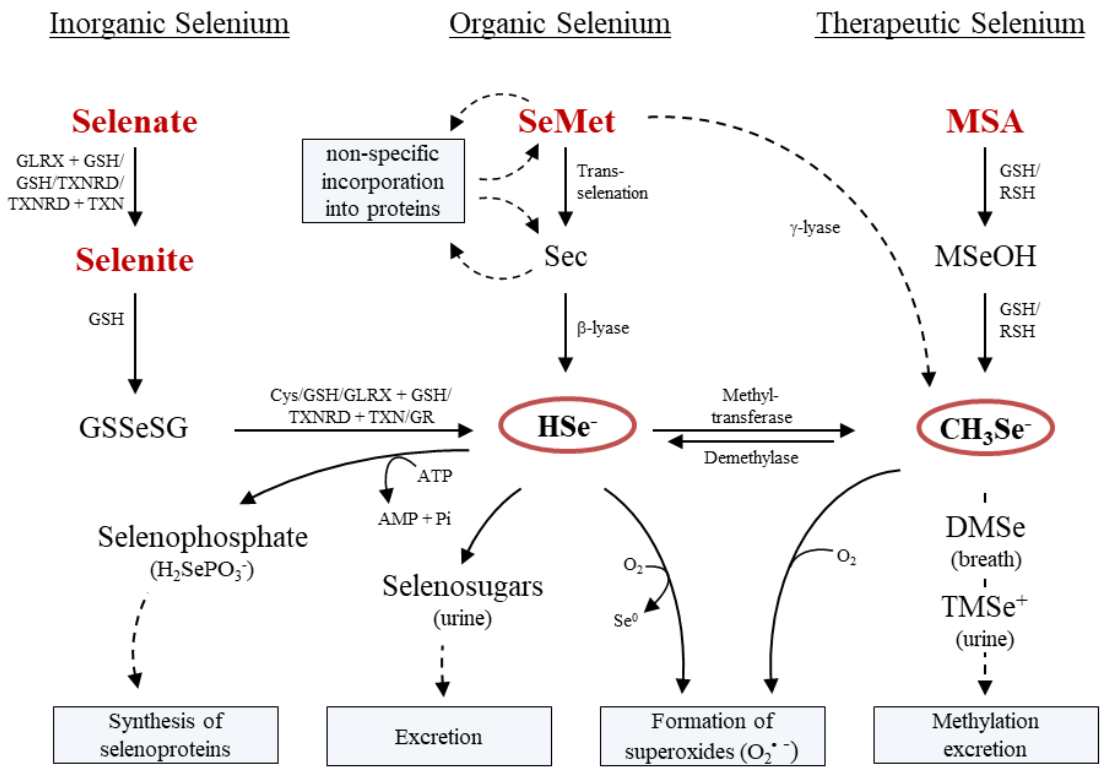

Figure 3: The metabolic pathways of distinct Se compounds.
Hydrogen selenide (HSe^-), the precursor for selenoprotein synthesis can be generated by all Se compounds but to different extents. The metabolite methylselenol (CH_3Se^-) is suggested to be the key metabolite mediating anti-cancer properties of Se. In the presence of oxygen, hydrogen selenide (HSe^-) and methylselenol (CH_3Se^-) generate superoxide anion radicals ($O_2^{\bullet -}$). Se can be excreted via kidneys and lungs. See text for more details.
Figure adapted and modified according to Fernandes et al.[22]
Cys, Cysteine; DMSe, Dimethylselenide; GSH, Glutathione; GR, Glutathione reductase; GLRX, Glutaredoxin; MSA, Methylseleninic acid; MSeOH, Methylselenenic acid; TXNRD, Sec, Selenocysteine; SeMet, Selenomethionine; Thioredoxin reductase; TXN, Thioredoxin; $TMSe^+$, Trimethylselenonium ion.

In the presence of oxygen extensive amounts of HSe^- can be harmful to cells by generation of superoxide anion radicals ($O_2^{\bullet-}$) and elemental Se (Se^0)[23]. For excretion of Se, two distinct pathways are available: **(i)** the selenosugar, and **(ii)** the methylation pathway. The biological significance of the first one is relatively uncertain, whereas the methylation pathway is considered as the main pathway to detoxify Se[24–26]. The resulting compounds methylselenol (CH_3Se^-, $MeSe^-$) and dimethylselenide (DMSe) are excreted via exhalation, whereas trimethylselenonium ions ($TMSe^+$) are the major Se metabolites found in urine[27–29]. The respective methylation reactions are enzyme-dependent and utilize methyl-groups provided by *S*-adenosylmethionine (SAM)[30]. Thus, a high consumption of SAM because of high Se intake could be a possible mechanism responsible for Se toxicity.

Insufficient Se intake results in rapid loss of selenoproteins with e.g. oxidoreductase properties. In contrast, Se oversupply might disrupt the cellular redox homeostasis via generation of superoxide anion radicals or the formation of thiol-reactive Se compounds[31]. These pro-oxidant effects differ depending on the Se compound used. Selenite and methylseleninic acid (MSA) are known to modulate the cellular redox state, whereas similar effects have not been attributed to e.g. SeMet.

1.2.1 The metabolism of selenate and selenite

The most intensively studied inorganic Se compounds include sodium selenite (oxidation state of Se: +IV) and sodium selenate (oxidation state of Se: +VI). Dietary selenate is absorbed within the gastrointestinal tract (GIT) by sharing a sodium-dependent transport system with sulfates, whereas selenite is primarily absorbed through passive diffusion[32–35]. Furthermore, selenate reaches peripheral tissues in an unmodified state[36]. In contrast, the highly reactive selenite reacts with thiols of proteins and peptides within the GIT leading to the formation of selenotrisulfides (RSSeSG) and selenodiglutathione (GSSeSG)[37]. Unlikely to oxidized glutathione (GSSG), GSSSeSG is an excellent substrate for the mammalian thioredoxin reductase (TXNRD)[38]. Thus, further reduction of GSSeSG via the TXNRD-Thioredoxin system and/or free thiols yields HSe^- (**Figure 3**). Selenate can be directly taken up into liver tissues, whereas selenite is rapidly absorbed by erythrocytes, reduced to HSe^- and transported bound to albumin to the liver[39].

1.2.2 The metabolism of selenomethionine

Selenomethionine (SeMet), the main Se compound in foods, is more readily absorbed within the small intestine when compared to selenate and selenite[40,41]. Intermediary, SeMet is

transformed to Sec via *trans*-selenation analogous to the pathway responsible for the formation of cysteine (Cys) from methionine (Met) (*trans*-sulfuration)[42,43]. The obtained Sec undergoes lysis catalyzed by the selenocysteine Se-conjugate β-lyase (β-lyase), resulting in HSe^- release[44,45]. Further, SeMet can be cleaved by the enzyme methionine-γ-lyase (γ-lyase) leading to the formation of methylselenol. However, to which extent this occurs in mammalian tissues needs to be clarified as at least in supernatants obtained from rat liver tissues β- but not γ-lyase activities could be detected[46]. Moreover, SeMet can be non-specifically incorporated into proteins substituting for its sulfur analog Met and thus stored until the respective proteins are degraded[47] (**Figure 3**). Herein, the extent of SeMet incorporation into proteins depends on its dietary dosage. Food supplements used in clinical trials contain about 90 % of Se in form of SeMet[41]. Met has been proposed to have beneficial effects on health since it serves as a Cys precursor. Cys, in turn, is essential for GSH synthesis and acts as a redox buffer under conditions of oxidative stress[48,49]. Recently it could be shown that SeMet exhibits peroxidase activities contributing to the rapid and efficient removal of cellular hydrogen peroxides (H_2O_2)[50].

Protein-bound Met can be easily oxidized by H_2O_2 to methionine-sulfoxides that are subsequently reduced by methionine-sulfoxide reductases (MSRB)[51]. Thus, Met functions as an H_2O_2 scavenger thereby protecting critical amino acid residues of the respective proteins against oxidation. Similarly, SeMet can be oxidized to the corresponding selenoxide but at a rate 10 to 1000-fold higher compared to the oxidation rate for Met[52]. Moreover, the reduction of oxidized SeMet proceeds non-enzymatically under consumption of GSH as the reductive equivalent[53,54]. Therefore, into proteins incorporated SeMet might be superior over its sulfur analog Met in scavenging biological radicals.

1.2.3 The metabolism of methylseleninic acid (MSA)

The synthetic Se compound MSA, initially generated to study the *in vitro* effects of Se, is a precursor of methylselenol ($MeSe^-$, CH_3Se^-) (**Figure 3**). $MeSe^-$ is considered as the metabolite mediating the anti-carcinogenic effects of Se[55,56]. Direct comparison of several Se compounds revealed that those directly entering the mono-methylated Se pool are more potent in reducing the tumor growth in mice compared to those that mainly yield HSe^-[57]. MSA is converted to $MeSe^-$ via non-enzymatic processes using reducing equivalents in form of cellular thiols, e.g. GSH. In addition, MSA can be processed catalyzed by enzymes[58,59]. In comparison to MSC that can be converted to $MeSe^-$ only in the presence of β-lyase, the bioavailability of MSA is far less efficient. Moreover, *in vivo* studies that compared the stability of MSC and MSA

revealed that in contrast to MSC MSA does not reach organs in an intact state. Thus, MSA is excellent for studying Se effects *in vitro* as not all cell lines exert β-lyase activities, whereas MSC is the best methylselenol source in most organs/tissues[58].

1.3 Selenium and selenoproteins

At the recommended level of ~70 µg per day, Se exhibits anti-oxidant, anti-inflammatory, and chemopreventive potentials. The physiological functions of Se are mediated by so-called functional selenoproteins that contain the 21st proteinogenic amino acid Sec within their primary protein structure[60]. The co-translational insertion of Sec during protein biosynthesis is far more complicated and complex as it is for the other 20 proteinogenic amino acids, e.g Cys.

1.3.1 Biosynthesis of selenoproteins

Proteins containing Sec are present in all three lines of evolution: eubacteria, archaea, and eukarya. Sec insertion into proteins is unique and has some unusual features compared to "normal" protein biosynthesis (**Figure 4**).

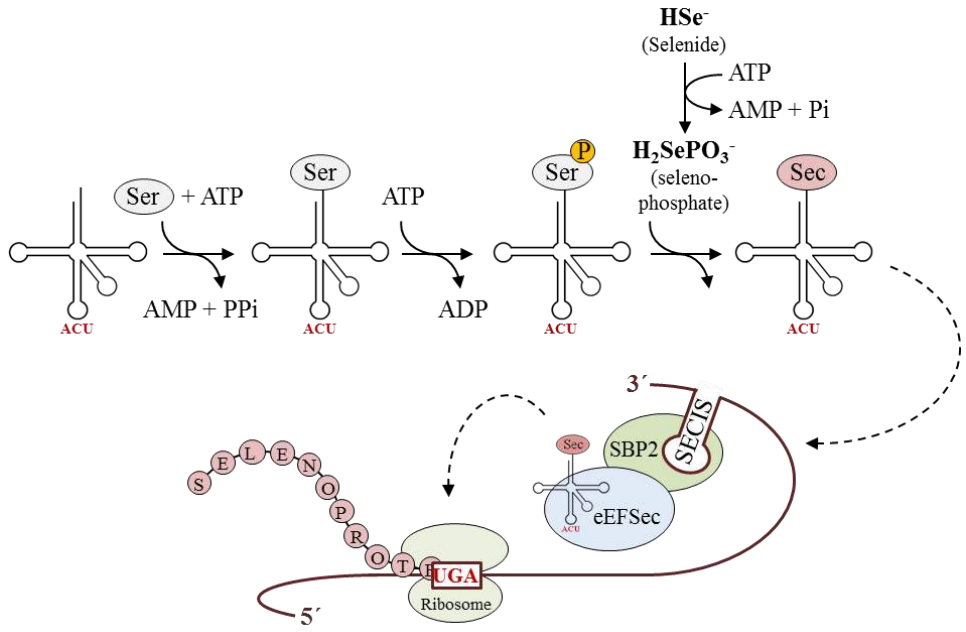

Figure 4: Schematic representation of selenoprotein biosynthesis.
First, tRNA[Ser]Sec is loaded with serine to form Ser-tRNA[Ser]Sec which is further transformed to O-phosphoseryl-tRNA[Ser]Sec. Next synthesis of Sec-tRNA[Ser]Sec is synthesized using selenophosphate which was formed under ATP consumption from selenide. To recode the UGA base triplet for Sec, *cis*-acting factors (SECIS), as well as *trans*-acting factors (SBP2, eEFSec), are essential. The complex composed of SBP2, eEFSec and Sec-tRNA[Ser]Sec is recruited to the ribosome.
Figure adapted and modified according to Brigelius-Flohé et al.[60]
SBP2, Selenium-binding protein 2; Sec, Selenocysteine; SECIS, Sec insertion element; Ser, Serine; eEFSec, Eukaryotic elongation factor, selenocysteine-tRNA-specific

Sec is encoded by the base triplet UGA that normally functions as a signal to terminate protein biosynthesis[61]. To recode the UGA to a sense codon numerous accessory proteins, as well as specific sequences in the 3´UTR region of genes encoding for selenoproteins, are necessary[62]. The so-called Sec Insertion Element (SECIS) is a hairpin structure essential for recruiting factors that enable recoding and insertion of Sec into the growing polypeptide chain[63]. In contrast to other amino acids, Sec is directly synthesized at its own tRNA ($tRNA^{[Ser]Sec}$). Initially, serine (Ser) is attached to this tRNA, phosphorylated and converted to Sec. The used Se source is selenophosphate ($H_2SePO_3^-$) generated from HSe^- under consumption of ATP. Thus, Sec taken up by foods cannot be directly used but needs to be reduced to HSe^- prior to selenoprotein biosynthesis (**Figure 3**). The synthesis of selenoproteins is substantially driven by the availability of $tRNA^{[Ser]Sec}$ and therefore, not surprisingly, dependent on the cellular Se status. This means that the mRNA levels of different selenoproteins only imperfectly reflect the actual protein abundance. In addition, the biosynthesis of selenoproteins is hierarchically organized. Thus, upon Se shortage, some selenoproteins are synthesized at normal rates, whereas the synthesis of others is clearly diminished. For instance, GPX1 ranking low in the hierarchy of selenoproteins is only scarcely synthesized during insufficient Se availability, whereas the synthesis of others, e.g. GPX2, GPX4, TRXNDs, and DIOs, is only influenced by long-term Se depletion suggesting a more pronounced importance of these enzymes[64,65].

1.3.2 Why selenocysteine instead of cysteine?

As early as 1817 Berzelius stated that the novel element Se is “midway between sulfur and tellurium and has almost more characters of sulfur than of tellurium”. In fact, sulfur and Se share similar physical and chemical characteristics[66]. Thus, if comparing general properties of both elements, functional advantages of Se over sulfur might not become immediately obvious. Both elements have similar outer valence-shell electronic configurations and atomic sizes. In addition, their ionization potentials, bond energies, and electron affinities are practically identical[66]. Se and sulfur share the same oxidation states. Furthermore, their structures and functional groups are so similar that analogous compounds often co-crystallize[66,67]. Given the similarities between cysteine (Cys) and Sec, the specific metabolic requirements necessary for selenoprotein synthesis, and the high expenses of ATP during Sec insertion into a protein (**Figure 4**), the question arises: What are the benefits of Se over sulfur? In biological systems, the biochemistry of Se and sulfur differs in at least two aspects. Sulfur-compounds are metabolized to more oxidized states, whereas Se compounds are

converted to more reduced states[68]. In addition, the weaker bond to hydrogen results in higher acidity of the selenolate (R-SeH, R-Se^-) versus the thiolate (R-SH, RS^-). Thus, at physiological pH, the sulfur atom of Cys is predominantly protonated, whereas the Se atom of Sec is mainly found in the selenolate status. This results in a higher nucleophilicity of Sec compared to Cys[67,68]. These distinctions between Se and sulfur might explain the observation that Se compounds are more active against tumors compared to their corresponding sulfur analogs[69]. Furthermore, replacing the Sec residues of various selenoproteins by Cys significantly reduced the catalytic activity of the respective proteins. Vice versa, replacement of Cys by Sec in Cys-containing enzymes strongly enhanced their enzymatic activities[70,71]. However, this is not the case for TXNRD. Here, Sec was not necessary for the functional activity as insertion of Cys instead of Sec did not result in altered enzymatic activities[72]. This observation led to the conclusion that besides the gain of catalytic activity the redox properties of Se by itself are related to Se advantages[72]. Several selenoproteins exhibit peroxidase activity and as Se is both, a good nucleophile and electrophile, it easily redox-cycles between reduced and oxidized states avoiding the risk of irreversible over-oxidation[67]. Thus, the inclusion of Sec instead of Cys in enzymes with peroxidase functions might be a mechanism to protect the respective enzymes from irreversible inactivation. This hypothesis is strengthened by the notion that Cys-mutants of GPX1 exhibits less enzymatic activity compared to the Sec-containing wild type-enzyme, but in addition, it is also readily inactivated in the presence of its substrate H_2O_2[67].

1.3.3 Sec-containing proteins and their physiological roles

To date, approximately 100 different selenoprotein families have been discovered. *Aureococcus anophagefferens,* a unicellular brown alga, exhibits with 59 selenoproteins the largest and most diverse selenoproteome[73]. Analysis of the human genome revealed the presence of 25 genes encoding for selenoproteins[15]. *In vivo* labeling of rats with ^{75}Se uncovered the existence of more than 25 selenium-containing proteins or protein subunits[74]. Most of the selenoenzymes exhibit peroxidase and/or oxidoreductase properties and are therefore necessary for maintaining the cellular redox homeostasis. Selenoproteins are directly or indirectly involved in the detoxification of H_2O_2, they provide assistance in repairing oxidized metabolites, are part of the protein folding machinery within the endoplasmic reticulum (ER), and are involved in degradation processes of misfolded proteins. Until now the best-characterized selenoproteins belong to the family of the glutathione peroxidases

(GPX), thioredoxin reductases (TXNRD) and deiodinases (DIO). An overview of selenoproteins and their functions is given in **Table 1.**

Table 1: Overview of mammalian selenoproteins and their functions.

Selenoprotein ***(Symbol/ synonyms)***	**Enzymatic activity**	**Function**	**Expression/ localization**
		Glutathione (GPX) peroxidase family	
Glutathione peroxidase 1 *(GPX1/ Cytosol GPX, GSHPX1)*	Glutathione:hydrogen-peroxide oxidoreductase[75]	Reduction of hydrogen peroxides, anti-oxidant[75]	Ubiquitous; cytosol[75]
Glutathione peroxidase 2 *(GPX2/ Gastrointestinal GPX, GSHPX-GI)*	Glutathione:hydrogen-peroxide oxidoreductase[76]	Reduction of hydrogen peroxides; anti-oxidant; mucosal homeostasis[76,77]	Gastrointestinal tract, cytosol[78]
Glutathione peroxidase 3 *(GPX3/ Plasma GPX)*	Glutathione:hydrogen-peroxide oxidoreductase[79]	Reduction of hydrogen peroxides; anti-oxidant[79,80]	Extracellular, plasma[81]
Glutathione peroxidase 4 *(GPX4/ Phospholipid hydroperoxide GPX, PHGPX)*	Glutathione:hydrogen-peroxide oxidoreductase, also accepts lipid hydrogen peroxides[82]	Reduction of lipid hydrogen peroxides; spermatogenesis; cerebral embryogenesis[82,83]	Ubiquitous, cytosol, mitochondria, nucleus[83]
Glutathione peroxidase 6 *(GPX6)*	Unknown	Unknown	Embryos, Olfactory epithelium[15]
		Thioredoxin reductase (TXNRD) family	
Thioredoxin reductase 1 *(TXNRD1/ TR1, TRXR1)*	Thioredoxin disulfide reductase; NADPH: disulfide oxidoreductase[84,85]	Reduction of oxidized thioredoxin, selenite, selenodiglutathione, hydro-peroxides; DNA synthesis; regulation of signaling pathways, e.g. ASK1, TXNIP, PTEN[80,86–88]	Ubiquitous, cytosol, nucleus[84,85]
Thioredoxin reductase 2 *(TXNRD2/ TR3, TRXR2)*	Thioredoxin disulfide reductase[89]	Reduction of oxidized thioredoxin[89]	Mitochondria[90]
Thioredoxin-glutathione reductase *(TXNRD3/ TR2, TRXR3, TGR)*	Thioredoxin glutathione disulfide reductase[91]	Reduction of thioredoxin and glutathione; sperm maturation[91,92]	Cytosol, endoplasmic reticulum, mitochondria, nucleus[91,92]
		Deiodinase family	
Iodothyronine deiodinase 1 *(DIO1/ D1)*	5´-deiodination of T4 and rT3[93,94]	Systemic thyroid hormone biosynthesis (conversion of T4 to bioactive T3; inactivation of T3)[93]	Liver, kidney, thyroid; plasma membrane, active center cytosolic[95]
Iodothyronine deiodinase 2 *(DIO2/ D2)*	5´-deiodination of T4[93,94]	Local thyroid hormone biosynthesis (conversion of T4 to bioactive T3)[95]	CNS, brain, thyroid, bone, adipose tissue, skeletal muscle, heart; endoplasmic reticulum, active center cytosol[95]
Iodothyronine deiodinase 3 *(DIO3/ D3)*	Deiodination of the inner tyrosyl-ring of T4[93–95]	Inactivation of T4 and T3 (formation of rT3)[95]	CNS, brain, placenta, uterus, skin; plasma membrane, cell surface[95]
		Putative oxidoreductases	
Selenoprotein H *(SELENOH/ SELH)*	Unknown	Mitochondrial biogenesis; tumor suppressor functions; regulation of redox homeostasis; induction of phase II enzymes[96,97]	Ubiquitous; nucleus
Selenoprotein M *(SELENOM/ SELM, SEPM)*	Unknown	Interaction partner of Galectin-1; cytosol calcium regulation; neuroprotection[98,99]	Brain; endoplasmic reticulum, golgi apparatus[100]
Selenoprotein O *(SELENOO/ SELO)*	Unknown	Mitochondrial redox regulation[101]	Ubiquitous; mitochondria[101]

Selenoprotein ***(Symbol/ synonyms)***	**Enzymatic activity**	**Function**	**Expression/ localization**
Selenoprotein T *(SELENOT/ SELT)*	Unknown	Intracellular calcium regulation; control of glucose homeostasis, insulin secretion, neuroprotection[102–104]	Highly induced in nervous, endocrine and metabolic tissues; endoplasmic reticulum membrane[104,105]
Selenoprotein V *(SELENOV/ SELV)*	Unknown	Interacts with OGT; spermatogenesis[106]	Testis[106]
Selenoprotein W *(SELENOW/ SELW)*	Unknown	Glutathione-dependent antioxidant; involved in muscle growth and differentiation; involved in cell immunity[107–109]	Ubiquitous, highly expressed in muscle, heart, spleen, and brain; cytosol[109]
		Others	
Selenoprotein I *(SELENOI/ SELI)*	Ethanolaminphospho-transferase[110]	Biosynthesis of phospholipid phosphatidylethanolamine, formation and maintenance of vesicular membranes[110]	Ubiquitously, highly expressed in the cerebellum; endoplasmic reticulum[110]
Selenoprotein K *(SELENOK/ SELK)*	Palmitoylation of CD36 and the Calcium channel protein IP3R[111,112]	Regulation of calcium-mediated activation of immune cells; ER stress-induced apoptosis protection; Association to the ERAD complex[112–115]	Ubiquitous, high expression in immune cells; endoplasmic reticulum[114]
Selenoprotein N *(SELENON / SELN)*	Unknown	Redox regulation; regulation of ATP2A2 activity; regulation of intracellular calcium homeostasis, embryonic muscle development[116–118]	Ubiquitous; endoplasmic reticulum[116,119]
Selenoprotein S *(SELENOS/ SELS, SEPS1)*	Unknown	Involved in ERAD mediated degradation of C99; cooperation partner of SELNOK; peroxidase activity[120–122]	Endoplasmic reticulum[122]
Selenoprotein F *(SELENOF/ selenoprotein 15, SEP15)*	Thiol-disulfide oxidoreductase[123]	Quality control of protein folding in ER[124,125]	Endoplasmic reticulum[125]
Selenoprotein P *(SELENOP/ SeP, SELP, SEPP)*	Oxidoreductase[126]	Selenium transport in plasma; anti-oxidant activity[126,127]	Extracellular[126]
Methionine sulfoxide reductase B1 *(MSRB1/ SELR, SELX, SEPX1)*	Peptide methionine-*R*-sulfoxide reductase	Reduction of methionine (R)-sulfoxide back to methionine; regulation of protein activity by PTMs; role in innate immunity by reducing oxidized actin[128–130]	Cytoskeleton, Cytoplasm, Nucleus[129,131]
Selenophosphate synthetase *(SEPHS2 / SPS2)*	Synthesis of selenophosphate from selenide and ATP[132]	Biosynthesis of selenocysteine necessary for selenoprotein synthesis[132]	Cytosol[132]

1.3.3.1 The glutathione peroxidase family

In mammals, eight different glutathione peroxidases (GPX1 – 8) exist, whereas only five are selenium-dependent (GPX1, GPX2, GPX3, GPX4, GPX6; **Table 1**). The remaining three contain a conserved Cys instead of Sec. With the exception of GPX4, that is a monomeric enzyme, all others are homotetrameric[133–135].

The Sec residues of Se-containing GPXs are located *N*-terminal within a conserved catalytic triad, Sec-Gln-Trp[136]. GPXs exhibit a wide range of functions and depending on the respective isoform they are involved in detoxification of hydrogen peroxides, lipid peroxides or other organic peroxides. Thereby, the Sec residue is oxidized to a seleneninic acid intermediate (-Se-OH), that is reduced back at the expense of one molecule GSH. The formed

glutathionylated selenol (-Se-SG) is further reduced using a second GSH molecule, thereby restoring the enzyme´s Sec under production of GSSG. GSSG is reduced by the NADPH dependent enzyme glutathione reductase (GR)[137] (**Figure 5**). In case of GPX3, glutaredoxin (GLRX) or thioredoxin (TXN) can also be used as reducing equivalents. In addition, GPX4 oxidizes protein thiols during its catalytic cycle[138,139].

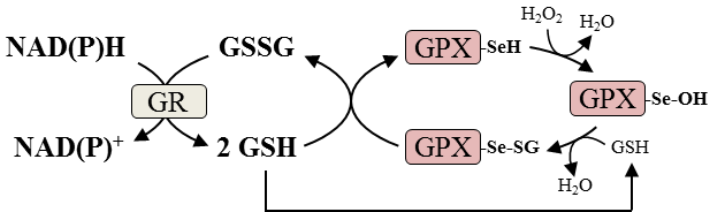

Figure 5: Reduction of H_2O_2 mediated by the GPX/GSH pathway.
In a first step, the selenol of Sec is oxidized to seleninic acid by H_2O_2 that is reduced back under consumption of 2 GSH molecules. The formed GSSG is recovered to 2 GSH by the NAD(P)H-Glutathione-GR-system.
Figure adapted and modified according to Bindoli et al.[137]
GPX, Glutathione peroxidase; GR, Glutathione reductase; GSH, Glutathione; GSSG, Glutathione disulfide.

Besides taking a role in the defense against oxidative damage and maintaining the cellular redox status, GPXs are indirectly involved in cellular redox signaling processes. H_2O_2 is considered to oxidize and thereby modulate the activity of several proteins, e.g. protein tyrosine phosphatase 1B (PTP1B) or the dual specific phosphatase PTEN, involved in the regulation of cellular metabolic pathways. Thus it is conceivable that high or low GPX expression, respectively, influences the activity of these enzymes by altering cellular H_2O_2 levels[140].

Taken together, GPXs play decisive roles in preventing the organism against oxidative damage, maintaining the cellular redox homeostasis, and regulation of redox signaling processes.

1.3.3.2 Glutathione peroxidase 2

Due to its high rank within the family of glutathione peroxidases (see chapter: **1.3.1**), GPX2 is thought to fulfill important functions besides detoxification of H_2O_2. One major part of the current work was specifically focused on this selenoprotein and is therefore described in more detail.

GPX2 is a selenoprotein highly enriched within the gastrointestinal tract (GIT) suggesting a critical role in preventing the GIT from damage mediated by reactive oxygen species (ROS)[141]. The expression of GPX2 is influenced by Se bioavailability. Furthermore, GPX2 is regulated by several transcription factors, e.g. the anti-oxidant response transcription factor

Nuclear factor (erythroid-derived 2)-like 2 (Nrf2)[142], supporting the role of GPX2 in maintaining the cellular redox homeostasis. Indeed, GPX1 and GPX2 double KO mice are highly susceptible to bacteria associated inflammation and cancer. This indicates an important role of oxidative stress in promoting colonic pathology and inflammation that can lead to the development of cancerous diseases[143]. Low expression of GPX2 is associated with advanced tumor status and an unfavorable clinical outcome of patients suffering from urothelial carcinomas[144]. In addition, GPX2 deficiency predisposed mice to UV-induced cutaneous squamous cell carcinomas (CSCC), indicating a role of this selenoprotein in cancer prevention[145]. Contradictory to studies showing a role of GPX2 in the prevention of cancer development, several studies indicate a pro-carcinogenic role of this selenoprotein. GPX2 was found to be highly expressed in different types of epithelium-derived tumors, including colorectal adenomas, colorectal cancers (CRCs), and mammary carcinomas[142]. Furthermore, clinical analyses have shown that GPX2 levels positively correlate with the differentiation and proliferation status in human colon tumor cohorts[146,147]. Moreover, high GPX2 expression levels were associated with early colorectal carcinoma recurrence[146] and were contributed to the poor prognosis of patients with hepatocellular carcinomas[148] as well as gastric cancers[147]. In addition, a significant association between GPX2 expression levels, lymph node (LN) metastasis, and vascular invasion was observed. Therefore, it could be speculated that GPX2 protects metastasizing tumors from oxidative damage by lowering intracellular H_2O_2 levels. In concert, GPX2 depleted tumor cells exhibited significantly higher ROS levels and were more sensitive to H_2O_2 induced cell death[146]. Moreover, GPX2 knockdown in rats and human cancer lines, respectively, resulted in growth inhibition[149] and induction of apoptosis[150]. Together, GPX2 plays a pivotal role regarding cancer development. Whether GPX2 has a protective and/or promoting role in tumor development remains an open question[142,151].

Within the GIT epithelium, GPX2 is mainly expressed in the crypt bases, where also stem cells are located, and gradually declines to the top of the crypts or villi[77]. Stem cells of the GIT continuously differentiate into two distinct cell lineages, the absorptive and the secretory one. Whereas enterocytes are adapted for metabolic and digestive functions, goblet cells and Paneth cells are specialized for maintaining barrier functions and supporting innate immunity of the epithelium. Enteroendocrine cells are hormone-secreting cells and represent a link between the neuroendocrine and digestive system[152]. The Wnt signaling pathway controls genes that are necessary for proliferation and differentiation processes of stem cells[153]. Interestingly, GPX2 is regulated via Wnt signals pointing out a role for GPX2 in continuous self-renewal of the intestinal epithelium[154]. Given that the Wnt pathway is constitutively

active in several colon cancer cells, the support of proliferation in these cells by GPX2 cannot be rated as anti-carcinogenic[77]. In addition, GPX2 contains binding sites for the transcription factor ΔNp63[150], a negative regulator of p53[155]. This supports the hypothesis that GPX2 might play pro-carcinogenic roles.

So far, the roles of GPX2 in self-renewal of the intestinal epithelium and therefore in maintaining mucosal homeostasis have not been sufficiently investigated. Recently *in vitro* analyses showed that GPX2 depleted tumor cells exhibited a stem cell-like phenotype[146]. In contrast, tumor cells displaying GPX2-mediated stress resistance exhibited similar characteristics to those of cancer stem cells, including vigorous proliferation, aggressiveness, and naïve differentiation[147].

1.4 Selenium and non-selenium proteins

Several studies demonstrated that the expression of several non-selenium proteins can be influenced by Se availability. The proteins upregulated under marginal Se levels include a large number of anti-oxidant and phase II enzymes (PIIE) coming along with a so-called Antioxidant Response Element (ARE) in their promoter regions[156,157]. The transcription factor Nrf2 has been recognized to transduce gene activation of PIIE as well as enzymes with antioxidant functions[158,159] by binding to the ARE in the promoter region of the respective genes[160,161]. Under basal conditions, Nrf2 is associated with the redox-sensitive Kelch-like ECH-Associated Protein 1 (Keap1). This complex inhibits Nrf2 action and leads to its ubiquitination and proteasomal degradation. ARE activation signals, e.g. oxidants, disrupt the Keap1-Nrf2-complex via the modulation/oxidation of redox-sensitive Cys residues of Keap1, thus leading to conformational changes of Keap1 and subsequent release of Nrf2 out of the inhibitory boundary. Further, active Nrf2 translocates into the nucleus and induces the transcription of ARE-containing enzymes[162] (**Figure 6**). Thus, manipulating the cellular redox-state through modulating Se availability significantly influences the adaptive response of Nrf2-regulated genes[163]. Besides two selenoproteins, GPX2 and TXNRD1, several other proteins are affected by Nrf2 activity, including glutathione *S*-transferases (GSTs) and NAD(P)H dehydrogenase [quinone] 1 (NQO1). Furthermore, proteins carrying foreign substances and their metabolites, as well as key enzymes of GSH biosynthesis (e.g. GCL, GR), are regulated via the Nrf2/ARE pathway[164]. GSH functions as the co-substrate for GPXs and is therefore indispensable for GPXs activity (**Figure 5**). Furthermore, GSH influences together with glutaredoxins (GLRX) the functions/activities of proteins by altering their

cysteine-redox state via reversible *S*-glutathionylation. Thus, GSH plays a role in redox-signaling processes[165].

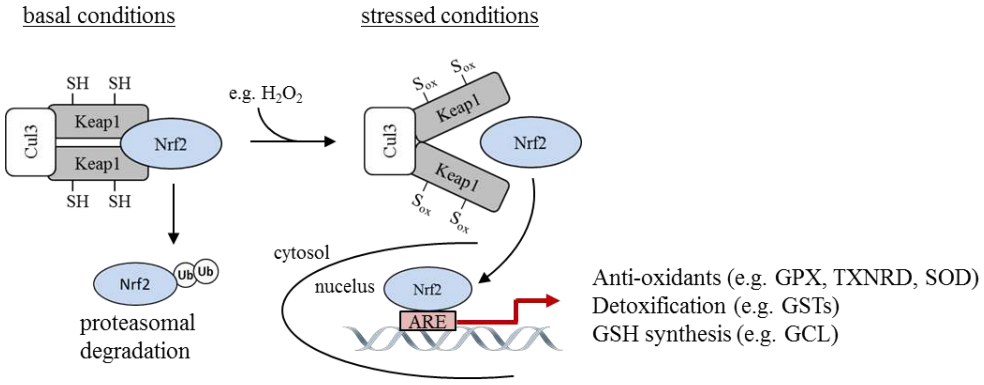

Figure 6: The Nrf2 pathway.
Under basal conditions, Nrf2 is bound to Keap1 and marked for proteasomal degradation via ubiquitination (Ub). In the presence of high H_2O_2 levels or electrophiles, Keap1 is redox-modified at redox-sensitive cysteine residues leading to changes in its conformation. Thus, Nrf2 is released from its inhibitory boundary to Keap1, translocates into the nucleus and induces the transcription of genes containing an ARE within their promoter regions. Figure adapted and modified according to Lennicke et al.[162].
ARE, Anti-oxidant response element; CUL3, Cullin 3; GCL, Glutamate cysteine ligase; GPX, Glutathione peroxidase; GSH, glutathione; GST, Glutathione *S*-transferase; Keap1, Kelch-like ECH-associated protein 1; Nrf2, Nuclear factor erythroid 2-related factor 2; SOD, Superoxide dismutase; TXNRD, Thioredoxin reductase; Ub, Ubiquitin.

As increased Nrf2 activity enhances the expression of components of the pentose phosphate pathway (PPP) that generates NAD(P)H equivalents necessary for the GSH-based anti-oxidant system, the energy metabolism is interconnected with the redox homeostasis[166]. This is in accordance with the observation that combined inhibition of glycolysis, the PPP, and thioredoxin increases oxidative stress and cytotoxicity in tumor cell lines[167]. Furthermore, Nrf2 knockdown suppressed tumor growth, inhibited cell proliferation and promoted apoptosis[168,169]. In contrast, Nrf2 played a critical role in protecting against colitis/inflammation-associated neoplastic transformation. Herein, Nrf2 knockout mice had significantly greater incidence, and colorectal tumor sizes compared to their WT counterparts[170]. High levels of ROS have been identified as a possible mechanism in the influence of inflammation on cancer development. In line with this, Nrf2-deficient mice were more susceptible to DSS-induced colitis[171]. These data indicate that Nrf2 may play a role in the regulation of inflammation as Nrf2 mediates the induction of anti-oxidant response enzymes. Thus, Nrf2 might be critical in the prevention of inflammation-triggered development of cancers, whereas established tumors might benefit from high Nrf2 levels. Given that several cancers exhibit high Nrf2 levels associated with enhanced tumor

progression and chemotherapy resistance, whereas the lack of Nrf2 has opposite effects, Nrf2 represents a promising target for cancer therapies[172].

1.5 Selenium, selenoproteins and human health

Adequate Se supply is necessary for proper function of selenoproteins, the normal function of the immune system as well as the thyroid metabolism and is therefore indispensable for human health. In humans, a Se undersupply is associated with loss of immune competence, male infertility, an increased susceptibility to viral infections, as well as an increased risk to develop cancerous diseases[173–175]. Good Se sources include seafood, meat, cereals, and Brazil nuts. However, Se concentrations in plants, and therefore meat, are highly dependent on the Se content in the soil. Consequently, Se intake of humans varies widely depending on the area and furthermore on the bioavailability of different Se compounds[176] (see chapter: **1.2**). According to the D-A-CH (D, Germany; A, Austria; CH, Switzerland) society, reference values for adequate Se supply were calculated from persons living in regions of China that are poor in Se. There, approximately 1 µg Se per kg body weight each day was necessary to maximize the plasma levels of selenoprotein P (SELENOP). Using the calculated reference body weights of European humans (women 60 kg, men 70 kg), the resulting estimated reference values for the daily Se intake are 60 µg for women and 70 µg for men[177]. On average, the actual Se intake in Europe is around 40 µg per day and thus only suboptimal, whereas with 93 to 134 µg Se per day the Se status is higher in the U.S.A.[174]. Even severe Se deficiency is rare in humans, the intake of Se-containing supplements (10 to 200 µg Se/day) is popular in European countries and the U.S. due to its potential health benefits[178]. However, the dose range for Se that is health beneficial is narrow and follows a U-shaped curve. Thus, Se intake above the recommended level might exert adverse side-effects even below the "tolerable upper intake level" of 300 to 450 µg Se per day[178,179]. Together, Se is a trace element with a tight therapeutic window resulting in a balancing act between essentiality and toxicity (**Figure 7**).

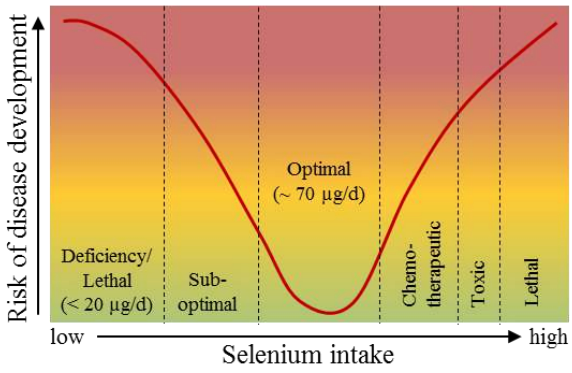

Figure 7: Dose-dependent health effects of Se.
For details see text. Figure adapted and modified according to Fernandes et al.[22].

1.5.1 The bad side – The selenium and diabetes story

Type II Diabetes (T2D) is the most common form of Diabetes and caused by a dysfunctional insulin secretion and peripheral insulin resistance resulting in hyperglycemia[180]. Oxidative stress is highly associated with complications and the pathogenesis of T2D[181,182]. As part of anti-oxidant selenoproteins, Se counteracts oxidative stress. Therefore, Se might be protective against the development of T2D but the relationship between Se and T2D is highly complex and the existing literature contradictory. On the one hand, over-expression of GPX1 in pancreatic islets protects β-cells, stimulates pancreatic β-cell gene expression and improves insulin secretion[183,184]. On the other hand, high Se doses might counteract peripheral insulin signaling, which is critical for proper regulation of glucose homeostasis[140,185]. Thus, several studies were conducted to analyze the effects of Se levels on T2D but the results are conflicting: A few case-control-studies proposed an inverse relationship between the Se status and the prevalence of diabetes[186–189]. For instance, a longitudinal study conducted in the U.S.A. reported higher Se levels in toenails to be associated with a lower risk for T2D[186]. In contrast, various animal and human studies provided evidence that a long-term Se oversupply might increase the risk to develop metabolic disorders[190–193]. Two cross-sectional studies, National Health and Nutrition Examination Survey (NHANES) III[192] and NHANES 2003-2004[194] reported a positive correlation between high Se intake and T2D. Furthermore, a secondary analysis of the NPC study, a placebo-controlled randomized trial designed to investigate the effects of Se oversupply (200 µg in form of Se yeast per day) in preventing skin cancer, revealed an increased T2D risk by high Se intake[191]. Furthermore, a positive but non-significant association between Se supplementation (200 µg SeMet per day) and T2D was found in the Selenium and Vitamin E Cancer Prevention Trial (SELECT)[193,195]. However, no correlations were reported by other epidemiological studies and clinical trials[182,196–199]. Although a recently published Cochrane Review reflected the inconsistency of trial results[200]. In addition to the effects of Se supply on T2D, the above mentioned NHANES III and NHANES 2003-2004 studies analyzed the relationship between Se and hyperlipidemia and reported that subjects with highest Se levels in plasma also exhibited highest plasmatic levels of triglycerides (TG). Moreover, total cholesterol levels, as well as levels of High-Density Lipoprotein (HDL) cholesterol and Low-Density Lipoprotein (LDL) cholesterol correlated positively with serum Se levels[192,194]. Similar to T2D data, the available studies regarding Se and hyperlipidemia are rather inconsistent. Whereas two investigations found no effect of Se supplementation[201,202], others could show that intake of 100 µg and 200 µg Se per day,

respectively, in form of Se-yeast decreases total as well as non-HDL cholesterol levels in plasma[203].

In this context, the indiscriminate use of Se supplements in individuals and populations with adequate-to-high Se intake cannot be justified. Although there seems to be a link between selenoproteins and glucose metabolism, the observed relationship between Se and T2D based on clinical trials is more complex and needs more mechanistic studies.

1.5.2 Selenium and cancer – a long-lasting discussion

Since 1969 the discussion about the role of Se in cancer prevention and or therapy is ongoing. Several prospective studies indicate a positive correlation between Se intake and Se status, respectively, on the risk of several types of cancer, including lung, bladder, colorectal, liver, esophageal, thyroid and prostate[174]. The Nutritional Prevention of Cancer (NPC) trial revealed that an intake of 200 µg Se in form of SeMet had no effect on the primary endpoint non-melanoma skin cancer but resulted in a significant reduction of the mortality and incidence of prostate (52 %), lung (26 %), and colorectal (54 %) cancers[204]. However, the correlation between Se and cancer risk is rather inconsistent between different clinical trials[205]. In addition, the Selenium and Vitamin E Cancer Prevention Trial (SELECT) was early terminated as it failed to detect an effect of Se supplementation on the onset of prostate cancer[195]. There exist several hypotheses about the anti-carcinogenic effects of Se, including the anti-oxidant functions of selenoproteins, the involvement of regulation of protein folding in the endoplasmic reticulum, Se effects on DNA stability, regulation of cell cycle, as well as the stimulation of apoptosis and necrosis[206–209]. Most of the effects are related to functions of selenoproteins. However, increasing the activity of selenoproteins appears to be only one of many ways by which Se contribute to normal cell growth and function. Besides the role of Se and selenoproteins in maintaining the cellular redox homeostasis, further pathways could be identified that are affected by Se supply. These include amongst others the Wnt pathway, Nrf2, and nuclear factor NF-kappa B (NF-kB) signaling, as well as protein kinase B (AKT) and forkhead protein (FOXO) activities[163,208,210,211]. Together, the mechanisms by which Se might exhibit its anti-carcinogenic effects are not well understood. In addition, epidemical data and interventional trials have shown a clear role of Se compounds in the prevention as well as therapy of certain kinds of cancers, whereas others failed to detect a beneficial effect of Se supplementation. Different malignant cells have their specific biological characteristics and eventually different properties to utilize Se. Thus, Se might have effects on some types of cancers whereas not on others. Moreover, the inconclusive variability in chemoprevention/

chemotherapeutic efficacy which does not correlate with Se levels alone, reveals that the effects mediated by Se differ between distinct Se compounds[212]. In conclusion, the effects of Se on cancer depend on the chemical form, the given dose, and finally on the type of cancer.

So far, regarding Se and cancer, the research has been mainly focused on chemoprevention. The primary idea is based on the indirect anti-oxidant properties of Se that protect cells against oxidative damage. In contrast, the rationale for chemotherapeutic effects of Se differs and assumes that Se-compounds at higher concentrations might turn into pro-oxidant agents. Tumor cells, due to accelerated glycolysis, are characterized by increased levels of reactive oxygen species (ROS) making them more vulnerable to oxidative stress when compared to non-malignant cells[213]. Consequently, Se-compounds with pro-oxidant properties have great potential as anti-cancer agents as highlighted in recent studies showing that selenite induces apoptosis in sarcomatoid malignant mesothelioma cells or non-small lung cancer cell by formation of ROS[213,214].

Several studies suggested that methylselenol is the key metabolite mediating the anti-cancer effects of Se. Thus, the cytotoxic efficacy of MSA was reported for human lung, prostate, and breast tumor models [212,215–218]. Furthermore, MSA significantly reduced tumor growth in prostate and colon cancer xenograft models without affecting the general behavior of the mice, inducing genotoxic side effects, or leading to signs of systemic toxicity[57,219–221]. More recently it could be shown that MSA inhibited mammary tumorigenesis as well as the formation of pulmonary metastasis in mice[222,223]. In combination therapies, MSA enhanced the sensitivity of triple-negative breast cancer to paclitaxel, reversed resistance to cisplatin in ovarian cancer cells and exhibited synergistic induction of apoptosis with cisplatin[224–226]. In addition, comparing the impact of different Se-compounds on tumor growth revealed a superior tumor growth inhibitory effect of MSA over SeMet and selenite *in vivo*[57]. Thus, it was concluded that methylselenol might mediate the anti-cancer effects of selenium[57]. However, the underlying mechanisms of these observations are not yet well understood.

1.5.3 Selenium and anti-cancer immunity

Much attention has been focused on the anti-proliferative effects of Se on various normal and neoplastic cell types. However, dietary Se supplementation can also enhance the expression of various humoral and cellular immune responses, which play roles in anti-cancer immunity. The first reference to selenium´s possible role in immune functions was given in 1959, only two years after the finding that Se is an essential trace element. McConnell at al. injected ^{75}Se into dogs and recognized that the isotope was incorporated into leucocytes[227]. In fact, immune

cells express several selenoproteins. The most abundant selenoproteins in T cells are GPX1, GPX4, SELENOP, SELENOK, and SELENOF[228,229], which are involved in redox-regulation, quality control in the ER and regulation of cellular Ca^{2+} homeostasis. Therefore it is hardly surprising that the Se status affects immune responses[230–235].

Experimental animal studies have demonstrated that an insufficient Se status results in a decreased capacity of T cells to respond to stimulation with antigens (Ag) or mitogens, and to differentiate into cytotoxic lymphocytes (CTLs)[236–238]. Furthermore, the authors showed that lymphocytes from animals maintained on a Se-supplemented diet had a greater ability to destroy tumor cells than lymphocytes from animals maintained on the normal diet[236]. Besides the effects of Se on immune cells involved in eliminating cancer cells, just a few studies exist regarding Se and anti-tumor immunity. For proper anti-cancer immune responses, transformed cells need to be recognized as "foreign" by effector cells of the innate and adaptive immune system. However, tumor cells developed several strategies to escape from this immune surveillance[239]. For instance, the abundance of NKG2D ligands on the cell surface of neoplastic cells is frequently downregulated[240–242]. NKG2D ligands, which are non-classical MHC class I like molecules[243], can be recognized by different subtypes of immune cells, including NK cells, $CD8^+$ T cells, and some activated $CD4^+$ T cells via NKG2D receptor interactions[244,245]. Thus, downregulation of NKG2D ligands renders the cells invisible to NKG2D receptor positive effector cells. This phenomenon is associated with poor prognosis and impaired immuno-therapeutic benefits in many human cancers[246]. Regarding Se, it could be shown that the methylselenol-generating Se compound MSA enhances the expression of the NKG2D ligands MIC/A and B on tumor cells and thereby might enhance their recognition by NKG2D receptor-expressing immune effector cells[247,248]. Interestingly this effect is restricted to methylselenol-generating Se compounds as similar effects were not observed when cells were treated with selenite or selenomethionine[248]. These data clearly highlight differences in the action of distinct Se compounds. As methylselenol-generating Se compounds are known to exhibit superior effects over anti-oxidant Se compounds regarding inhibition of tumor growth, one could speculate that shaping anti-tumor immunity and "boosting" the immune system might play an important role in anti-tumor properties of Se.

2 Objectives

As described in the chapter “introduction” it is well established that the essential trace element Se possess a significant impact on the cellular redox status via **(i)** functional anti-oxidant selenoproteins, **(ii)** the generation of reactive oxygen species (ROS) by Se oversupply, and **(iii)** the modulation of the activity of non-selenoproteins involved in the antioxidant response. Thus, Se bioavailability might not only affect the functional activity of selenoproteins but also signaling processes that are sensitive to changes in the cellular ROS levels. For instance, cancer cells exhibit due to their high metabolic rate permanently high levels of H_2O_2 resulting in persistent upregulation of redox-sensitive signaling pathways that are involved in differentiation and proliferation processes. Thus, manipulating the cellular redox status by either enhancing or decreasing intra-cellular H_2O_2 levels, interrupt e.g. anti-apoptotic pathways in cancers. Further, several redox-sensitive pathways have been described that, when altered, might lead to the development of metabolic disorders, e.g. T2D. Thus, modulation of the cellular redox status via the bioavailability of Se impacts signaling cascades that are involved in disorder development. However, distinct Se compounds exhibit significant differences in their intestinal absorption rate, their bioavailability, as well as their intermediary metabolism. Thus, depending on the Se doses and the Se compound given, different impacts on the cells might occur. Since the discussion started that Se supplementation might have several health benefits, the Se supplement market boomed remarkably. Se is consumed by many individuals as a promising health promoting agent without controlling total intake or discriminating between distinct Se compounds. Based on this background and the reported conflicting results regarding beneficial or even harmful effects of Se oversupply, the overall aim of the current project was to characterize the cellular activities of different Se derivatives thereby addressing the following questions:

(i) Do distinct Se compounds differentially affect the cellular redox status in murine organs?

(ii) Do distinct Se compounds differentially influence the murine energy metabolism and if so are these metabolic alterations related to changes in the cellular redox status?

(iii) Which role plays the selenoprotein GPX2 in maintaining mucosal homeostasis related to the overall energy metabolism?

(iv) What are the underlying mechanisms, which contribute to the observed anti-cancer properties of pro-oxidant Se compounds?

3 Summary of the articles

3.1 Article I - Individual effects of different selenocompounds on the hepatic proteome and energy metabolism of mice.

Claudia Lennicke, Jette Rahn, Anna P. Kipp, Bilijana P. Dojčinović, Andreas S. Müller, Ludger A. Wessjohann, Rudolf Lichtenfels, and Barbara Seliger

Selenium exerts its physiological role mainly via the activity of Se-containing enzymes. Selenoproteins fulfill different functions including maintenance of the cellular redox status. For the optimal activity of selenoproteins a daily intake of ~ 70 µg Se is recommended. However, several studies point out beneficial effects of Se oversupply regarding cancer prevention. In contrast, a high Se status has also been associated with the development of metabolic disorders. In general, the results of distinct studies are inconsistent and controversial. Importantly, the metabolic pathways of distinct Se compounds differ significantly resulting in downstream metabolites differing in their biological activity. Thus, in this manuscript, we addressed the question if the inconsistencies of the published studies might be a result of distinct effects mediated by distinct Se compounds. Therefore, we directly compared the effects of not only different Se concentrations (deficient, < 20 µg Se/kg diet; adequate, 150 µg Se/kg diet; high, 750 µg Se/kg diet) but also different Se compounds (sodium selenite, sodium selenate, selenomethionine) on parameters of the energy metabolism in mice. Furthermore, parameters of the cellular redox capacity were analyzed and correlated to metabolic alterations. We show that indeed not only Se doses but also the used Se compounds differentially affect biological processes. Most strikingly, SeMet oversupply resulted in a unique response pattern that might be contributed to its non-specific incorporation into body proteins. The findings of this study become practically relevant when considering Se as a supplement.

The detailed information regarding material, methods, results, and discussion is found in **article I**:
C. Lennicke, J. Rahn, A.P. Kipp, B.P. Dojčinović, A.S. Müller, L.A. Wessjohann, R. Lichtenfels, B. Seliger, Individual effects of different selenocompounds on the hepatic proteome and energy metabolism of mice, Biochim. Biophys. Acta - Gen. Subj. 1861 (2017) 3323–3334. doi:10.1016/j.bbagen.2016.08.015.

3.2 Article II - Altered protein expression pattern in colon tissue of mice upon supplementation with distinct selenium compounds

Jette Rahn, **Claudia Lennicke**, Andreas S. Müller, Ludger A. Wessjohann, Rudolf Lichtenfels, and Barbara Seliger

Several studies suggest preventive roles of Se in colon cancer development. Given that the intestine is the first line internal organ confronted with Se after food intake and that the intestine expresses high levels of the selenoprotein GPX2, the aim of the study was to analyze the impact of different Se concentrations as well as distinct Se compounds on **(i)** the anti-oxidant capacity and **(ii)** proteomic changes of colon tissues of mice. The colon tissues analyzed in this experiment originate from the same mice described in **article I**. In this manuscript, we show that the antioxidant capacity in colonic tissues is only marginally affected by different Se compounds. This contrasts with results obtained from liver tissues (**article I)** where Se compounds and Se doses, respectively, exerted differential effects. For instance, targets of the transcription factor Nrf2 were markedly upregulated in response to Se deficiency in liver tissues, whereas similar effects were not observed in colon tissues. This indicates **(i)** differences in the general redox capacity within both organs that might be due to a general higher baseline oxidative microenvironment of colon tissues compared to livers, and **(ii)** tissue-specific actions of Se. Furthermore, the protein expression patterns of colon tissues were markedly altered dependent on either the doses of Se supplementation or the type of the given Se compound. Herein, the protein abundance of calcium-activated chloride channel regulator 1 (CLCA1) was markedly enhanced in response to selenite supply compared to the other Se derivatives. Given that CLCA1 is a protein secreted by goblet cells within the gastrointestinal tract (GIT), these results indicate an altered mucosal homeostasis mediated by specific Se compounds and/ or the activity of Se-dependent proteins. Together, these data led to a better understanding of bioactive roles of Se in colon tissues.

The detailed information regarding material, methods, results, and discussion is found in **article II**
J. Rahn, **C. Lennicke,** A.P. Kipp, A.S. Müller, L.A. Wessjohann, R. Lichtenfels, B. Seliger, Altered protein expression pattern in colon tissue of mice upon supplementation with distinct selenium compounds, Proteomics. 17 (2017). doi:10.1002/pmic.201600486.

3.3 Article III - Loss of epithelium-specific GPx2 results in aberrant cell fate decisions during intestinal differentiation

Claudia Lennicke, Jette Rahn, Claudia Wickenhauser, Rudolf Lichtenfels, Andreas S. Müller, Ludger A. Wessjohann, Anna P. Kipp, and Barbara Seliger

The selenoprotein GPX2 is predominantly found in the crypt base of the gastrointestinal tract (GIT) but is also expressed in several types of cancers. The co-occurrence of GPX2 with components of signaling pathways, such as e.g. Wnt, and the regulation of GPX2 by several transcription factors involved in cell differentiation and proliferation processes reveals a role of GPX2 in the prevention and/or development of diseases including cancer. Furthermore, data obtained from **article II** revealed a specific role of selenite treatment in colon tissues. Therefore, the aim of the present study was to analyze and identify roles of GPX2 within the colons of mice using GPX2 KO models and the respective WT mice.

In the study, a diminished CLCA1 expression pattern in GPX2 KO mice was found. TCGA data analysis revealed high CLCA1 mRNA expression levels in normal colon tissues but reduced ones in colon tumor sections. Furthermore, we found that the transcript levels for markers of several cell types (e.g. enteroendocrine cells, stem cells) were reduced in GPX2 KO mice compared to the WT counterparts. This was associated with decreased mRNA levels of intestinal hormones. Together, we found that GPX2 might be important for the modulation of cell fate decisions in murine intestinal epithelium and thus plays a role in mucosal homeostasis (**Figure 8**). These data gave novel insights about the association of the cellular redox status, the activity status of GPX2 and mucosal homeostasis.

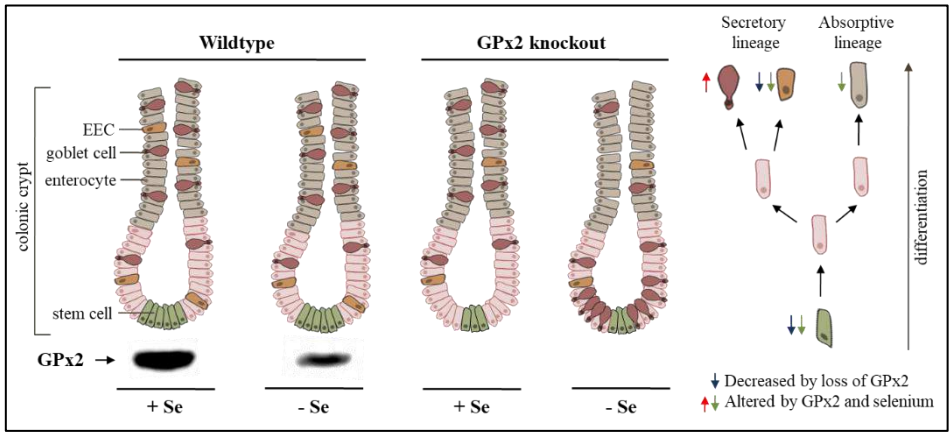

Figure 8: GPX2 is involved in cell fate decisions within the GIT.
Within the GIT, GPX2 is mainly localized at crypt bases, where also stem cells are located. The data of the current study indicate that GPX2 modulates the expression of several proteins involved in differentiation and proliferation processes, leading to an altered distribution pattern of secretory and absorptive cell types in colon tissues..EEC, Enteroendocrine cell.

The detailed information regarding material, methods, results, and discussion is found in **article III**:
C. Lennicke, J. Rahn, C. Wickenhauser, R. Lichtenfels, A.S. Müller, L.A. Wessjohann, A.P. Kipp, B. Seliger, Loss of epithelium-specific GPx2 results in aberrant cell fate decisions during intestinal differentiation, Oncotarget. (2018).

3.4 Article IV - Modulation of MHC class I surface expression in B16F10 melanoma cells by methylseleninic acid

Claudia Lennicke, Jette Rahn, Jürgen Bukur, Falko Hochgräfe, Ludger A. Wessjohann, Rudolf Lichtenfels, and Barbara Seliger

Mono-methylated Se compounds might play a role in cancer therapeutic applications. Methylselenol-generating Se compounds can kill tumor cells through distinct mechanisms including the generation of superoxide radical anions and induction of apoptosis. Furthermore, methylselenol has been shown to affect the innate immune response by modulating the expression of NKG2D and HLA-E ligands, indicating that Se might improve immunotherapeutic strategies. Interestingly, methylselenol-generating Se compounds are superior over other Se compounds, e.g. SeMet, in inhibiting cancer growth. However, the underlying mechanisms are rather insufficiently understood. Therefore, this study aimed to explore the anti-tumor effects of MSA, a direct precursor of methylselenol, using B16F10 melanoma cells as model. Non-toxic doses of MSA altered the proteome profile of the melanoma cells. Interestingly, ER-resident proteins involved in the MHC class I assembly were upregulated. Furthermore, redox proteomic analysis revealed that MSA activated proteins playing a role in the oxidative folding of MHC class I molecules. In line, MHC class I abundance on the cell surfaces of the tumor cells was enhanced in response to MSA and DMDSe, but not altered by SeMet and selenite. Furthermore, MSA treatment of B16F10 cells enhanced the expression of major components of the antigen processing machinery (APM) (**Figure 9**), of the IFNγ signaling pathway, and of the anti-oxidant response system. Together, these data suggest that MSA might affect the malignant phenotype of melanoma cells and enhances the expression of MHC class I APM components by partially mimicking IFNγ signaling. This provides a novel mechanism for the chemotherapeutic potential of methylselenol-generating Se compounds.

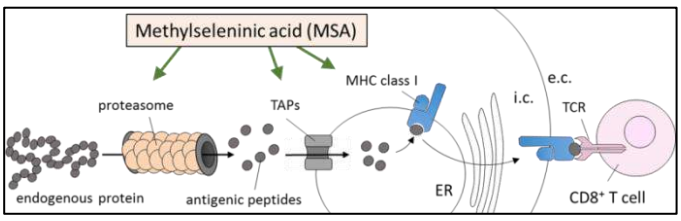

Figure 9: MSA enhances the expression of components involved in MHC class I assembly.
The presentation of intracellular antigenic peptides by MHC class I molecules is the result of a series of reactions. First, endogenous proteins are degraded by the proteasome. The resulting peptides are translocated via the heterodimeric transporter associated with antigen presentation (TAP1, TAP2) into the endoplasmic reticulum (ER) lumen and loaded onto MHC class I molecules. The trimeric peptide–MHC class I complexes are then released from the ER and transported via the Golgi to the cell surface for antigen presentation to $CD8^+$ T cells. ER, Endplasmic reticulum; e.c., extra-cellular; i.c.; intra-cellular.

The detailed information regarding material, methods, results, and discussion is found in **article IV**: C. Lennicke, J. Rahn, J. Bukur, F. Hochgräfe, L.A. Wessjohann, R. Lichtenfels, B. Seliger, Modulation of MHC class I surface expression in B16F10 melanoma cells by methylseleninic acid., Oncoimmunology. 6 (2017) e1259049. doi:10.1080/2162402X.2016.1259049.

4 Published articles

4.1 Article I - Individual effects of different selenocompounds on the hepatic proteome and energy metabolism of mice.

Individual effects of different selenocompounds on the hepatic proteome and energy metabolism of mice.

Claudia Lennicke, Jette Rahn, Anna P. Kipp, Bilijana P. Dojčinović, Andreas S. Müller, Ludger A. Wessjohann, Rudolf Lichtenfels, and Barbara Seliger

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Individual effects of different selenocompounds on the hepatic proteome and energy metabolism of mice

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ABSTRACT

Background: Selenium (Se) exerts its biological activity largely via selenoproteins, which are key enzymes for maintaining the cellular redox homeostasis. However, besides these beneficial effects there is also evidence that an oversupply of Se might increase the risk towards developing metabolic disorders. To address this in more detail, we directly compared effects of feeding distinct Se compounds and concentrations on hepatic metabolism and expression profiles of mice.

Methods: Male C57BL6/J mice received either a selenium-deficient diet or diets enriched with adequate or high doses of selenite, selenate or selenomethionine for 20 weeks. Subsequently, metabolic parameters, enzymatic activities and expression levels of hepatic selenoproteins, Nrf2 targets, and additional redox-sensitive proteins were analyzed. Furthermore, 2D-DIGE-based proteomic profiling revealed Se compound-specific differentially expressed proteins.

Results: Whereas heterogeneous effects between high concentrations of the Se compounds were observed with regard to body weight and metabolic activities, selenoproteins were only marginally increased by high Se concentrations in comparison to the respective adequate feeding. In particular the high-SeMet group showed a unique response compromising higher hepatic Se levels in comparison to all other groups. Accordingly, hepatic glutathione (GSH) levels, glutathione S-transferase (GST) activity, and GSTpi1 expression were comparably high in the high-SeMet and Se-deficient group, indicating that compound-specific effects of high doses appear to be independent of selenoproteins.

Conclusions: Not only the nature, but also the concentration of Se compounds differentially affect biological processes.

General significance: Thus, it is important to consider Se compound-specific effects when supplementing with selenium.

1. Introduction

During the last two decades the essential trace element selenium (Se) has gained considerable attention as a potential cancer preventive agent [1] leading to a marketing boom of Se supplements [2]. Se is an integral part of selenoproteins, which contain the redox-active amino acid selenocysteine (SeCys) in their catalytically active center [3,4]. The best characterized selenoproteins like glutathione peroxidases (GPx) and thioredoxin reductases (TrxR) play key roles in detoxifying hydrogen peroxide, lipid peroxides and in the reduction of cellular disulfides generated by interactions of free radicals and other oxygen-derived species as part of the normal cellular metabolism. Further selenoproteins are

Abbreviations: AKT, protein kinase B; ARE, anti-oxidant response elements; CCB, colloidal Coomassie Blue; DIGE, difference gel electrophoresis; ERK, extracellular regulated kinase; Fasn, fatty acid synthase; Gck, glucokinase; Gpx/GPx, glutathione peroxidase; GS, glutathione synthetase; G6pc, glucose-6-phosphatase; GSH, glutathione; GST, glutathione S-transferase; Hprt, hypoxanthine guanine ribosyl transferase; Keap1, Kelch-like ECH-associated protein 1; NQO1, NAPH dehydrogenase 1; Nrf2, NF-E2-related factor 2; Pdha, hepatic pyruvate dehydrogenase; PTEN, phosphate and tensin homolog; PTP, protein tyrosine phosphatase; RPL13a, ribosomal protein L13a; Se, selenium; SeMet, selenomethionine; SOD, superoxide dismutase; TG, triglyceride; Txnrd/TrxR, thioredoxin reductase.

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involved in the modulation of the immune response, inflammatory processes as well as chemoprevention [5].

Insufficient Se intake results in a rapid and selective downregulation of several selenoproteins like the ubiquitously expressed GPx1, which impairs the anti-oxidant capacity of a cell. In contrast, high Se concentrations might directly affect the cellular redox balance by inducing the formation of thiol-reactive selenocompounds and by generating oxygen-free radicals [6]. This pro-oxidant effect is supposed to differ depending on the used selenocompound; e.g. selenite, selenocystine, methyseleninic acid, and methylselenocysteine are known to modulate the cellular redox state and are therefore termed "redox active selenocompounds" [7]. Thus, the correlation of the cellular redox status with the Se supply follows a U-shape curve. Based on this strong relationship, it has been shown that the Se status also directly modulates the expression of non-selenoproteins via e.g. redox-sensitive transcription factors like the NF-E2-related factor 2 (Nrf2) [8]. Under physiological conditions, the redox-sensitive Kelch-like ECH-associated protein 1 (Keap1) restrains Nrf2 in the cytosol, whereas oxidative stress disrupts the Keap1/Nrf2 complex leading to the nuclear translocation of Nrf2. This results in the activation of genes carrying anti-oxidant response elements (ARE) within their promoters like glutathione S-transferases (GST) and enzymes of the glutathione (GSH) biosynthesis pathway [9].

Furthermore, the modification of redox-sensitive phosphatases is supposed to be the cause of insulin-mimetic and anti-diabetic properties of supranutritional Se concentrations [10–12], but also insulin-antagonistic and even pro-diabetic effects of an over-supply of Se compounds have been observed and discussed [13–15]. Upon binding to its receptor, insulin initiates a signaling cascade, which is accompanied by a burst of hydrogen peroxide (H_2O_2) that acts as a second messenger by modifying redox-sensitive cysteine residues [10,16]. It has been demonstrated that the activity of the protein tyrosine phosphatase 1B (PTP1B), which is a negative regulator of the insulin signaling pathway, could be modulated via Se availability in a redox-dependent manner [17]. Phosphorylation of protein kinase B (AKT) links the insulin signaling cascade to the energy metabolism. Supra-nutritional intake of Se led to an increased AKT phosphorylation in visceral adipose tissues of pigs [18] and to a delayed insulin-induced phosphorylation of AKT and forkhead box protein 1a/3 (FoxO1a/3) in myocytes [19]. Mice overexpressing GPx1 develop insulin resistance, hyperlipidemia and obesity [20], whereas mice with decreased GPx1 activity and consequently increased cellular levels of reactive oxygen species (ROS) are more sensitive to insulin [21]. In this context it is discussed that enhanced levels of anti-oxidant enzymes may lead to an "over-scavenging" of H_2O_2, which is involved in and necessary for redox signaling pathways including the insulin signaling cascade [22] thereby resulting in the deregulation of glycolysis, gluconeogenesis and lipogenesis. Regarding the insulin-mimetic, anti-diabetic or even pro-diabetic effects of Se supply a recently published Cochrane review discussed the inconsistency of results obtained from different trials [23]. A possible explanation for such discrepant data could be the use of different Se compounds.

The current idea is that selenomethionine (SeMet), the major form of Se in foods, is faster absorbed in the small intestine when compared to inorganic forms, such as selenate and selenite [24–26]. Furthermore, SeMet does not only provide Se for selenoprotein synthesis, but can also be non-specifically incorporated into proteins instead of methionine [27]. This allows Se to be stored in the organism and to be released by normal protein turn-over [28], but can also give rise to toxic effects once functional methionine is substituted in enzymes. The inorganic Se compound selenate shares a sodium-dependent transport system with sulfates, whereas selenite is mainly absorbed by passive diffusion [29,30].

Under conditions of an increased availability of inorganic selenocompounds an enhanced Se excretion in form of methylated selenocompounds or selenosugars occurs, while SeMet is slowly metabolized and rather accumulates in the body. Furthermore, selenite is supposed to have direct effects on the cellular redox status, which in contrast has not been attributed to SeMet.

Based on this information the present study aimed to directly compare the effects of long-term feeding with different Se compounds and doses on metabolic health of mice. Therefore, parameters of the insulin-regulated energy metabolism were analyzed and correlated to the hepatic selenoprotein expression and the anti-oxidant capacity of the liver. In addition, hepatic Nrf2 target gene expression was analyzed in response to different Se compounds and concentrations. New Se-sensitive proteins are identified using a proteome approach followed by mass spectrometry.

2. Material and methods

2.1. Animals and diets

Four-week old healthy male C57BL/6J mice (Harlan Laboratories, Netherlands) kept under conventional conditions (room temperature 22 ± 1 °C, humidity 50 ± 10%) with free access to their respective diets and autoclaved tap water were used. The experiments were approved by the ethical committee of the Martin Luther University Halle-Wittenberg (42502-2-1187MLU). Mice were randomly assigned to seven experimental groups of 8 individuals/group. The groups were fed with diets supplemented either with adequate (ad; 150 μg Se/kg diet) or supra-nutritive (high, hi; 750 μg Se/kg diet) Se concentrations in the form of selenite, selenate and SeMet, respectively, whereas the control group received a Se-deficient diet (-Se). The diets were based on torula yeast and Se-deficient wheat and with the exception of the Se contents composed according to the National Research Council (NRC) recommendations for rodents (Table S1). Se content of the diets was confirmed by ICP-MS (−Se < 20; ad-selenite 165 ± 1.9; hi-selenite 768 ± 3.1; ad-selenate 157 ± 1.9; hi-selenate 762 ± 2.3; ad-SeMet 161 ± 2.7; hi-SeMet 765 ± 3.4 μg Se/kg diet). After the 20 weeks feeding period and 4 h of feeding deprivation, mice were decapitated under CO_2 anesthesia. Blood was collected in heparinized tubes and centrifuged (15 min, 4 °C, 2.000 × *g*). Plasma was stored at −80 °C until further analysis. Liver tissue samples were excised, snap frozen in liquid nitrogen, and stored at −80 °C until further use.

2.2. Determination of plasma glucose and insulin concentrations

Glucose concentrations were measured in plasma samples collected from tail veins using a glucometer according to the manufacturer's instructions. Plasma insulin levels were assayed using a mouse insulin ELISA kit (Mercodia, Uppsala, Sweden). The HOMA-IR was calculated using glucose and insulin concentrations obtained after 4 h of food withdrawal using the following formula: plasma glucose [mg/dl] × fasting insulin [ng/ml]/405 [31].

2.3. Determination of triglyceride concentrations in plasma

The total triglyceride (TG) content of plasma samples was determined using a commercially available kit (Fluitest TG, Analyticon Diagnostics, Lichtenfels, Germany) according to the manufacturer's instructions.

2.4. Se analysis in liver tissues using inductively coupled plasma mass spectrometry (ICP-MS)

The digestion of the samples for ICP-MS studies was performed on an Advanced Microwave Digestion System (ETHOS 1, Milestone, Italy) using an HPR-1000/10S high pressure segmented rotor. Determination of the Se content in liver tissues of mice was performed by ICP-MS using the Thermo Scientific iCAP Qc ICP-MS (Thermo Scientific, Bremen, Germany) with the operational software Qtegra. For ^{80}Se determination the instrument was adjusted for optimum performance in gas H_2/He mixture in Collision Cell Technology (CCT) mode using the supplied auto-tune protocols (Thermo Scientific, Bremen, Germany) [32].

2.5. Real-time RT-PCR

RNA was isolated using the acid guanidinium thiocyanate-phenol-chloroform extraction method [33] and then subjected to PCR analysis as recently described [34]. Briefly, 1 μg RNA was reverse transcribed into cDNA using the *RevertAid First Strand cDNA Synthesis Kit* and *Oligo dT primers* (Thermo Scientific) according to the manufacturer's protocol. Real-time PCR was performed using SYBR Green I as fluorescent reporter. Target-specific primers (Table S2) were designed using the program *Primer3* [35] and validated on agarose gels. Amplification data were analyzed with the Rotor-Gene 6000™ series software (Qiagen, Hilden, Germany) using the method according to Pfaffl [36]. Amplifications of Ribosomal Protein L13a (Rpl13a) and hypoxanthine guanine ribosyl transferase (Hprt) were used for normalization. The data are represented as relative mRNA expression levels as x-fold of the Se-deficient group.

2.6. Preparation of tissue homogenates

For analysis of hepatic GPx, TR, NADPH quinone dehydrogenase 1 (NQO1), and GST activity liver tissue lysates were prepared in Tris buffer (100 mM Tris, 300 mM KCl, 0.1% Triton X-100, pH 7.6; Calbiochem® protease inhibitor cocktail II (Merck Millipore, Darmstadt, Germany)) using a tissue lyser (Qiagen). Lysates for the determination of hepatic superoxide dismutase (SOD) activity were prepared in 0.05 M potassium phosphate buffer containing protease inhibitor (ROCHE, Basel Switzerland). For Western blot analyses tissue lysates were prepared in RIPA buffer (50 mM Tris, 150 mM $NaCl_2$, 0.5% DOC, 1% NP-40, 0.1% SDS) containing protease and phosphatase inhibitors (HALT™, Thermo Scientific). All lysates were centrifuged for 30 min at 14.000 ×g and 4 °C. Supernatants were stored at −80 °C until further analyses.

2.7. Western blot analysis

Western blot analysis was performed as recently described [37]. For immune detection the primary (monoclonal or polyclonal) antibodies, purchased form Cell Signaling Technology (Danvers, MA, USA), were anti-phospho-AKT^{Ser473}, anti-phospho-AKT^{Thr308}, anti-AKT (#9916); anti-phospho-ERK1/2 (#4370), anti-phospho-PTEN$^{Ser380/Thr382/383}$ (#9549), and anti-GAPDH (#2118). The secondary HRP-coupled anti-rabbit immunoglobulins (#7074) were purchased from Cell Signaling Technology.

2.8. Determination of enzyme activities

Total GPx activity was measured in plasma and liver samples according to the method of Lawrence and Burk [38], which was modified for 96-well microtiter plates as recently described using H_2O_2 as substrate [39]. TrxR catalyze the formation of 5′-thionitrobenzene (TNB) by using the substrate 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB). The consumption of NADPH was spectrophotometrically determined at 412 nm for 2 min at 25 °C as previously described [40]. Total SOD activity was determined according to the method of Marklund and Marklund [41]. For determination of MnSOD activity, 1 mM KCN was added to the assay buffer, which selectively inhibits the Cu/ZnSOD. NQO1 activity was determined as previously described [8]. GST activity was spectrophotometrically measured using the substrate 1-chloro-2,4-dinitrobenzene (CDNB) in the presence of reduced glutathione according to the protocol of Habig and co-authors [42]. The native activity of PTP was determined by analyzing paranitrophenyl phosphate hydrolysis, which could be monitored at 410 nm [17].

2.9. GSH assay

Total GSH (reduced and oxidized) concentrations were determined in plasma and liver homogenates according to a standard procedure coupled to GSH reductase and DTNB [43]. The concentrations were calculated using a GSSG standard curve ranging from 1 to 10 nM GSH equivalent/ml.

2.10. Specific labeling of reduced cysteine residues

Labeling of reduced cysteine residues was performed using the thiol reactive S-300 fluorescence Dye (NH DyeAgnostics, Halle, Germany) according to the manufacturer's protocol with the exception that the reducing step with TCEP prior to the labeling procedure was omitted in order to label only reduced cysteine residues. Lysates of liver tissues were prepared under nitrogen overlay in 7 M urea, 2 M thiourea, 4% CHAPS, 30 mM Tris (pH 7.5) using a grinding kit (Readyprep mini grinders, Bio-Rad Laboratories GmbH, Munich, Germany). 5 μg of total protein was labeled with S-300 at 37 °C for 1 h. The reaction was stopped by adding one volume of the labeling buffer containing 2% DTT. After one-dimensional SDS PAGE separation, gels were scanned using a FUJI FLA-500 scanner (FujiFilm GmbH, Düsseldorf, Germany) and total fluorescence per lane was quantified and normalized to post fluorescence scanning performed on colloidal Coomassie Blue staining (CCB, Applichem, Darmstadt, Germany) [44].

2.11. Proteome analysis by difference gel electrophoresis (DIGE) and mass spectrometry

Four biological liver samples from each group were 3-times washed in cold PBS, subsequently grinded (Readyprep mini grinders, Bio-Rad Laboratories GmbH, Munich, Germany) and solved in DIGE lysis buffer (30 mM Tris, 7 M urea (Applichem, Darmstadt, Germany), 2 M thiourea (Sigma, Deisenhofen, Germany), 4% (w/v) CHAPS (Applichem), pH 8.5). After sonification and centrifugation (13.000 ×g, 90 min, 15 °C) supernatants were collected and stored at −80 °C before protein concentrations were determined as previously described [45]. DIGE analysis was performed using the minimal labeling approach according to the manufacturer's instructions (NH DyeAGNOSTICS) with the exception that 25 μg of protein were labeled with 100 pmol G-dye. Isoelectric focusing using Immobiline DryStrips pH 3–10 NL (18 cm, GE Healthcare) and second dimension SDS separation were done as previously described [46]. After separation the gels were subsequently washed in distilled water, fixed with 10% acetic acid (Carl Roth GmbH and Co. KG, Karlsruhe, Germany) and 40% EtOH (Sigma-Aldrich Chemie GmbH, Munich, Germany) followed by a wash in distilled water. Gels were scanned as previously described [47] and gel image analysis was carried out with the Delta2D software package (Decodon GmbH, Greifswald, Germany) according to the manufacturer's guidelines. Protein spots were considered as differentially expressed if a 1.5-fold altered expression level was observed. In addition, the spots of interest were statistically analyzed with the Student's *t*-test and only those selected for mass spectrometric identification with a p value <0.05. For mass spectrometric (MS) analysis a preparative gel was loaded with an amount of 500 μg protein and spots of interest were subsequently picked, in gel digested (DigestPro, Intavis, Cologne, Germany) and further analyzed by MS using the matrix-assisted laser-desorption/ionization time-of-flight (MALDI-TOF) mass spectrometer (ultrafleXtreme™, Bruker Daltonics Inc., Bremen, Germany) [48]. The PMF dataset analysis was performed using the MASCOT software package (Matrix Science, Dauhaim, USA). In addition the functional classification of the target structures was based on literature backsearches thereby relying on Pubmed entries.

2.12. Statistical analysis

Mean values were calculated from results of 8 animals/group or 4 animals/group in the case of the proteome analyses and given as means ± their standard error of mean (S.E.M.). SPSS 20 software was used to analyze significant differences within the groups, therefore

Table 1
Body weights (bw), feed intake, and feed conversion ratio (FCR) of mice fed with different Se compounds and Se concentrations.

		selenite		selenate		SeMet	
	-Se	ad	hi	ad	hi	ad	hi
Body weight (g)							
Initial	16.6 ± 0.27	17.1 ± 0.30	16.4 ± 0.26	16.7 ± 0.41	17.0 ± 0.16	16.9 ± 0.41	16.5 ± 0.37
Final	33.7 ± 1.57	36.4 ± 2.07	38.0 ± 1.05*	36.1 ± 1.21	38.7 ± 1.57*[a]	37.2 ± 1.15	34.0 ± 1.14[b]
Total weight gain	17.1 ± 1.47	19.2 ± 1.91	21.6 ± 1.10*[a]	19.4 ± 1.11	21.7 ± 1.53*[a]	20.4 ± 1.26	17.5 ± 1.26[b]
Total feed intake (g)	433 ± 14.2	473 ± 12.2*	481 ± 4.66*	476 ± 10.4*	480 ± 11.0*	485 ± 4.83*	487 ± 13.9*
Total FCR (mg bw/g feed)	39.5 ± 3.25	40.4 ± 3.59	44.8 ± 2.16[b]	40.6 ± 1.89	45.3 ± 3.29[b]	42.0 ± 2.74	35.9 ± 2.47[a]

Values are given as means ± S.E.M. (n = 8). Different small letters within a line indicate significant differences within the Se supplemented groups, levels marked with * indicate significant differences compared to the -Se group ($p < 0.05$; one-way ANOVA). ad, adequate (150 μg Se/kg diet); hi, high (750 μg Se/kg diet)

one-way ANOVA was implemented after asserting the normality of distribution (Shapiro-Wilk test) and the homogeneity of variance (Levene test). If variances were homogeneous the least significant (LSD) test was used, otherwise the Games-Howell test was employed. Differences between the groups were considered to be significantly different at $p < 0.05$.

3. Results

3.1. A high SeMet intake resulted in lower body weight gain without affecting feed intake

Prior to the experiment the mean body weights of all mice were comparable between the seven groups. After 20 weeks of intervention mean body weights of the groups treated with hi-selenite ($p = 0.037$) or hi-selenate ($p = 0.017$) were significantly higher when compared to the Se-deficient group, while the mean body weight of the hi-SeMet group was lower compared to both the hi-selenate ($p = 0.024$) and hi-selenite ($p = 0.052$) groups. The low body weight of the Se-deficient group was accompanied by a lower total food intake when compared to Se-supplemented groups (Table 1). Total food intake within the Se-supplemented mice did not differ, but the feed conversion ratio (FCR; mg body weight gain/g food intake) was significantly higher in the groups with hi-selenite ($p = 0.036$) and hi-selenate ($p = 0.042$) supplementation when compared to the hi-SeMet group and did not differ between the other groups (Table 1).

3.2. Selenium status after feeding different Se compounds and concentrations

After 20 weeks of intervention hepatic Se concentrations were significantly higher in all Se supplemented groups when compared to the Se-deficient group ($p < 0.001$). Highest Se levels were observed in mice

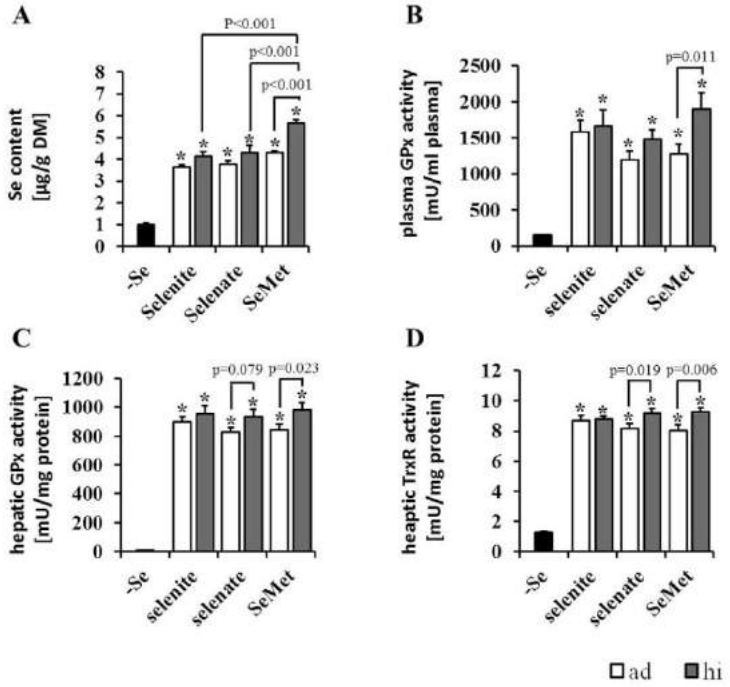

Fig. 1. Hepatic Se status following long-term supplementation with different Se compounds and Se concentrations. (A) Se concentrations were measured by ICP-MS, (B) plasma GPx, (C) total hepatic GPx and (D) total hepatic TrxR activities were spectrophotometrically determined as described in the Material and methods section. Values are given as means ± S.E.M. (n = 8). Significant differences were calculated by one-way ANOVA. *$p < 0.05$ vs. -Se. ad, adequate (150 μg Se/kg diet); hi, high (750 μg Se/kg diet). DM, dry matter.

treated with hi-SeMet when compared to the other Se-supplemented groups ($p < 0.01$). Additionally there was a trend for increased hepatic Se concentrations in the groups fed with diets containing hi-selenite ($p = 0.071$) and hi-selenate ($p = 0.052$) when compared to the corresponding adequately fed groups (Fig. 1). Plasmatic GPx activity dramatically decreased in the Se-deficient group ($p < 0.001$), whereas hi-SeMet supplementation resulted in the highest plasma GPx activities (Fig. 1). The mRNA expression of Gpx1, the most abundant selenoprotein in the liver, was dramatically reduced under Se deficiency ($p < 0.001$), while Se supplementation increased its mRNA levels. Also Txnrd1 transcription levels were decreased under Se deficiency, while no changes in the expression pattern of both genes were found within the Se-supplemented groups (Table S3). The mRNA expression patterns of Gpx1 and Txnrd1 were confirmed at their activity levels (Fig. 1). Both hepatic GPx and TrxR activities dramatically decreased in the Se-deficient group ($p < 0.001$). Within the supplemented groups, small differences between hi-selenate and hi-SeMet supplementation and the respective adequately fed groups were observed.

3.3. Up-regulation of anti-oxidant Nrf2 target genes during Se-deficiency

Since previous studies have shown that both low and high Se intakes can enhance the activity of the redox-sensitive transcription factor Nrf2 [49], the activity of NQO1, one of the best characterized Nrf2 target genes, was analyzed. NQO1 activity was increased in the Se-deficient group ($p < 0.001$) in comparison to the other feeding groups, while no changes were detected between the Se-supplemented groups (Fig. 2). Next to NQO1 also anti-oxidant enzymes are regulated via Nrf2, including MnSOD and several GSTs. While the MnSOD activity (Fig. 2) showed the same pattern as the NQO1 activity, total GST activity was differently modulated. Total GST activity was also highest in the Se-deficient group in comparison to the other groups, but the hi-SeMet group showed an intermediate response being significantly higher as all other supplemented groups ($p < 0.001$, Fig. 2). In order to determine the GST isoform responsible for this effect, mRNA expression levels of several isoforms were analyzed (Table S3). The most obvious candidates were GSTa4, GSTm1 and GSTpi1, also known to be regulated via Nrf2. GSTa4 and GSTm1 expression were down-regulated in all supplemented groups. However, in the hi-SeMet group only GSTpi1 expression did not significantly differ from the -Se group (Fig. 2) as shown for total GST activity. The mRNA expression patterns of GSTm1 and GSTpi1 were confirmed at the protein level (Table 2).

3.4. Impact of distinct Se compounds and Se concentrations on hepatic proteomic profiles

To gain further insights in the hepatic effects of feeding different Se compounds and concentrations, DIGE-based proteomic analyses were performed as an untargeted approach (Fig. 3). The consensus map across all seven experimental groups (4 mice/group, $n = 28$) was comprised of 821 distinct spots. Based on the average spot intensity within a given group the respective profiles were analyzed. Spots of interest were defined as regulated when the ratio with the Se-deficient group exceeded the factor 1.5, and when the corresponding *t*-test was defined as $p < 0.05$ (Table 2). In total 54 differentially expressed protein spots could be identified, which were subjected to MALDI-TOF-MS resulting in the identification of 28 protein species. Four protein spots contained two or more protein identities and were therefore excluded from further analysis. The identified proteins were grouped according to their cellular function(s) (Table 2). Two of the proteins identified are

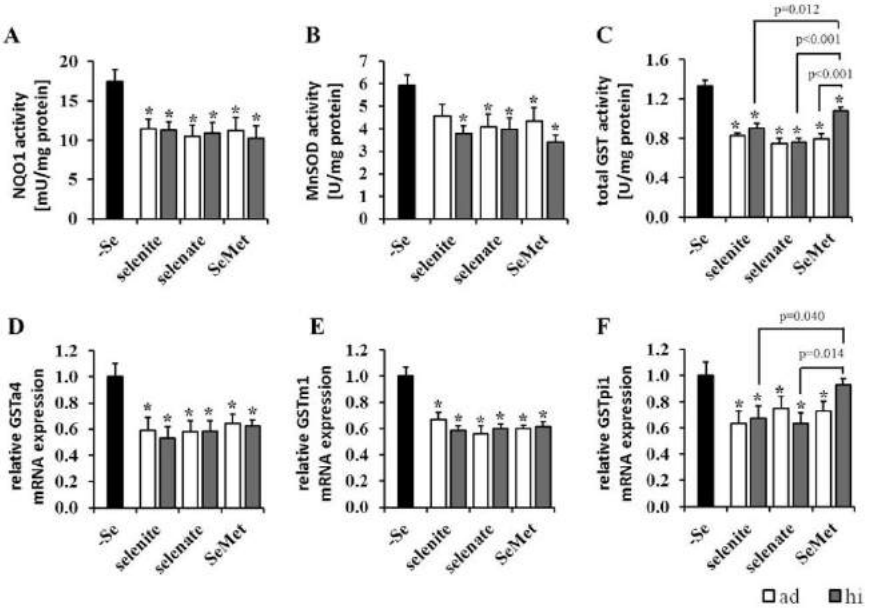

Fig. 2. Enzyme activities and mRNA expression of Nrf2 target genes following long-term supplementation with different Se compounds and Se concentrations. (A) NQO1, (B) MnSOD and (C) total GST activity; (D) GSTa4, (E) GSTm1 and (F) GSTpi1 mRNA expression in liver tissues of mice fed with different Se compounds and Se concentrations for 20 weeks. Enzyme activities were spectrophotometrically determined as described in the Material and methods section. mRNA levels were analyzed by qPCR, normalized to the reference genes Rpl13a and Hprt and depicted in relation to the Se-deficient group (-Se). Values are given as means ± S.E.M. ($n = 8$). Significant differences were calculated by one-way ANOVA. *$p < 0.05$ vs. -Se.ad, adequate (150 μg Se/kg diet); hi, high (750 μg Se/kg diet).

related to inflammation or immune modulatory processes. Nine of the proteins are involved in cell cycle, cell development and tumor metabolism. Six of the proteins are related to the intermediary metabolism and metabolic processes and three of the proteins exhibit other biological functions. These include tropomyosin beta chain, which is part of the cytoskeleton, brefeldin A-inhibited guanine nucleotide exchange protein 2, which promotes guanine-nucleotide exchange and is involved in protein transport processes and limbin, which modulates the hedgehog signaling pathway. Fourteen of the proteins were up-regulated (Table 2, indicated in red) and seven down-regulated (Table 2, indicated in green) in response to supplementation with one or several Se compounds when compared to the Se-deficient group. Out of the seven down-regulated proteins, GSTm1 and GSTpi1 were both represented in two independent protein spots (Fig. 3) suggesting not only a differential expression, but also post-translational modification(s) (PTMs). The other consistently down-regulated proteins (ferritin light chain 1 (FTL1), limbin and L-aspartate dehydrogenase) might reflect the Nrf2 response as known for FTL1 [50]. Interestingly, two proteins, brefeldin A-inhibited guanine nucleotide exchange protein 2 and carbamoyl-phosphate synthase, were down-regulated in the hi-selenite and hi-selenate groups, but up-regulated in the hi-SeMet group only. The underlying mechanism of this regulation pattern might be comparable to that controlling the GSTpi1 expression. Furthermore, three proteins (tropomyosin beta chain, membrane-associated progesterone receptor component 1, and NACHT, LRR and PYD domains-containing protein 6) showed an inverse expression pattern being up-regulated only in the hi-selenite group and down-regulated upon SeMet feeding. However, it is noteworthy that twelve proteins were consistently up-regulated in most of the feeding groups in comparison to Se deficiency (Fig. 3).

3.5. GSH levels and mRNA expression of key enzymes involved in GSH homeostasis

To test whether Nrf2 activation during Se deficiency also affected glutathione (GSH) homeostasis, plasma and hepatic levels of GSH were determined in all experimental groups. All Se-supplemented groups exhibited markedly lower plasma GSH concentrations in comparison to the Se-deficient group (Fig. 4). In liver tissues an inverse pattern was detected with the lowest GSH levels in the Se-deficient group, whereas all Se-supplemented groups, except for the hi-SeMet group, showed increased GSH levels. GSH levels in the hi-SeMet group were comparable to the Se-deficient group (Fig. 4). In order to study the molecular mechanisms underlying these effects, the mRNA expression levels of enzymes involved in GSH biosynthesis, reduction of GSSG, and GSH export were analyzed by RT-PCR (Table 3). These include the γ-glutamylcysteine ligase (GCL), composed of a catalytic (GCLc) and a modifier (GCLm) subunit, the GSH synthetase (GS) catalyzing the second step of GSH biosynthesis, the glutathione reductase (GR), and Mrp4, the main exporter of hepatic GSH into the plasma. With the exception of GS all enzymes are regulated by Nrf2. As expected GCLc, GR, and Mrp4 mRNA expression were elevated under Se-deficiency in comparison to Se-supplemented groups, while no differences were detected amongst Se-supplemented groups. The strongest up-regulation in the -Se group with a 5-fold change was observed for Mrp4 (Fig. 4). The

Table 2
Differentially expressed hepatic proteins in response to Se treatment.

Spot no.	Protein name	Acc no	Score (MS)	Sequence coverage [%]	No. of matched peptides	Mass	pI	selenite		selenate		SeMet	
								ad	hi	ad	hi	ad	hi
	Proteins involved in maintaining the redox homeostasis												
1	Glutathione S-transferase Mu 1 (GSTm1)	P10649	241	71	29	26.06	7.71	0.54	0.39	0.47	0.40	0.46	0.36
2	Glutathione S-transferase Mu 1 (GSTm1)	P10649	189	61	26	26.06	7.71	0.59	0.47	0.51	0.48	0.59	0.61
3	Glutathione S-transferase P1 (GSTpi1)	P19157	113	55	12	23.76	7.68	0.61	0.40	0.58	0.40	0.71	0.80
4	Glutathione S-transferase P1 (GSTpi1)	P19157	109	42	16	23.76	7.68	0.65	0.41	0.54	0.47	0.71	1.04
	Proteins related to inflammatory or immunomodulatory processes												
5	NACHT, LRR and PYD domains-containing protein 6 (NLRP6)	Q91WS2	58	16	11	98.76	8.75	1.83	3.25*	1.20	1.15	0.91	0.67
6	Fibrinogen gamma chain (FGG)	Q8VCM7	86	37	13	50.04	5.54	1.12	2.23*	0.77	1.21	0.66	1.45*
	Proteins related to cell cycle, cell development or tumor metabolism												
7	HIV Tat-specific factor 1 homolog (HTATSF1)	Q8BGC0	58	21	12	86.64	4.27	1.23	0.88	1.43	2.50	0.85	1.21
8	Protein NDRG2 (N-myc downstream regulated gene 2)	Q9QYG0	87	42	11	41.10	5.23	0.88	2.11*	0.84	2.17*	0.79	1.58*
9	40S ribosomal protein SA (RPSA)	P14206	88	35	9	32.93	4.80	1.13	2.26*	1.46	1.89	1.10	1.44
10	Protein FAM3C (Interleukin-like EMT inducer)	Q91VU0	56	27	7	25.02	8.52	1.48	3.36*	2.44	3.36	1.64	1.47
11	Omega-amidase NIT2	Q9JHW2	62	33	9	30.83	6.44	1.07	2.91	1.66	5.77*	0.78	0.95
12	Alpha/beta hydrolase domain-containing protein 14b (ABHD14B)	Q8VCR7	130	69	13	22.55	5.82	2.53	2.74	2.78	3.10	2.33	2.71
13	Putative hydrolase RBBP9	O88851	79	56	10	21.06	5.64	0.97	1.50	1.17	1.24	2.42	1.46
14	Membrane-associated progesterone receptor component 1 (PGRMC1)	O55022	87	31	9	21.79	4.57	0.98	1.46*	0.97	0.97	0.68	0.64
15	Structural maintenance of chromosomes protein 1A (SMC1A)	Q9CU62	64	19	26	143.7	7.51	2.07	1.45	1.75	1.33	1.83	1.60
	Proteins related to intermediary metabolism and metabolic processes												
16	Glycine N-methyltransferase (GNMT)	Q9QXF8	58	39	11	33.11	7.10	1.43	2.83*	2.03	3.09*	1.91	1.19
17	Carbamoyl-phosphate synthase [ammonia], mitochondrial (CPS1)	Q8C196	87	17	28	165.7	6.48	1.20	0.53*	0.73	0.60	0.92	1.49*
18	Electron transfer flavoprotein subunit alpha, mitochondrial (ETFA)	Q99LC5	58	23	9	35.33	8.62	1.71	1.80	1.64	1.64	1.09	0.80*
19	Putative L-aspartate dehydrogenase (ASPDH)	Q9DCQ2	87	40	12	30.47	6.45	0.45	0.82	0.73	0.81	0.40	0.65
20	Ferritin light chain 1 (FTL1)	P29391	102	44	10	20.84	5.66	0.78	0.41	0.87	0.62	1.28	1.22
21	Putative glycerol kinase 5 (GK5)	Q8BX05	63	14	10	60.34	6.84	1.16	1.52	1.97	1.68	1.01	0.98
	Proteins with other functions (cell adhesion, signal transduction, cytoskeleton)												
22	Tropomyosin beta chain (TPM2)	P58774	96	34	15	32.93	4.66	1.11	2.11*	0.69	1.26	0.73	1.18
23	Brefeldin A-inhibited guanine nucleotide-exchange protein 2 (ARFGEF2)	A2A5R2	66	14	23	204.56	6.12	1.47	0.53*	1.20	0.86	1.07	2.09*
24	Limbin (EVC2)	Q8K1G2	70	14	18	138.2	5.87	0.73	0.38	0.40	0.45	0.64	1.12

Values are given as means (n = 4) in relation to Se-deficient (-Se) mice. Red/green filled boxes indicate significant upregulated/downregulated hepatic proteins in relation to the -Se group. Levels marked with * indicate significant differences of hi Se intake to the ad Se intake within one indicated Se compound ($p < 0.05$; one-way ANOVA). ad, adequate (150 μg Se/kg diet); hi, high (750 μg Se/kg diet).

transcription of GCLm and GS was not or only marginally altered across the experimental groups. Thus, low GSH levels in plasma appear to be mediated by the reduced release of hepatic GSH due to the strong reduction in Mrp4 expression.

3.6. Modulation of the oxidation status of proteins by Se supplementation

To determine whether differences in hepatic GSH levels had an impact on the overall oxidative state of the hepatic proteome, protein lysates from each experimental group were individually labeled with a fluorescent dye specifically targeting free (reduced) thiol residues prior to their subsequent separation on a one-dimensional (1-DE) gel. As shown in Fig. 5, the overall oxidative state of liver proteins was specifically altered in the hi-selenite and hi-SeMet groups. The hi-selenite group showed a decreased fluorescence labeling efficiency when compared to the Se-deficient group suggesting that these proteins were more oxidized. In contrast, the samples from the hi-SeMet group demonstrated a more intense staining pattern indicating more free (reduced) thiols when compared to the Se-deficient ($p = 0.048$), hi-selenite ($p = 0.001$) and hi-selenate ($p = 0.037$) groups.

3.7. Effects of Se supplementation on the expression and activation of enzymes related to energy metabolism

Due to the effect of high-SeMet feeding on weight gain (Table 1) metabolic parameters after feeding different Se compounds and concentrations were characterized. Therefore, triglycerides (TG), fasting glucose, and fasting insulin levels were determined in plasma and the HOMA-IR score was calculated (Table 4). Plasma TG levels were significantly increased under supplementation with either selenite or selenate when compared to the Se-deficient group. Lowest TG concentrations were observed in mice of the hi-SeMet group in comparison to hi-selenite ($p < 0.001$), hi-selenate ($p < 0.001$) and ad-SeMet ($p = 0.018$). All Se-supplemented groups had higher glucose levels when compared to the Se-deficient group without differences within the supplemented groups. There was a trend for increased insulin levels in the hi-selenate group when compared to ad-selenate ($p = 0.069$) and hi-SeMet ($p = 0.059$) and even a significant effect in comparison to the hi-selenite group ($p = 0.033$). The resulting HOMA-IR score was also increased in the hi-selenate group when compared to the ad-selenate ($p = 0.069$), hi-selenite ($p = 0.021$) and hi-SeMet ($p = 0.032$) groups. In order to gain further insights into the molecular mechanisms leading to the observed metabolic changes, the transcriptional profiles of selected genes involved in glucose metabolism and fatty acid synthesis were determined in liver tissues (Table 4). To understand the increase in plasma glucose levels the mRNA expression of two glycolytic enzymes glucokinase (Gck) and pyruvate dehydrogenase (Pdha1), and of the gluconeogenic enzyme glucose-6-phosphatase (G6pc) was analyzed. Pdha1 was slightly decreased in the hi-SeMet group and unaffected in all other groups. In contrast, Gck was increased across all Se-supplemented groups when compared to the Se-deficient group, but stayed unaffected in the hi-SeMet group. Thus, up-regulation of Gck might explain the increased plasma glucose levels in most of the supplemented groups, but does not provide an explanation for higher glucose levels in the hi-SeMet group. In addition, transcript levels of G6pc were down-regulated in the hi-SeMet group indicating that gluconeogenesis was also not enhanced under these conditions. The triglyceride levels in plasma were up-regulated upon Se supplementation, but down-regulated in the hi-SeMet group. A similar expression pattern was observed for the fatty acid synthase (Fasn) and also mRNA levels of acetyl-CoA carboxylase (Acaca) tended to be affected into the same direction (Table 4). The correlation analysis of hepatic Fasn transcript levels and plasma TG levels defined a Pearson coefficient of 0.512 ($p < 0.001$). Phosphorylation of AKT at serine 473 and threonine 308 residues plays a key role in mediating insulin signaling thereby linking the insulin signaling cascade to the energy metabolism. In liver tissues, the threonine phosphorylation of AKT remained unchanged in response to Se supplementation, whereas an increased phosphorylation of AKT^{Ser473} was found in the hi-selenate ($p = 0.009$) and ad-

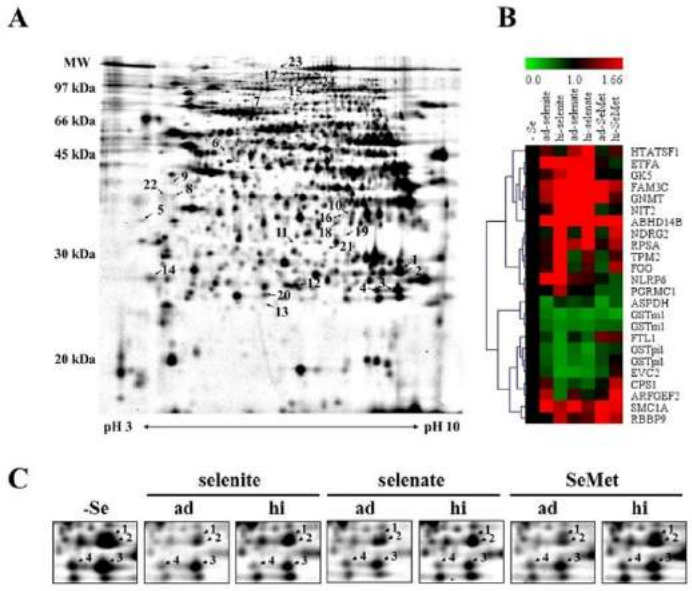

Fig. 3. Proteomic profiling of hepatic tissue samples. (A) Representative spot pattern of a hepatic proteome (hi-selenate) following separation by 2D-DIGE. Differentially expressed proteins which were identified by MALDI-TOF-MS as described in the Material and methods section are marked with numbers (1–24, see also Table 2). (B) Heatmap and cluster analyses regarding the expression profiles of the identified proteins. (C) Representative inserts showing the mapping of GST spots.

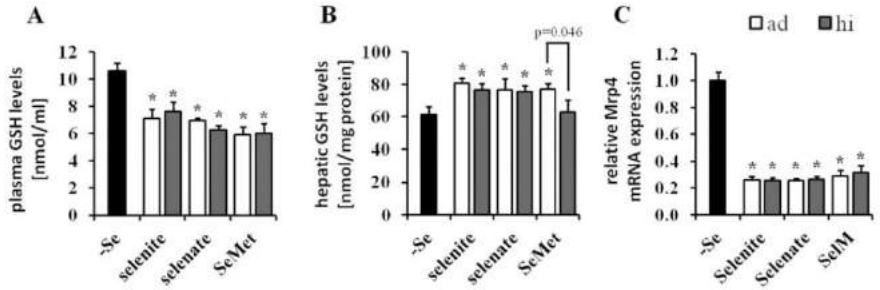

Fig. 4. GSH levels in plasma and liver tissues and hepatic Mrp4 mRNA expression. GSH levels in (A) plasma and (B) liver tissue lysates of mice were spectrophotometrically determined as described in the Material and methods section. In (C) mRNA expression levels of hepatic Mrp4 were determined by qPCR as described in the Material and methods section. Changes are depicted in relation to Se-deficient mice (-Se). Values are given as means ± S.E.M. (n = 8). Significant differences were calculated by one-way ANOVA. *p < 0.05 vs. -Se. ad, adequate (150 μg Se/kg diet); hi, high (750 μg Se/kg diet).

SeMet groups. In the hi-SeMet group, AKT^{Ser473} phosphorylation levels were comparable to that of the Se-deficient group. However, the phosphorylation pattern of PTEN and ERK1/2 remained unchanged (Fig. 6). Since PTP1B is involved in AKT signaling and has been reported to be regulated by Se [43], the PTP activity was determined in hepatic tissues of mice in response to Se supplementation (Fig. 6). There were no obvious differences in PTP activity between all feeding groups, only the PTP activity of the hi-selenate group was reduced in comparison to the ad-selenate ($p = 0.038$) and hi-SeMet ($p = 0.042$) groups.

4. Discussion

The current study aimed to directly compare effects of long-term feeding with three different Se compounds supplied at different concentrations. Previous feeding studies mostly either focused on one Se compound or only considered one concentration. As expected, the adequate concentration of all three Se compounds was sufficient to maximize hepatic selenoprotein expression as indicated by GPx and TrxR activities (Fig. 1) with very marginal additional effects of the respective supplemented diets. While hepatic Se levels were already saturated upon feeding adequate amounts of the inorganic Se compounds selenite and selenate, a SeMet supplementation further increased the hepatic Se content. SeMet differs from the other two Se compounds in that it can directly substitute for Met and thus can be non-specifically incorporated into proteins. It is discussed that SeMet acts as an unregulated Se storage, but SeMet might also have direct catalytic properties e.g. by removal of peroxides and protein oxidation products [51,52]. Although these potential features have not yet been shown in vivo, they might explain why the hi-SeMet group had less oxidized proteins (Fig. 5) and most often exerts distinct effects than the other supplemented groups.

Next to selenoproteins Nrf2 targets are regulated in response to the Se status [8,53]. Already in 1978 an enhanced GST activity during Se deficiency providing a "selenium-independent GPx activity" was described [54] suggesting that this up-regulation of mainly anti-oxidant Nrf2 target genes is an approach to compensate for the loss of the selenium-dependent GPxs and TRs. A similar compensation was also observed in our study, since there was no difference in the amount of oxidized proteins between mice with adequate or deficient Se diets, independent of the Se compound (Fig. 5). In contrast to all Nrf2 target genes analyzed with equally low expression levels in all supplemented groups, GSTpi1 showed another expression pattern. In line with total GST activity, GSTpi1 was also up-regulated in the hi-SeMet group reaching comparable levels as the -Se group (Fig. 2). Based on the higher Se content in the hi-SeMet group this effect appears to be independent from selenoproteins, but rather mediated by SeMet itself. Using rat hepatoma cells, a previous study has shown an increased GST activity in the presence of high concentrations of Se compounds like selenocysteine Se-conjugates, which was specifically mediated by the isoforms GSTa2 and GSTpi1 [55]. The authors suggested that the β-lyase-mediated generation of selenols is essential for GST induction. SeMet can also be a source for selenocysteine and selenols after being metabolized via the trans-sulfuration pathway. Interestingly, the proteome analysis identified additional proteins with a comparable expression pattern as GSTpi1, namely limbin, ARFGEF2, and CPS1. Further experiments are needed to clarify whether these proteins are regulated via the same mechanism in response to hi-SeMet feeding. An additional characteristic feature of the hi-SeMet group was the low hepatic GSH content, which was also low in the -Se group, but up-regulated in all other supplemented groups (Fig. 4). A putative accumulation of hepatic GSH levels due to the strong down-regulation of the GSH exporter Mrp4 should affect all supplemented groups to a similar extend. Thus, reduced GSH levels in both the hi-SeMet and -Se groups might be caused by a higher GSH consumption via GSTs. To clarify this point, more specific analysis including also the GSH/GSSG ratio need to be performed in the future.

In contrast to the hi-SeMet group, the most specific characteristic of the hi-selenite group was the increase in oxidized proteins only identified in this group. At the same time Nrf2 expression was not induced in this group indicating that the more oxidized conditions are not compensated by Nrf2. Thus, the hi-selenite induced redox shift might affect

Table 3
Influence of different Se derivatives on the gene expression of enzymes involved in GSH biosynthesis.

		selenite		selenate		SeMet	
Gene name	-Se	ad	hi	ad	hi	ad	hi
γ-Glutamylcysteine ligase, modifier	1.00 ± 0.07	0.86 ± 0.06	0.78 ± 0.05*	0.75 ± 0.09*	0.73 ± 0.05*	0.79 ± 0.02*	0.83 ± 0.05
γ-Glutamylcysteine ligase, catalytic	1.00 ± 0.11	0.99 ± 0.11	0.91 ± 0.06	0.91 ± 0.08	0.94 ± 0.10	0.88 ± 0.11	0.79 ± 0.13
Glutathione synthetase	1.00 ± 0.07	0.94 ± 0.07	0.91 ± 0.04	1.00 ± 0.07	0.92 ± 0.04	0.96 ± 0.06	1.08 ± 0.08
Glutathione reductase	1.00 ± 0.08	0.97 ± 0.06	0.83 ± 0.08	0.83 ± 0.05	0.81 ± 0.06*	0.70 ± 0.05*	0.92 ± 0.04

Values are given as means ± S.E.M. (n = 8) in relation to Se-deficient (-Se) mice. Levels marked with * indicate significant differences compared to the -Se group ($p < 0.05$; one-way ANOVA). ad, adequate (150 μg Se/kg diet); hi, high (750 μg Se/kg diet).

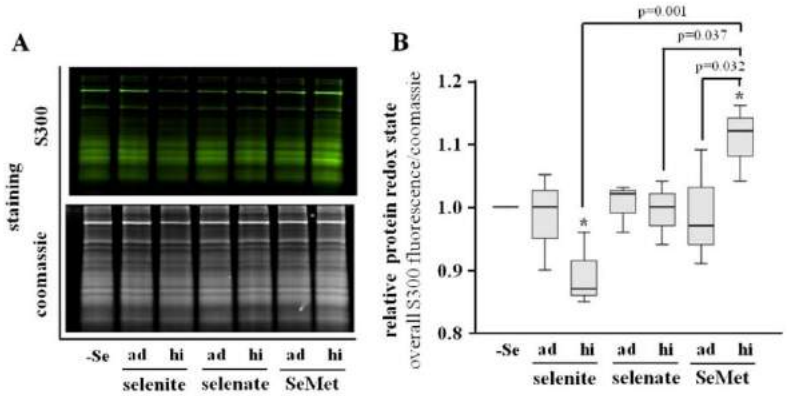

Fig. 5. Oxidative status of hepatic proteomes. (A) The level of reduced hepatic proteins was analyzed by trapping the free thiols with a specific thiol-reactive dye (S-300 dye) prior to separation by SDS-PAGE (n = 3). The overall fluorescence signal of each lane (A, upper panel) was normalized to the respective post-stained Coomassie intensity signal (A, lower panel). (B) Quantification of the protein redox state in relation to the -Se group. Data are given as means ± S.E.M. (n = 3). Significant differences were calculated by one-way ANOVA. *$p < 0.05$ vs. -Se. ad, adequate (150 μg Se/kg diet); hi, high (750 μg Se/kg diet).

different pathways than the one induced by Se deficiency. Three proteins were only up-regulated in the hi-selenite group, namely TPM2, PGRMC1, and NLRP6. Interestingly, NLRP6 is part of the intestinal inflammasome, which is essential for mediating interactions between the host and the intestinal microbiota. Inflammasome deficient mice have a higher risk to develop hepatic steatosis and non-alcoholic liver disease (NAFLD) [56], a condition associated with higher levels of oxidative stress. The second protein related to inflammatory processes was fibrinogen gamma chain (FGG) (Table 2), which also showed the highest fold-change in the hi-selenite group. FGG is up-regulated in hepatoma cells with nutrient overload as an in vitro model for NAFLD [57]. In a DSS-induced murine colitis model, high selenite feeding during acute colitis worsens the course of the disease, while this effect was not detected in the presence of high concentrations of SeMet [58]. These results indicate that high doses of selenite specifically modulate the inflammatory response, but underlying mechanisms need to be further studied.

In addition to redox-modulated effects, the impact of different Se compounds on the energy metabolism was analyzed within this study. At the end of the experiment lower body weights were observed under Se-deficient conditions in comparison to Se supplementation. This observation is in accordance with previous studies performed in rats and chicken [59,60] and can be largely explained by a lower total feed intake in the -Se group. The hi-SeMet group also had an impaired weight gain, which could not be explained by a reduced total feed intake (Table 1). As previously described, blood glucose as well as plasma TG levels were lower in the -Se group [59]. However, the plasma TG content was also reduced in the high-SeMet group and thus could provide an explanation for the lower weight gain of this group. Thus, SeMet supplementation mimics symptoms also observed under methionine restriction [61–64], such as reduced growth, but enhanced feed intake and reduced hepatic GSH levels. Therefore, it cannot yet be excluded that the effects observed in this study might at least partially result from an interference of SeMet with the methionine metabolism.

Plasma insulin levels were only enhanced in the hi-selenate group. Previously, selenate has been reported to exhibit insulin-mimetic properties [60,65,66]. The liver is the key organ for controlling the insulin-mediated metabolism. In the current study an enhanced phosphorylation of AKT^{Ser473} was found in the hi-selenate group, which appears to be the consequence of increased plasma insulin levels, even though the phosphorylation pattern of other insulin-related kinases like ERK1/2 remained unaffected. In addition, the PTP activity was decreased in the hi-selenate group in comparison to ad-selenate feeding, which has been previously shown using leptin receptor deficient db/db

Table 4
Influence of different Se compounds and Se concentrations on parameters of the hepatic energy metabolism and mRNA expression of metabolic proteins.

		selenite		selenate		SeMet	
	-Se	ad	hi	ad	hi	ad	hi
Plasma triglycerides (mg/dl)	70.7 ± 2.41	93.5 ± 7.00*	91.2 ± 3.75*^{b}	95.1 ± 6.98*	91.2 ± 8.20*^{b}	80.3 ± 2.72^{b}	60.9 ± 4.38^{a}
Plasma glucose (mmol/l)	8.68 ± 0.16	9.71 ± 0.53	10.0 ± 0.43*	10.4 ± 0.54*	10.2 ± 0.44*	10.2 ± 0.45*	9.49 ± 0.33
Plasma insulin [μU/ml]	70.0 ± 5.79	64.0 ± 5.62	55.2 ± 2.70^{b}	61.5 ± 5.18	96.2 ± 8.25^{a}	73.3 ± 4.89	60.2 ± 4.44
HOMA-IR	1.19 ± 0.09	1.20 ± 0.10	1.04 ± 0.10^{b}	1.23 ± 0.14	1.70 ± 0.10^{a}	1.45 ± 0.13	1.10 ± 0.14^{b}
Glycolysis							
Glucokinase	1.00 ± 0.09	1.51 ± 0.15*	1.25 ± 0.11	1.20 ± 0.10	1.36 ± 0.08*^{a}	1.24 ± 0.09	1.02 ± 0.14^{b}
Pyruvate dehydrogenase	1.00 ± 0.04	0.98 ± 0.05	0.94 ± 0.04	0.96 ± 0.07	1.03 ± 0.04^{a}	1.01 ± 0.05	0.87 ± 0.04^{b}
Gluconeogenesis							
Glucose 6P phosphatase	1.00 ± 0.07	0.97 ± 0.05^{b}	0.86 ± 0.06^{b}	0.91 ± 0.06	1.09 ± 0.06^{b}	0.92 ± 0.06^{b}	0.68 ± 0.07*^{a}
Fatty acid synthesis							
Acetyl-CoA carboxylase	1.00 ± 0.06	1.19 ± 0.10	1.18 ± 0.07	1.15 ± 0.03	1.27 ± 0.09*^{a}	1.19 ± 0.06^{a}	0.96 ± 0.06^{b}
Fatty acid synthase	1.00 ± 0.14	1.33 ± 0.18	1.18 ± 0.13^{b}	1.28 ± 0.13^{b}	1.75 ± 0.27*^{a}	1.31 ± 0.08	0.90 ± 0.12^{b}

Values are given as means ± S.E.M. (n = 8) in relation to Se-deficient (-Se) mice. Different small letters within a line indicate significant differences within the Se supplemented groups, levels marked with * indicate significant differences compared to the -Se group ($p < 0.05$; one-way ANOVA). ad, adequate (150 μg Se/kg diet); hi, high (750 μg Se/kg diet).

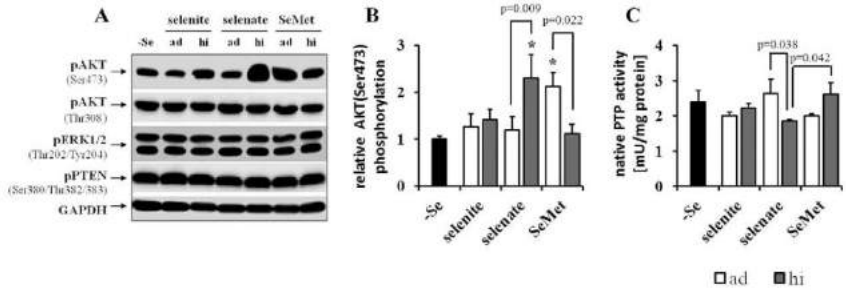

Fig. 6. Phosphorylation pattern and activity of key enzymes involved in the insulin-regulated energy metabolism. (A) Representative phosphorylation status of protein kinases in liver tissues following the 20 week feeding period were determined by Western blot analysis as described in the Material and methods section. (B) Quantification of the AKT^{Ser473} phosphorylation status across the various experimental groups in relation to the -Se group. (C) Total PTP activity was determined under non-reducing conditions as described in the Material and methods section. Data are given as means ± S.E.M. Significant differences were calculated by one-way ANOVA. *$p < 0.05$ vs. -Se. ad, adequate (150 μg Se/kg diet); hi, high (750 μg Se/kg diet); ns, not significant.

mouse, which spontaneously develop obesity and type 2 diabetes [12]. It has been postulated that selenite, the cellular metabolite of selenate, and not selenate itself modifies and thereby inactivates PTPs directly [65]. However, in the current study feeding selenite neither had an effect on PTP activity nor on AKT phosphorylation. In contrast to Mueller and Pallauf [12] also no decreased PTP activity was detected in the Se-deficient group. Next to the hi-selenate group the ad-SeMet group showed comparable effects with more AKT^{Ser473} phosphorylation and equally low PTP activity, but under this condition insulin levels were unmodified. Still, the comparable effects of feeding hi-selenate and ad-SeMet might indicate that Se-Met is a more potent modulator of the metabolic state than selenate. PTPs, in particular PTP1B, counteract the insulin-regulated metabolism by blocking the insulin-stimulated tyrosine phosphorylation of the insulin receptor [67]. PTP1B is known to be a redox-sensitive protein [68] although we did not find a correlation between a high redox status and low PTP activity. In the hi-selenate and ad-SeMet group with the lowest PTP activity no changes in oxidized proteins were detected.

The specific phosphorylation of AKT^{Ser473} mediated by mTOR2 increased glycolysis and lipogenesis in mice [69]. In our study higher plasma TG levels were detected under hi-selenate supplementation (hi pAKT status) when compared to levels detected in response to hi-SeMet supplementation (lower pAKT status). This was further accompanied by an increased mRNA expression of FAS in the hi-selenate group and decreased levels in the hi-SeMet group. In addition, the mRNA expression of the glycolytic enzymes glucokinase (GK) and pyruvate dehydrogenase (PDH) significantly differed between these two experimental groups indicating a lower glycolysis rate in the hi-SeMet group in comparison to hi-selenate. Moreover, the proteomic profiling of liver tissue specimens led to the identification of several proteins, which are involved in the intermediary metabolism. One of those was the putative glycerol kinase 5 (GK5), which is involved in the biosynthesis of TG by synthesizing glycerol-3-phosphate from glycerol and ATP. GK5 was up-regulated in the hi-selenite and in both selenate groups when compared to the Se-deficient group.

5. Concluding remarks

The present study provides evidence that the effects of high concentrations of different Se compounds markedly differ from each other, which might be due to the fact that higher concentrations are not predominantly needed for selenoprotein synthesis. Only high levels of SeMet further increase the hepatic Se status indicating that this Se compound or its metabolites might have effects independent of selenoproteins, which might be mediated by the unspecific incorporation into proteins instead of methionine. In addition, Se oversupply affected the expression and activity of enzymes involved in the regulation of the energy metabolism, but the underlying mechanisms of the different Se compounds have to be clarified in the future. Thus, it is important to consider compound-specific effects when supplementing with selenium. Based on this study selenite and selenate might have advantages over supplementing with SeMet.

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Transparency document

The Transparency document associated with this article can be found, in online version.

Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbagen.2016.08.015.

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4.2 Article II - Altered protein expression pattern in colon tissue of mice upon supplementation with distinct selenium compounds

Altered protein expression pattern in colon tissue of mice upon supplementation with distinct selenium compounds

Jette Rahn, **Claudia Lennicke**, Andreas S. Müller, Ludger A. Wessjohann,
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RESEARCH ARTICLE

Altered protein expression pattern in colon tissue of mice upon supplementation with distinct selenium compounds

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The essential trace element selenium (Se) is controversially discussed concerning its role in health and disease. Its various physiological functions are largely mediated by Se incorporation in the catalytic center of selenoproteins. In order to gain insights into the impact of Se deficiency and of supplementation with different Se compounds (selenite, selenate, selenomethionine) at defined concentrations (recommended, 150 μg/kg diet; excessive, 750 μg/kg diet) in murine colon tissues, a 20-week feeding experiment was performed followed by analysis of the protein expression pattern of colon tissue specimens by 2D-DIGE and MALDI-TOF MS. Using this approach, 24 protein spots were identified to be significantly regulated by the different Se compounds. These included the antioxidant enzyme peroxiredoxin-5 (PRDX5), proteins with binding capabilities, such as cofilin-1 (COF1), calmodulin, and annexin A2 (ANXA2), and proteins involved in catalytic processes, such as 6-phosphogluconate dehydrogenase (6PGD). Furthermore, the Se compounds demonstrated a differential impact on the expression of the identified proteins. Selected target structures were validated by qPCR and Western blot which mainly confirmed the proteomic profiling data. Thus, novel Se-regulated proteins in colon tissues have been identified, which expand our understanding of the physiologic role of Se in colon tissue.

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Abbreviations: **ABHD14B**, alpha/beta hydrolase domain-containing protein 14B; **CaM**, calmodulin; **CLCA1**, calcium-activated chloride channel regulator 1; **COR**, coronin; **CRC**, colorectal cancer; **GAPDH**, glyceraldehyde 3-phosphate dehydrogenase; **Gpx/GPx**, glutathione peroxidase; **HMG-CoA/HMGCS**, 3-hydroxy-3-methylglutaryl-CoA synthase; **hnRNP**, heterogeneous nuclear ribonucleoprotein; **IPS**, internal protein standard; **KEAP1**, kelch-like ECH-associated protein 1; **PRDX**, peroxiredoxin; **qPCR**, quantitative PCR; **Se**, selenium; **–Se**, Se-deficient; **Sec**, selenocysteine; **SeMet**, selenomethionine; **Txnrd1/TR1**, thioredoxin reductase 1

Colour Online: See the article online to view Figs. 2, 3, and Table 2 in colour.

1 Introduction

Selenium (Se), as part of the 21st proteinogenic amino acid selenocysteine (Sec), is incorporated into functional selenoproteins via a complex molecular mechanism. The Sec

Significance of the study

The impact of diet supplementation with selenium compounds is still controversially discussed. This is largely due to conflicting results regarding its either beneficial or detrimental role in health and disease. However, since many of these studies used either different types of Se compounds or differed in the applied doses, the resulting data are hardly comparable. In order to gain novel insights into the role that Se supplementation has on the regulation of the (overall) protein expression pattern in murine colon tissues side-by-side long-term feeding experiments in groups of C57BL/6J mice were performed with either adequate or high doses of selenite, selenate, and SeMet, respectively, whereas the control group received a Se-deficient diet. This experimental strategy allowed to monitor and to compare the impact of the most frequently applied Se compounds at distinct doses on the regulation at the transcriptional as well as at protein expression level of selected selenoproteins next to their impact on the general proteomic profile.

insertion depends in particular on the presence of the Sec Insertion Sequence (SECIS) element in the 3' UTR of the selenoprotein coding sequence [1, 2]. So far 25 genes encoding for selenoproteins have been identified in humans, whereas the rodent genome encodes for 24 selenoproteins [3]. Glutathione peroxidases (GPx) and thioredoxin reductases (TR) involved in antioxidative defense and in maintenance of the intracellular redox homeostasis represent the two best characterized selenoprotein families [2]. Besides other functions selenoproteins act in oxidative stress defense and therefore an adequate (ad) Se supply might be a useful strategy to prevent human diseases such as metabolic disorders or even cancer linked to impaired antioxidative stress responses. Next to the supplemented Se concentrations also the Se compounds used might have different impacts on diseases, since a distinct bioavailability and biological activity of the different Se compounds exist [4]. The most commonly used inorganic Se supplements are selenite and selenate, whereas the organic selenomethionine (SeMet) is the major Se compound of Se-enriched yeast [5]. The uptake and metabolism of Se compounds depend on its respective chemical form with lower absorption rates of inorganic when compared to organic forms such as SeMet [2,6,7]. While the uptake of selenate and SeMet occurs through an active transcellular transport, selenite is passively absorbed [6, 8, 9]. Furthermore SeMet, but not selenate can be unspecifically incorporated into proteins instead of methionine [10] allowing a storage of Se in the body.

Selenoproteins are differentially located within cellular compartments as well as differentially expressed in tissues, e.g. GPx4 is highly expressed in the testis and in the intestinal epithelium GPx2 expression is enriched in rodents [11]. Next to selenoproteins, it was also shown that the expression of nonselenoproteins can be influenced upon the availability of Se. For instance microarray analysis demonstrated an activation of the transcription factor (TF) Nrf2 (nuclear factor erythroid 2 [NF-E2]-related factor 2) under Se deficiency conditions. This TF regulates the transcription of enzymes involved in the antioxidant response and in phase II detoxification processes including GPx2 [12]. Due to antioxidant and anti-inflammatory [13] functions of GPx2 its expression has been associated with tumorigenesis, in particular in colorectal cancer (CRC) [14]. CRC is associated with a significant death rate at least in Western countries, in particular for elderly people diagnosed with metastatic disease [15]. Taking further into account that its tumorigenesis is frequently associated with and promoted by a pro-inflammatory tumor micromilieu including the generation of reactive oxygen species (ROS) it seems reasonable to bolster the antioxidant response capacity as a potential preventive strategy even by supplementation with Se.

Using 2DE-based proteomics the influence of Se compounds on cancer cells, e.g. prostate cancer lines [16, 17], HeLa cells [18] and Caco-2 cells [19], on rat plasma and on different rodent tissues has been previously investigated [20–22]. However, in all these studies only effects of single selenocompounds have been evaluated. Therefore, this study aimed to directly compare different concentrations of different selenocompounds in one mouse feeding experiment. Employing comparative 2D-DIGE we previously reported that selenite, selenate, and SeMet supplementations differentially affect the expression of hepatic proteins involved in metabolism, inflammation, and cell-cycle regulation [23]. In order to gain more insights into the impact of selenite, selenate, and SeMet in recommended and supranutritive levels in comparison to a Se-deficient group (–Se) protein expression profiles of murine colon tissues were compared by 2D-DIGE followed by MALDI-TOF MS and further validated by quantitative PCR (qPCR) and Western blot analyses.

2 Materials and methods

2.1 Animals and treatment

Male C57BL/6J mice were obtained from Harlan (Horst, Netherlands) and kept under conventional conditions with free access to their respective diets and autoclaved tap water. The feeding experiment was approved by the ethical committee of the Martin Luther University Halle-Wittenberg (42502-2-1187MLU). Seven experimental groups were generated. In each group eight animals were randomly assigned.

The groups were fed with diets supplemented with either adequate (ad; 150 μg Se/kg diet) or supra-nutritive (high, hi; 750 μg Se/kg diet) Se concentrations in form of selenite, selenate, and SeMet, respectively, whereas the control group received a Se-deficient diet (–Se) as previously described [23].

2.2 Sample collection and preparation

After a feeding period of 20 weeks mice were decapitated under CO_2 anesthesia and colon tissue specimens were excised, snap frozen in liquid nitrogen, and stored at −80°C until further use. The colon lysates for GPx and TR activity assays were prepared in a 1:25 w/v dilution in Tris buffer (100 mM Tris (Applichem, Darmstadt, Germany), 300 mM KCL, 0.1% Triton X-100 (Carl Roth GmbH, Germany), protease inhibitor cocktail II (Calbiochem, Merck Millipore, Darmstadt, Germany), pH 7.6) using a tissue lyser (Qiagen, Hilden, Germany) for 2 × 2 min at 30 Hz. These lysates were centrifuged for 30 min at 14 000 rpm and 4°C, supernatants collected and stored at −80°C until further analysis. Protein concentrations were determined with the Bradford method as previously described [24]. For the proteomic analysis, colonic tissue samples were washed thrice in ice-cold PBS, subsequently grinded (Readyprep mini grinders, Bio-Rad Laboratories GmbH, Munich, Germany) and then lysed in DIGE lysis buffer (30 mM Tris, 7 M urea (Applichem), 2 M thiourea (Sigma, Deisenhofen, Germany), 4% w/v CHAPS (Applichem), pH 8.5). After sonication (SONOPULS HD 2070, BANDELIN electronic GmbH & Co. KG, Berlin, Germany) and centrifugation (13 000 rpm, 90 min, 15°C) supernatants were collected and stored at −80°C.

2.3 GPx and TR enzyme activities

Total GPx activities was measured by the indirect spectrophotometrically method coupled to glutathione reductase using the method of Lawrence and Burk [25], which was modified for 96-well microtiter plates [26]. TR catalyze the formation of 2-nitro-5-thiobenzoic acid (TNB) by using the substrate 5,5′-dithio-bis (2-nitrobenzoic acid) (DTNB) and consumption of NADPH [27], which was spectrophotometrically determined at 412 nm in a plate reader (Tecan Group Ltd., Maennedorf, Switzerland) for 2 min at 25°C as previously described [28].

2.4 Proteome analysis

2.4.1 DIGE labeling, 2DE, and software evaluation

The minimal DIGE labeling approach was performed with the SERVA Lightning SciDye Set (SERVA Electrophoresis GmbH, Heidelberg, Germany) according to the manufacturer's instructions. In brief, 25 μg protein of each sample was labeled with 200 pmol of either Sci3 or Sci5. In addition, 25 μg of protein composed of an equal mixture of all samples serving as an internal protein standard (IPS) were labeled with 200 pmol of Sci2. Isoelectric focusing (IEF) using Immobiline DryStrips pH 3–10 NL (18 cm, GE Healthcare, Germany) and second-dimension SDS-PAGE separation were performed as previously described [29]. After separation the gels were briefly washed in distilled water, fixed for 45 min in fixing solution (10% acetic acid, 40% EtOH), and after further washing steps in distilled water scanned with a laser scanner (Fujifilm FLA-5100, FUJIFILM Europe GmbH, Germany) as recently described [30]. The Delta2D software package version 4.6 (Decodon GmbH, Greifswald, Germany) was used for gel image analysis according to the manufacturer's guidelines. Proteins were defined as differential expressed when they reached a cut-off value set at a change of at least 1.5-fold (increased or decreased) and a p-value of <0.05 between any of the seven experimental groups.

2.4.2 Spot picking, in-gel digestion, and MS analysis

Similarly to the analytic gels, preparative gels were generated, but loaded with 500 μg of protein/gel and after 2D separation stained with CCB (Applichem) as previously described [31]. Respective picking lists were created in Delta2D and imported into the Easy4Sience software (Herolab GmbH, Wiesloch, Germany) prior to automatic spot picking via a spot picker (Spot Hunter, Herolab). The spots were directly picked into 96-well *reaction plates* (Intavis, Cologne, Germany) and subsequently destained in a robot-assisted way, in-gel digested, purified with ZipTips (C18, Merck Millipore), and spotted (DigestPro, Intavis) onto a MALDI target plate (MTP 384 ground steel, Bruker Daltonics Inc., Bremen, Germany). The steps of digestion and spotting are listed in the Supporting Information Table 1. The MALDI-TOF MS analysis was performed on an ultrafleXtreme™ mass spectrometer (Bruker Daltonics Inc.) in positive reflector mode using an accelerating voltage of 25 kV and the spectra were calibrated externally using Bruker's peptide calibration standard II (Bruker Daltonics Inc.). The PMF dataset analyses were performed using the MASCOT search engine (Matrix Science, Dauhaim, USA) with the following parameter settings: (i) *mus musculus* sequences; (ii) fixed modification, carbamidomethylation of cysteines; (iii) variable modification, oxidation of methionine; (iv) cleavage enzyme, trypsin; (v) a maximum of one missed cleavage was allowed; and (vi) the mass tolerance (monoisotopic) was 50.0 ppm. When the MOWES score exceeded 55 according to the MASCOT-defined significance threshold for false-positive events at $p < 0.05$, the proteins were assigned. To classify the identified proteins the PANTHER GO software [32] was used and the protein interaction analysis was performed with STRING [33].

2.5 Real-time PCR (qPCR)

RNA was isolated using the acid guanidinium thiocyanate-phenol-chloroform extraction method [34] and subjected to PCR analysis as recently described [35]. Briefly, 1 μg of RNA was reverse transcribed into cDNA using the *RevertAid First Strand cDNA Synthesis Kit* and *Oligo dT primers* (Thermo Scientific) according to the manufacturer's protocol. SYBR Green I was used as fluorescent reporter during the qPCR analysis. Target-specific primers (Supporting Information Table 2) were designed with the tool Primer-BLAST [36]. Amplification data were analyzed with the Rotor-Gene 6000™ series software (Qiagen) using the method according to Pfaffl [37]. Amplifications of ribosomal protein L13a (RPL13a) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were used for normalization. The data are represented as relative mRNA expression levels as x-fold of the –Se group.

2.6 Western blot analysis

Western blot analyses were performed as recently described [35]. Thirty μg total protein per lane were separated on 12% SDS polyacrylamide gels and transferred onto nitrocellulose membrane (GE Healthcare), which were subsequently processed as previously mentioned [35]. For immunodetection the primary (monoclonal or polyclonal) antibodies anti-Cofilin (#5175) and anti-GAPDH (#2118) were purchased from Cell Signaling Technology (CST, New England BioLabs GmbH, Frankfurt, Germany) and the anti-Calmodulin antibody (ab45689) was obtained from Abcam (Abcam plc, Cambridge, UK). The anti-GPx2 antiserum [38] was kindly provided by Regina Brigelius-Flohé/Anna P. Kipp from the German Institute of Human Nutrition Potsdam-Rehbrücke (Nuthetal, Germany). The relevant secondary HRP-coupled anti-rabbit antibodies were purchased from CST and Dako (Dako Deutschland GmbH, Hamburg, Germany).

2.7 Statistical analysis

Mean values were calculated from results of four independent animals/group or eight independent animals/group in the case of qPCR analysis and enzyme activities and given as means ± their SEM. SPSS 22 software (IBM, USA) was used to analyze significant differences within the groups; therefore one-way ANOVA was applied after declaring the normality of distribution (Shapiro-Wilk test) and the homogeneity of variance (Levene test). If variances were homogenous the least significant difference (LSD) test was used, otherwise the Games-Howell test was employed. Differences between the groups were considered to be significant at a p-value of < 0.05.

3 Results

3.1 Impact of different Se compounds on mRNA expression levels and enzyme activities of antioxidant selenoproteins in colon tissues

The enzyme activities of GPx and TR are established markers of the Se status within tissues [39]. Both activities were significantly enhanced in all Se-supplemented groups when compared to the –Se group after the 20-week of feeding experiment. In addition, the ad-selenate supplemented group exhibited significantly lower colonic GPx activities when compared to the ad-selenite group (Fig. 1A and B). Due to the fact that the GPx activities are composed of the GPx1 and GPx2 activity the protein expression of GPx2 was determined by Western blotting. The GPx2 protein expression was threefold increased by ad-selenite, fourfold increased by ad-selenate and ad-SeMet when compared to the Se deficiency group. Furthermore, the hi-SeMet group exhibited the highest GPx2 protein expression levels resulting in a fivefold increase compared to the –Se group and showed a significant upregulation of the GPx2 protein level when compared to ad-SeMet, hi-selenite, and hi-selenate supplemented groups (Fig. 1C and D). After Se supplementation, the Gpx1 mRNA levels were significantly increased when compared to the –Se group, while Txnrd1 was slightly, but non-significantly upregulated in the Se-supplemented groups when compared to the –Se group. The Gpx2 mRNA levels remained unchanged (Table 1).

3.2 Identification of novel Se-regulated proteins via a gel-based proteomic approach

To identify proteins, which were differentially expressed upon supplementation with different Se compounds, 2D-DIGE coupled with MALDI-TOF MS analysis was performed. In total, 825 protein spots were detected on the synthetic fused image in Delta2D (Decodon), from which 40 protein spots were significantly differentially expressed (1.5-fold change ($p < 0.05$)). A total of 23/40 single protein characters were identified by MALDI-TOF MS (Fig. 2A, Table 2, Fig. 3A). Thus 2/40 protein spots contained multiple protein identities and were excluded from further analysis. The identified proteins were classified according to their molecular function (Table 2, Fig. 2B) and involvement in biological processes (Fig. 2C). Most of the proteins exhibit binding capabilities (44%) followed by catalytic activities (39%). In terms of the implicated biological process according to the GO classification most of the proteins were classified in cellular processes (25%), metabolic processes (24%), or cellular localization (17%). Some proteins, which share the GO term "binding", e.g. calpain small subunit 1 (CSS1), cofilin-1 (COF1), coronin (COR)1A), and COR1B interact with each other as demonstrated in the interaction network generated with STRING (Fig. 3B). In three cases, at least two isoforms of a given

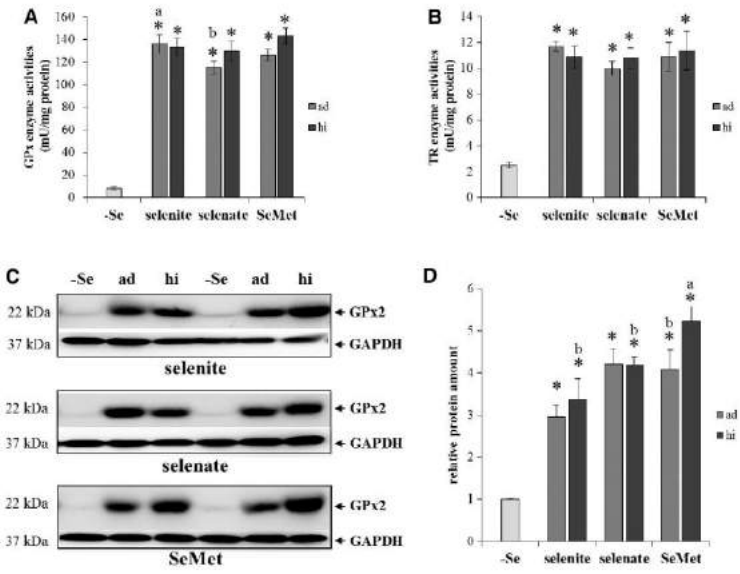

Figure 1. Influence of different Se supplements on enzyme activities and protein expression levels of colonic selenoproteins. Total enzyme activities of (A) GPx and (B) TR were spectrophotometrically determined as described in Section 2. (C) Representative protein expression levels of GPx2 after selenite (upper panel), selenate (middle panel), and SeMet (lower panel) supplementation if compared to the –Se group. Western blot analysis was performed as described in Section 2. (D) Relative quantification of GPx2 expression levels normalized to GAPDH and related to –Se. Data represent means ± SEM. *Significant differences in comparison to the –Se group and different superscripted small letters demonstrate significant differences within Se-supplemented groups ($p < 0.05$). ad, adequate (150 μg/kg diet); hi, high (750 μg/kg diet).

protein identity were identified to be regulated by Se supplementation, such as heterogeneous nuclear ribonucleoprotein L (hnRNP) L and hnRNP K, COR1A and COR1B, as well as cytoplasmic hydroxymethylglutaryl-CoA synthase (HMG-CoA) S1 and mitochondrial HMG-CoA S2. The expression levels of both hnRNP proteins were significantly downregulated in the hi-SeMet supplemented groups when compared to the –Se group, while the supplementation with selenite or selenate showed no significant effects. Furthermore, the HMG-CoA S1 isoform was significantly downregulated in the hi-selenate and hi-SeMet groups when compared to the –Se group, whereas the HMG-CoA S2 isoform was significantly decreased in the hi-selenite group if compared to the –Se group and ad-selenite group (Table 2).

3.3 Validation of selected protein structures identified by 2D-DIGE

The mRNA and protein expression levels of selected target structures defined as regulated in response to supplementations with different Se compounds in colon tissues were analyzed by qPCR and/or Western blot analyses. As shown in Table 3, the mRNA expression levels of peroxiredoxin 5 (Prdx5) and Cofl1 were not altered, but the COF1 protein expression levels significantly increased in the hi-selenite and ad-selenate supplemented groups when compared to the ad-SeMet supplemented group. In addition, hi-SeMet supplementation enhanced the COF1 protein expression level when compared to the adequate SeMet-supplemented group

Table 1. Influence of different Se compounds on gene expression of antioxidative selenoproteins in colon tissues of mice

	–Se	selenite		selenate		SeMet	
		ad	hi	ad	hi	ad	hi
mRNA expression							
Glutathione peroxidase 1 (Gpx1)	1.00 ± 0.11	4.82 ± 0.13*	4.82 ± 0.17*	4.56 ± 0.24*	4.86 ± 0.19*	4.96 ± 0.21*	5.15 ± 0.20*
Glutathione peroxidase 2 (Gpx2)	1.00 ± 0.18	0.95 ± 0.13	1.02 ± 0.15	0.77 ± 0.14	0.95 ± 0.15	0.85 ± 0.10	1.20 ± 0.15
Thioredoxin reductase 1 (Txnrd1)	1.00 ± 0.15	1.42 ± 0.16	1.28 ± 0.18	1.42 ± 0.13	1.31 ± 0.18	1.29 ± 0.17	1.28 ± 0.11

qPCR analysis was performed as described in Section 2. Changes of mRNA levels in the Se-treated groups are presented in relation to –Se group. Values are given as mean ± SEM comprising eight animals per group. ad, adequate (150 μg/kg diet); hi, high (750 μg/kg diet).
*Significant differences compared to –Se group ($p < 0.05$).

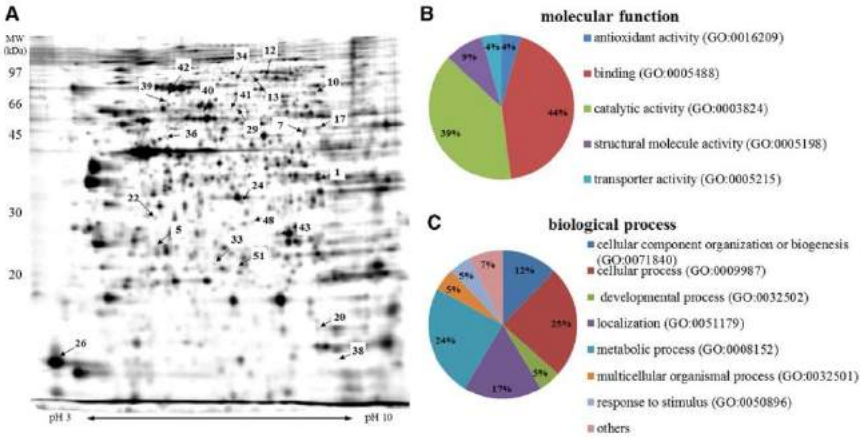

Figure 2. Spot pattern of the differentially regulated proteins from murine colon tissues in response to Se supplementation. (A) Representative spot pattern of a colon tissue proteome (sample –Se). 2D-DIGE was performed as described in Section 2. Identified spots are marked with numbers (see also Table 2). Classification of the differentially expressed proteins according to (B) molecular function and (C) biological process in colon tissue. The classification is based on the PANTHER.org software tool. The term others is comprised of the GO terms: biological regulation (GO:0065007), immune system process (GO:0002376), and locomotion (GO:0040011).

($p = 0.069$) (Fig. 4A). In line with the results from the 2D-DIGE analysis the mRNA levels of Anxa2 were significantly downregulated in the ad-SeMet group when compared to the −Se group (Table 2). On the other hand, the Anxa2 mRNA levels were significantly downregulated in the ad-selenite group when compared to the –Se group (Table 3), while the protein expression levels remained unchanged (Table 2). Among the panel of identified proteins, alpha/beta hydrolase domain-containing protein 14B (ABHD14B), prelamin-A/C (LMNA), and calcium-activated chloride channel regulator 1 (CLCA1) exhibited a strong upregulation of the respective protein levels obtained from the 2D-DIGE analysis across all Se-supplemented groups when compared to the –Se group, which could at least be confirmed by qPCR analyses (Table 3). Moreover, the protein expression level of calmodulin (CaM), a calcium binding protein, was significantly decreased in the ad-selenite and hi-selenate groups if compared to –Se group (Fig. 4B).

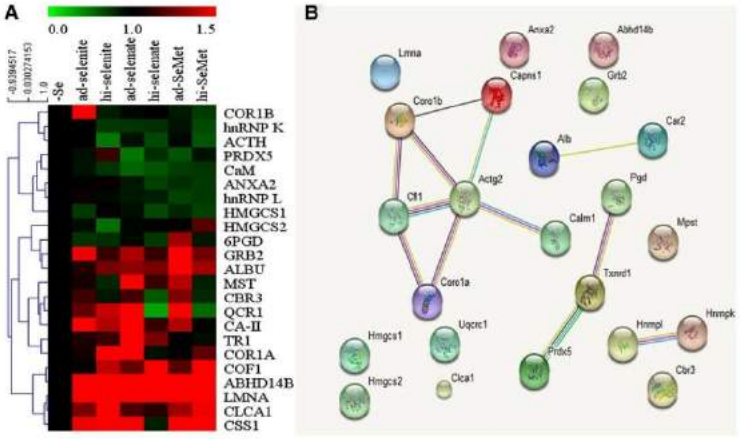

Figure 3. Cluster analysis of differentially expressed colonic proteins. (A) Heat map and hierarchal clustering analyses and (B) protein interaction network plot of the colon tissue proteome. The heat map and the hierarchal clustering were generated by using the MultiExperiment Viewer (MeV, The Institute for Genomic Research (TIGR)) and the interaction network was created with STRING. ad, adequate (150 μg/kg diet); hi, high (750 μg/kg diet).

Table 2. Differentially expressed protein in colon tissues of mice after treatment with different Se compounds

No	Protein name	Gene	Uniprot ID	Mass (Da)	p/	MOWSE score	Peptides matched	Sequence coverage (%)	selenite		selenate		SeMet	
									ad	hi	ad	hi	ad	hi
antioxidant activity (GO:0016209)														
38	Peroxiredoxin-5, mitochondrial (PRDX5)	Prdx5	P99029	22 226	9.10	78	8	41	0.90	1.14	0.48	0.77	0.62	0.90
binding (GO:0005488)														
1	Annexin A2 (ANXA2)	Anxa2	P07356	38 937	7.55	133	17	52	1.03	1.03	0.87	0.69	0.78	0.72
5	Calpain small subunit 1 (CSS1)	Capns1	O88456	28 559	5.41	59	9	41	1.48	4.36*	2.02	0.83	1.59	2.91
10	Heterogeneous nuclear ribonucleoprotein L (hnRNP L)	Hnrnpl	Q8R081	64 550	8.33	74	13	19	1.04	0.93	0.87	0.84	0.68	0.73
39	Heterogeneous nuclear ribonucleoprotein K (hnRNP K)	Hnrnpk	P61979	51 230	5.39	56	11	22	1.00	0.73	0.75	0.78	0.89	0.65
20	Cofilin-1 (COF1)	Cfl1	P18760	18 776	8.22	107	9	46	1.03	1.13	1.23	1.67*	1.20	1.53*
29	Coronin-1A (COR1A)	Coro1a	O89053	51 641	6.05	61	9	18	1.07	1.58	1.90	0.90	1.03	1.21
42	Coronin-1B (COR1B)	Coro1b	Q9WUM3	54 505	5.54	85	13	20	1.55	0.85*	0.94	0.99	0.86	0.71
26	Calmodulin (CaM)	Calm1	P62204	16 827	4.09	67	7	42	0.89	0.75	0.55	0.62	0.71	0.74
33	Growth factor receptor-bound protein 2 (GRB2)	Grb2	Q60631	25 336	5.89	72	12	36	1.48	1.14	1.37	1.13	1.81	1.25*
34	Serum albumin (ALBU)	Alb	P07724	70 700	5.75	65	13	26	1.09[a]	1.26	1.30	1.27	1.61[b]	1.34
catalytic activity (GO:0003824)														
7	6-Phosphogluconate dehydrogenase, decarboxylating (6PGD)	Pgd	Q9DCD0	53 726	6.81	65	15	32	0.75[a]	0.77	0.94	0.73	1.34[b]	0.87*
36	Cytochrome b-c1 complex subunit 1, mitochondrial (QCR1)	Uqcrc1	Q9CZ13	53 446	5.81	215	26	45	1.22	1.41	2.55	0.32*	1.93	0.53
40	Hydroxymethylglutaryl-CoA synthase, cytoplasmic (HMG-CoA S1)	Hmgcs1	Q8JZK9	58 160	5.65	64	10	20	0.73	1.03	0.91	0.66	0.80	0.69
17	Hydroxymethylglutaryl-CoA synthase, mitochondrial (HMG-CoA S2)	Hmgcs2	P54869	57 300	8.66	95	20	30	1.23	0.51*	0.95	1.02	1.02	1.20
24	3-Mercaptopyruvate sulfurtransferase (MST)	Mpst	O99J99	33 231	6.11	97	9	22	1.07	0.80	1.52	1.14*	1.38	0.84*
41	Thioredoxin reductase 1, cytoplasmic (TR1)	Txnrd1	Q9JMH6	68 239	7.42	62	10	22	1.09	1.14	1.55	1.26	0.98	0.91
43	Carbonic anhydrase 2 (CA-II)	Ca2	P00920	29 129	6.49	57	8	41	1.90	1.36	1.91	1.13*	1.37	0.97
48	Carbonyl reductase [NADPH] 3 (CBR3)	Cbr3	Q8K354	31 333	6.15	100	12	50	1.15	0.87	1.16	0.59*	1.29	0.79
51	Alpha/beta hydrolase domain-containing protein 14B (ABHD14B)	Abhd14b	Q8VCR7	22 551	5.82	58	7	42	1.95	3.03*	2.33	3.10	2.33	3.09
structural molecule activity (GO:0005198)														
13	Prelamin-A/C (LMNA)	Lmna	P48678	74 478	6.54	89	19	32	1.50	2.23*	1.50	1.50	1.85	1.86
22	Actin, gamma-enteric smooth muscle (ACTH)	Actg2	P63268	42 249	5.31	56	8	19	0.99	0.48*	0.90	0.67	0.95	0.61*
transporter activity (GO:0005215)														
12	Calcium-activated chloride channel regulator 1 (CLCA1)	Clca1	Q9D726	100 807	5.67	107	19	23	1.26	1.69	1.17	1.11	1.34	1.48

Values are given as means ($n = 4$) in relation to Se-deficient (–Se) mice. Red/green filled boxes indicate significant upregulated/downregulated proteins in relation to the –Se group.
*Significant differences of hi Se intake to the ad Se intake within one indicated Se compound ($p < 0.05$).
ad, adequate (150 µg Se/kg diet); hi, high (750 µg Se/kg diet).

Table 3. Validation of selected differentially expressed proteins in mice colon tissues after Se treatment via qPCR

gene	–Se	selenite		selenate		SeMet	
		ad	hi	ad	hi	ad	hi
Prdx5	1.00 ± 0.12	0.93 ± 0.05	1.05 ± 0.06	0.96 ± 0.07	0.91 ± 0.09	0.89 ± 0.08	1.00 ± 0.05
Anxa2	1.00 ± 0.13	0.69 ± 0.09*	0.81 ± 0.10	0.77 ± 0.05	0.75 ± 0.09	0.70 ± 0.05*	0.80 ± 0.07
Cofl1	1.00 ± 0.06	1.00 ± 0.11	1.06 ± 0.11	1.09 ± 0.08	1.05 ± 0.08	1.06 ± 0.08	1.09 ± 0.08
Pgd	1.00 ± 0.18	0.74 ± 0.07*	0.82 ± 0.10	0.81 ± 0.08	0.91 ± 0.10	0.97 ± 0.07	0.92 ± 0.11
Abhd14b	1.00 ± 0.12	1.43 ± 0.11	1.33 ± 0.26	1.33 ± 0.13	1.65 ± 0.25*	1.46 ± 0.10	1.53 ± 0.08*
Lmna	1.00 ± 0.09	1.34 ± 0.06*	1.22 ± 0.05*	1.24 ± 0.05*	1.26 ± 0.07*	1.17 ± 0.05*	1.23 ± 0.04*
Clca1	1.00 ± 0.24	2.29 ± 0.32*	2.14 ± 0.34*	1.99 ± 0.20*	2.77 ± 0.25*	2.59 ± 0.34*	2.72 ± 0.43*

qPCR analysis was done as described in Section 2. Changes of mRNA levels in the Se-treated groups are presented in relation to –Se group. Values are given as mean ± SEM comprising eight animals per group. *Significant differences compared to –Se group ($p < 0.05$). ad, adequate (150 μg/kg diet); hi, high (750 μg/kg diet).

4 Discussion

Se exhibits its physiological functions mainly by its catalytic function in the active center of selenoproteins. The activities of selenoproteins in particular of GPx are accepted biomarkers of tissue Se status [39]. As demonstrated in the current study total GPx activity in colon was more than tenfold downregulated in the –Se group when compared to the Se-supplemented groups indicating severe Se deficiency in the control group. In addition, the mRNA levels of Gpx2 were not altered (Table 1), which is in line with the current opinion of an existing hierarchy in selenoprotein synthesis, whereby Gpx2 ranks at a high position [40]. The highest GPx2 protein expression levels were observed in the hi-SeMet group. This might be explained by its very effective absorption rate through the active transcellular transport [41] as well as the organic nature of SeMet.

Next to antioxidative selenoproteins, the antioxidative enzyme PRDX5 was identified to be regulated in response to Se supplementation. Prdx5, Txnrd1, as well as Gpx2 are targets of the transcription factor Nrf2 [12, 42], the major regulator of the cellular antioxidant repsonse and phase II detoxification. Under physiological conditions, Nrf2 is bound through the protein kelch-like ECH-associated protein 1 (KEAP1) and localized in the cytoplasm. The release of Nrf2 from KEAP1 is induced under oxidative stress leading to the translocation of Nrf2 into the nucleus, where it binds to the antioxidant response element (ARE) within the promotor regions of its target genes and activates the expression of several cytoprotective genes [43]. In our study, PRDX5 was significantly downregulated following ad-selenate and ad-SeMet supplementation when compared to the Se group (Table 2). This result is supported by a previous study showing that PRDX2 and PRDX4 were downregulated in human prostate cancer cells in response to supplementation with 40 μmol/L selenite [17] and further suggests that Se deficiency induces oxidative stress and Nrf2 activation. This hypothesis was further strengthened by data from an independent microarray analysis demonstrating the regulation of several Nrf2 target genes under Se deficiency in colon tissues, in which next to Gpx2 and Txnrd1 Pgd was mentioned as Nrf2 target [12]. In this study, the Pgd gene was rather upregulated under SeMet supplementation when compared to Se deficiency conditions. The current analysis of murine colon tissues showed a slight, but not significantly increased PGD protein expression level in the ad-SeMet supplemented group when compared to the –Se (Table 2). In contrast, the ad-selenite group revealed significantly reduced Pgd mRNA levels accompanied by a slight reduction in the PGD protein expression level in the ad-selenite group when compared to the –Se group (Tables 2 and 3) demonstrating a different impact of the Se compounds used on the PGD expression.

Recently, a significantly downregulation of GSTm1 and GSTp1 transcript and protein levels as well as GST enzyme activities were detected in liver tissues of the same mouse strain in response to Se supplementation when compared to the corresponding Se-deficient group [23], which was also demonstrated in rat liver tissues by Blum and coworkers [44]. Moreover, the mRNA level and enzyme activity of the Nrf2 target NAD(P)H dehydrogenase [quinone] 1 (NQO1) was significantly downregulated in the Se-supplemented groups when compared to the –Se group in liver [23]. However, these findings could not be observed in the colon (data not shown) indicating that there might be differences in the general redox-capacity between murine liver and colon. Interestingly, ABHD14B, a transcription factor known as CCG1 (cell cycle arrest in G1)-interacting factor B (CIB) [45], was significantly upregulated in colon and liver tissues in response to Se supplementation when compared to –Se (Tables 2 and 3) [23]. Although the function of ABHD14B has not yet been fully characterized, it is involved in phase II conjugation processes especially in sulfonation (reactome.org). Sulfonation, a key posttranslational modification process [46], is important for the metabolism of several exogenous and endogenous compounds [47]. Furthermore, it has been associated with tumorigenesis and patients' survival [46]. In the gastrointestinal tract sulfonation is essential since mucins, which represent the major macromular component of the gastrointestinal mucus, hold sulfonated carboanhydrates [48]. A reduced sulfomucin amount is related to gastrointestinal diseases, e.g. cancer

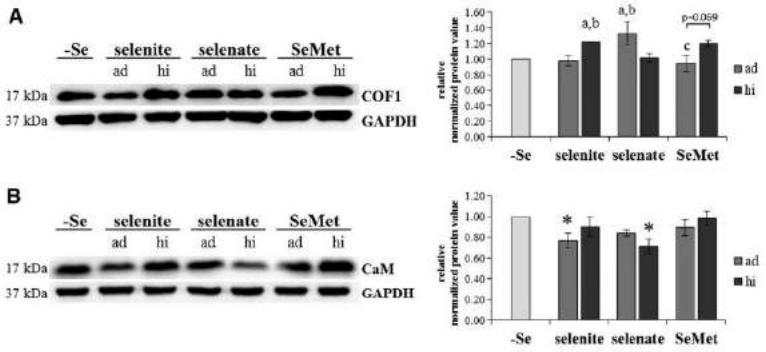

Figure 4. Validation of selected protein targets received by 2D-DIGE proteomic analysis. Protein amounts of (A) COF1 and (B) CaM determined by Western blot analysis as described in Section 2. Whereas the left panels illustrate representative Western blot signals displaying one sample/group, the corresponding right panels demonstrate the relative quantitation of the given protein levels normalized to GAPDH and related to –Se, respectively. Data represent means ± SEM. *Significant differences in comparison to the –Se group and different superscripted small letters demonstrate significant differences within Se-supplemented groups ($p < 0.05$). ad, adequate (150 μg/kg diet); hi, high (750 μg/kg diet).

and inflammatory bowel disease [48, 49]. However, while Se supplementation is able to enhance the expression of sulfonate group conjugating sulfotransferases [50], the possible regulation of ABHD14B under Se supply still needs to be investigated in more detail. Proteins identified in the current study exhibiting binding capabilities (44%, Fig. 2B) were further validated. COF1, a member of the ADF (actin depolymerizing factor)/cofilin protein family plays an essential role in controlling actin dynamics, catalyzing actin polymerization as well as depolymerization, and is involved in multiple cellular processes and located at different sites [51]. The COF1 protein expression levels were upregulated by supplementation with Se (Table 2; Fig. 4A) confirming the results of Fu and coworkers, who analyzed HeLa cells treated with 40 μmol/L selenite by a 2DE-based analysis [18]. In addition treatment of the prostate cancer cell line LNCaP with SeMet resulted in an upregulation of COF2, another ADF/cofilin family member [16], indicating that this protein family is regulated by Se. Moreover, the calcium (Ca^{2+})-binding protein CaM was downregulated in colon tissue by Se supply when compared to the –Se group, especially by selenate supplementation (Table 2; Fig. 4). CaM is a well-known Ca^{2+} sensor in non-muscle cells [52, 53] and involved in several cellular processes due to the regulation of downstream targets, such as the Ca^{2+}/CaM-dependent protein kinase family [54] or the activation of ion channels, such as the calcium-activated chloride channel TMEM16a [55]. Hi-selenite and hi-SeMet supplementation caused enhanced CLCA1 protein expression when compared to the Se-deficient group. CLCA1, a protein secreted by goblet cells within the gastrointestinal tract is a direct regulator of TMEM16a, whereas the activation of this calcium-activated chloride channel by secreted CLCA1 occurs in a paracrine fashion [56]. Moreover, CLCA1 may function as a tumor suppressor as its loss is associated with tumorigenic processes, at least in CRC [57–59]. In addition, the protein expression of the Ca^{2+}-dependent ANXA2, a well-known phospholipid-binding protein and redox sensor [60] was significantly downregulated by hi-selenate and both SeMet supplementations when compared to the –Se group (Table 2). Interestingly, downregulation of ANXA2 is beneficial for amelioration of gut inflammation by suppressing TNF-alpha cleavage, but promoting the cleavage of epidermal growth factor receptor (EGFR) ligands, such as amphiregulin (AREG) and proheparin-binding EGF-like growth factor (HB-EGF) resulting in the induction of cell proliferation and mucosal repair [61]. Moreover, ANXA2 downregulation might also play a role in preventing CRC development by limiting tumor related processes, such as cancer cell adhesion, neovascularization, invasion, and metastasis formation [62]. Thus, our findings seem to support the concept of Se supplementation as a tumor-protective option.

Within the study using 2D-DIGE coupled with MALDI-TOF-MS, a number of Se-regulated proteins have been identified in murine colon based on supplementation studies over a period of 20 weeks. In combination with data from a previous study, there seems to exist a difference in the general redox status of colon and liver tissues, which might at least to some degree be related with a higher baseline oxidative micromilieu in colon tissues, but might also depend on the given Se concentration and compound. In line with this hypothesis, the three Se compounds revealed distinct impacts on the respective protein expression pattern, but its final proof is beyond the scope of this initial proteomic profiling study. Common targets are involved in Ca^{2+} homeostasis, but also their specific role in combination with the influence of oxidative stress requires further investigation.

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4.3 Article III - Loss of epithelium-specific GPx2 results in aberrant cell fate decisions during intestinal differentiation

Loss of epithelium-specific GPx2 results in aberrant cell fate decisions during intestinal differentiation

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Loss of epithelium-specific GPx2 results in aberrant cell fate decisions during intestinal differentiation

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ABSTRACT

The selenoprotein glutathione peroxidase 2 (GPx2) is expressed in the epithelium of the gastrointestinal tract, where it is thought to be involved in maintaining mucosal homeostasis. To gain novel insights into the role of GPx2, proteomic profiles of colonic tissues either derived from wild type (WT) or GPx2 knockout (KO) mice, maintained under selenium (Se) deficiency or adequate Se supplementation conditions were established and analyzed. Amongst the panel of differentially expressed proteins, the calcium-activated chloride channel regulator 1 (CLCA1) was significantly down-regulated in GPx2 KO versus WT mice regardless of the given Se status. Moreover, transcript levels of the isoforms CLCA2 and CLCA3 showed a similar expression pattern. In the intestine, CLCA1 is usually restricted to mucin-producing goblet cells. However, although -SeKO mice had the highest numbers of goblet cells as confirmed by significantly enhanced mRNA expression levels of the goblet cell marker mucin-2, the observed expression pattern suggests that GPx2 KO goblet cells might be limited in synthesizing CLCA1. Furthermore, transcript levels of differentiation markers such as chromogranin-1 (Chga) for enteroendocrine cells and leucine-rich repeat-containing G-protein coupled receptor 5 (Lgr5) for stem cells were also downregulated in GPx2 KO mice. Moreover, this was accompanied by a downregulation of the mRNA expression levels of the intestinal hormones glucagon-like peptide 1 (Glp1), ghrelin (Ghrl) and somatostatin (Sst). Thus, it seems that GPx2 might be important for the modulation of cell fate decisions in the murine intestinal epithelium.

INTRODUCTION

Selenium is an essential trace element and modulates via functional selenoproteins a broad spectrum of key biological processes including immune responses, cellular differentiation, redox regulation and maintenance of cellular redox homeostasis [1–3]. For decades Se has been discussed to have beneficial effects in the prevention of different cancer types, including colorectal carcinoma (CRC) [4–6]. The surface of the mammalian gastrointestinal tract (GIT) is self-renewed every few days and is one of the highest proliferative tissues in the organism [7]. The rapid regeneration of the GIT surface is fueled by the proliferation of stem cells located at the intestinal crypt base and their upward migration and differentiation. The differentiated intestinal epithelial cells could be classified as goblet cells, paneth cells, enteroendocrine cells, and tuff cells, which all are derived from the secretory lineage or enterocytes derived from the absorptive lineage. Whereas enterocytes are adapted

for metabolic and digestive functions, the other cell types are specialized for maintaining the barrier function of the epithelium and supporting innate immunity. In addition, the hormone-secreting enteroendocrine cells represent a link between the neuroendocrine system and various hormone regulators of the digestive function [8–10]. The signaling pathways Wnt/β-catenin, Notch, bone morphogenic protein (BMP), and epidermal growth factor (EGF) are involved in the control of continuous proliferation and differentiation of the intestine crypt cell populations [11]. Inhibition of the Notch pathway regulates the enterocyte-secretory cell switch and mediates the differentiation into the absorptive cell lineage [12].

The selenoprotein glutathione peroxidase 2 (GPx2) first identified as an epithelium-specific enzyme of the GIT maintains the redox homeostasis by detoxifying hydrogen peroxides (H_2O_2) as shown in a CaCo2 cell culture model [13]. *In vitro* studies suggest that GPx2 exhibits anti-inflammatory properties and inhibits the migration of tumor cells, but also supports the growth of transformed intestinal cells [14]. This is in line with the observation that GPx2 is not only expressed in the GIT, but also in various tumor cells of epithelial origin, in the premalignant Barrett´s esophagus leading to esophageal adenomas, in colorectal adenomas and CRCs [15–17]. The expression of GPx2 can be regulated by several transcription factors (TF) which have been also shown to be involved in the mediation of proliferation and differentiation, such as the nuclear factor (erythroid-derived 2)-like 2 (Nrf2), homeobox protein Nkx3.1, β-catenin/TCF, delta-np63 and the STAT family [18–21].

Using a mouse model of inflammation triggered carcinogenesis (azoxymethane (AOM) and dextran sodium sulfate (DSS)) it could be shown that GPx2 knockout (KO) mice developed more tumors, which was also correlated with a more severe DSS-mediated colitis [22]. However, in a mouse model mimicking sporadic CRC (AOM only) GPx2 KO mice developed fewer preneoplastic lesions than WT mice under both Se-deficient and Se-supplemented conditions [23]. Thus, these data suggest an important role of GPx2 in carcinogenesis, which substantially differs depending on the contribution of inflammatory processes to carcinogenesis.

Given that GPx2 is mainly localized at crypt bases, where stem cells are located and that the absence of GPx2 is associated with enhanced apoptosis of crypt epithelia cells [24] the aim of the current study was to identify new candidate proteins, which might be regulated by GPx2 and/or the Se status. Therefore, colonic proteome profiles of GPx2 KO and WT mice fed with either Se deficient or Se adequate diets were analyzed by applying the 2D-difference gel electrophoresis (DIGE) technique. Our data indicate that GPx2 modulates the expression of several proteins involved in differentiation and proliferation processes of this tissue type, in particular CLCA1 and Pax4, leading to an altered distribution pattern of secretory cell types in the colon.

RESULTS

Redistribution of selenium into other selenoproteins upon loss of GPx2

Western blot analysis targeting the GPx2 protein abundance confirmed the GPx2 KO, while a ~3-fold increased GPx2 protein expression level was detected in the colon of +SeWT mice compared to the corresponding -SeWT mice (Supplementary Figure 1). In line with these data both the systemic selenium status as indicated by defining the plasma GPx activities (Figure 1A) along with the hepatic GPx activity (Figure 1B) and the local Se status in the colon as measured by GPx (Figure 1C) and TrxR activities (Figure 1E) were substantially decreased in the -Se groups. As described before, GPx2 KO mice maintained on the +Se diet exhibited a higher total GPx activity in the colon as well as in liver when compared to the respective WT group. This can be attributed to an increased GPx1 expression upon knockout of GPx2 [24], while GPx4 activity was unaffected by the loss of GPx2 (Figure 1D). In contrast to the GPx activity, total TrxR activity was increased in the GPx2 KO group only under -Se conditions (Figure 1E).

GPx2 KO alters the proteome profile of colonic tissues of mice

To gain further insights into the function of GPx2 the proteomic profiles of murine colonic tissues from the four experimental groups were compared using the 2D DIGE technology. In total 895 protein spots were detected on the resulting consensus gel (fused image), from which 53 were differentially regulated across the four groups and therefore subjected to MALDI-TOF MS. 19 unique protein IDs could be identified and are listed according to their involvement in cellular processes in Table 1 (see also Supplementary Figure 2): Out of those, six differentially expressed proteins are related to cell differentiation or proliferation, namely acidic leucine-rich nuclear phosphoprotein 32 family member B (AN32B), transgelin-2 (TAGL2), stathmin (STMN1), prelamin A/C (LMNA), heat shock protein HSP 90-beta (HSP84) and calcium-activated chloride channel regulator 1 (CLCA1). The expression of some proteins appeared to be dependent on the given Se status: The dual specificity protein phosphatase 3 (DUSP3) and peroxiredoxin-6 (PRDX6) were downregulated under Se supply, while the protein abundance of the selenoprotein thioredoxin reductase 1 (TR1) [25] was enhanced under these conditions. Furthermore, the GPx2 expression status had an influence on the protein expression pattern of CLCA1 and prelaminin A/C (LMNA9), which were both downregulated in the GPx2 KO groups.

To identify the most relevant candidates out of the 19 proteins associated with GIT disorders, an *in*

Table 1: The GPx2 KO influences the protein expression pattern in colonic tissue of mice

No	protein name	gene	Uniprot ID	mass (kDa)	pI	score	sequence coverage (%)	-Se WT	-Se KO	+Se WT	+Se KO
	Cell differentiation and proliferation										
1	Acidic leucine-rich nuclear phosphoprotein 32 family member B (AN32B)	Anp32b	Q9EST5	31.23	3.89	60	24	1.00	0.60#	0.57*	0.48
2	Transgelin-2 (TAGL2)	Tagln2	Q9WVA4	22.55	8.39	58	46	1.00	1.20	0.52*	0.53*
3	Stathmin (STMN1)	Stmn1	P54227	17.26	5.76	55	44	1.00	1.07	0.50*	0.56*
4	Calcium-activated chloride channel regulator 1 (CLCA1)	Clca1	Q9D7Z6	100.81	5.67	63	22	1.00	0.59#	1.34	1.18*
5	Prelamin A/C (LMNA)	Lmna	P48678	74.48	6.54	62	27	1.00	0.75	1.61	1.17*
6	Heat shock protein HSP 90-beta (HSP84)	Hsp90ab1	P11499	83.57	4.97	95	22	1.00	0.87	1.40*	1.14
	Signal transduction										
7	Protein kinase C gamma type (PKCγ)	Prkcg	P63318	79.56	7.27	56	16	1.00	1.52#	0.68*	0.67*
8	Dual specificity protein phosphatase 3 (DUS3)	Dusp3	Q9D7X3	20.69	6.07	64	50	1.00	1.18	0.57*	0.56*
9	Calmodulin (CaM)	Calm1	P62204	16.83	4.09	58	53	1.00	0.74	1.27	0.96#
	Cell metabolism										
10	Cytochrome c1, heme protein, mitochondrial (CY1)	Cyc1	Q9D0M3	35.53	9.24	72	32	1.00	1.53#	0.61	0.61*
11	Cytochrome c1, heme protein, mitochondrial (CY1)	Cyc1	Q9D0M3	35.53	9.24	84	35	1.00	1.34	0.16*	0.19*
12	NADH dehydrogenase [ubiquinone] iron-sulfur protein 3, mitochondrial (NDUS3)	Ndufs3	Q9DCT2	30.3	6.67	60	22	1.00	1.22	0.58*	0.61*
13	Carbonyl reductase [NADPH] 3 (CBR3)	Cbr3	Q8K354	31.33	6.15	85	39	1.00	1.07	0.53*	0.54*
	Redox homeostasis										
14	Peroxiredoxin6 (PRDX6)	Prdx6	O08709	24.97	5.71	72	38	1.00	1.36	0.65	0.79*
15	Thioredoxin reductase 1, cytoplasmic (TR1)	Txnrd1	Q9JMH6	68.24	7.42	62	22	1.00	1.08	1.51*	1.39
	Others (transport; metal ion binding; cytoskeleton organization)										
16	Transthyretin (TTHY)	Ttr	P07309	15.88	5.77	61	44	1.00	1.34#	0.62*	0.71*
17	Cytosolic Fe-S cluster assembly factor NUBP2 (NBP 2)	Nubp2	Q9R061	29.90	6.07	58	37	1.00	1.13	0.54*	0.58*
18	Tropomyosin alpha-1 chain (TPM1)	Tpm1	P58771	32.72	4.69	80	29	1.00	0.93	1.26	1.45*
19	Tropomyosin alpha-1 chain (TPM1)	Tpm1	P58771	32.72	4.69	91	34	1.00	1.19	1.37	1.55

Values are given as means ($n = 4$) in relation to the –Se WT group. Levels marked with # indicate significant differences between WT and KO within the same Se level and levels marked with * indicate significant differences between WT and KO versus the respective group maintained on –Se conditions. ($p < 0.05$; one-way ANOVA). +Se, selenite supplementation (150 µg Se/kg diet); score, Mascot Probability Based Scoring.

silico analysis of their RNA expression levels in human colorectal adenocarcinoma samples was performed (TCGA-COADREAD, $n = 434$). Some of the proteins found to be differentially expressed either by the genotype or by the selenium status were strongly altered in primary tumors compared to normal solid tissues (Figure 2). In particular, Clca1, a protein secreted by goblet cells and known to be involved in the regulation of cell proliferation and differentiation [26] shows a strong down-regulation in tumor samples versus normal tissues. In the proteomic profiling we found decreased expression levels of Clca1 in the two GPx2 KO groups when compared to their respective WT animals (Table 1), which were confirmed at both protein (Western blotting) and mRNA (qPCR) levels (Figure 3A and 3B). Transcription analysis of different Clca isoforms showed that next to Clca1 also Clca2 and Clca3 mRNA levels were decreased in the GPx2 KO groups, whereas Clca4 mRNA expression pattern were not affected (Figure 3C–3E).

Furthermore, Kaplan-Meier analysis were performed to evaluate the correlation between the survival of CRC patients with primary tumors (TCGA-COADREAD, $n = 380$) and the corresponding expression levels of Clca1, Clca2 and Clca3. Herefore, CRC patients were subdivided into a group with high expression (> 50 %) and a group with low expression (< 50 %) rates of the respective genes. The overall survival (OS) of CRC patients with high Clca1 or Clca2 expression levels is higher than that of patients with low Clca1 or Clca2 expression levels, whereas the expression levels of Clca3 showed no correlation with the patients' OS rate (Figure 4). However, in this data set no correlation between GPx2 and Clca isoforms was found.

Increased goblet cell numbers in Se deficient GPx2 KO mice

Since Clca1 is known to be expressed and secreted by goblet cells in colonic tissues [26], histological analyses were performed to understand whether the decreased Clca1 mRNA and protein expression levels observed within the GPx2 KO groups result from decreased goblet cell numbers. However, whereas GPx2 KO mice maintained on the -Se diet showed increased numbers of PAS-positive goblet cells in comparison to WT mice (Figure 5A, upper row and Figure 5B), no

such differences between genotypes were found under +Se conditions. Furthermore, not only goblet cell numbers, but also goblet cell localization within the colonic crypts was changed as a result of GPx2 loss. In -SeWT, +SeWT and +SeKO groups goblet cells were equally distributed throughout whole crypts, whereas in the -SeKO group they accumulated at the crypt base (Figure 5A). These data suggest that the -Se KO epithelium is characterized by an aberrant goblet cell localization (Figure 5A).

CLCA1 expression does not correlate with goblet cell numbers

Previous studies indicated a co-localization of CLCA1 and mucus-producing goblet cells [26]. Herein, the CLCA1-positive cells do not clearly overlap with goblet cells stained by PAS/AB. In particular, the high number of goblet cells located at the crypt base of the -SeKO group rather lack CLCA1 expression (Figure 5A, lower two rows; Figure 5C). Interestingly, the total number of enterocytes per colonic crypt was solemnly decreased in the –SeKO group (Figure 5D). Taken together this data indicates a dysbalance between the secretory and the absorptive lineage in colon tissues caused by loss of GPx2 in combination with Se deficiency.

A GPx2 knockout caused an altered expression of differentiation markers of several intestinal cell types

In order to determine whether the enhanced goblet cell formation observed by the combination of Se deficiency along with the loss of GPx2 affects other cell types, the mRNA levels of cell-type specific differentiation markers were determined (Figure 6). In parallel to the enhanced goblet cell formation, the mRNA expression levels of mucin-2 (Muc2), a glycoprotein synthesized and secreted by goblet cells, were significantly increased in mice of the -SeKO group when compared to all other groups, in which no differences regarding the Muc2 expression levels were found (Figure 6A). In addition, the GPx2 KO significantly altered the expression pattern of markers associated with several cell types present in colon tissues, including chromogranin-A (Chga) (enteroendocrine cells) and leucine-rich repeat-containing G-protein coupled receptor 5 (Lgr5) (stem cells). The mRNA levels of Chga and Lgr5 were clearly significantly downregulated in both GPx2 KO groups when compared to the respective WT groups (Figure 6B and 6D). GPx2 KO mice maintained on the +Se diet exhibited significantly higher expression levels of both markers

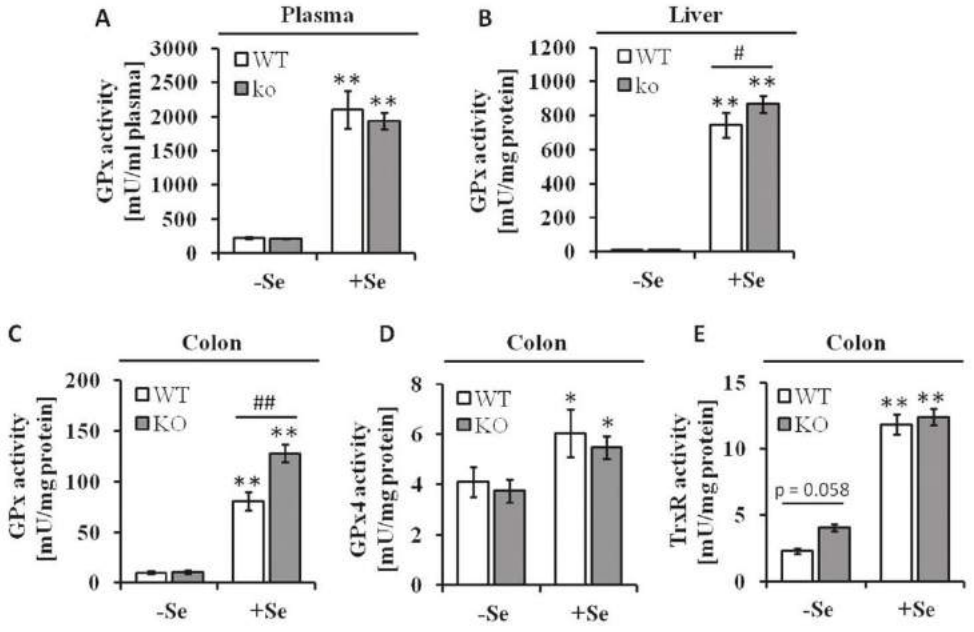

Figure 1: Se status following eight weeks of feeding with different Se concentrations. Enzyme activities of (**A**) GPx in plasma, (**B**) GPx in liver and (**C**) in colon lysates, (**D**) GPx4 and (**E**) TrxR in colon tissues were spectrophotometrically determined as described in the Material and Methods section. Values are given as means ± S.E.M. ($n = 9$). Significant differences were calculated by one-way ANOVA. *$p < 0.05$; **$p < 0.01$ vs. the respective -Se group; ##$p < 0.01$ WT vs. KO within the same Se supply level.

when compared to the -Se GPx2 KO group. In contrast, the expression levels of lysozyme (paneth cells) were neither affected by the GPx2 genotype nor by the given Se status (Figure 6C).

Since the loss of GPx2 is associated with decreased Chga expression levels, a common marker for enteroendocrine cells, we asked whether this might be associated with altered expression levels of intestinal hormones. Therefore, the mRNA expression levels of the intestinal hormones glucagon-like peptide 1 (Glp1), ghrelin (Ghrl), tryptophan 5-hydroxylase 1 (Tph1), and somatostatin (Sst) were analyzed. Glp1 expression was solely down-regulated in the GPx2 KO group maintained on the -Se diet, whereas all the other analyzed hormones showed decreased expression levels in the GPx2 KO groups when compared to the corresponding WT groups (Figure 6E–6H). Intestinal hormone producing cells display a connection between the neuroendocrine and the digestive system and play key roles in regulation of food intake, energy expenditure, glucose and lipid metabolism [27]. In the current study, GPx2 KO mice exhibited altered expression patterns of intestinal hormones and slightly reduced weight gains over the eight weeks feeding period (Supplementary Figure 3).

Transcription factors involved in modulating differentiation processes

To gain further insights into the underlying mechanisms leading to the increased goblet cell formation in the -SeKO group, the mRNA expression pattern of important mediators of signaling pathways involved in differentiation processes were analyzed. The Notch pathway regulates the expression of the TF atonal homolog 1 (Atoh1), which determines cell fate in the intestine. The transcription factor hairy and enhancer of split 1 (Hes1), a key target of Notch signaling, is important for the differentiation of intestinal absorptive cells, whereas Atoh1 plays a reciprocal role and positively promotes the secretory lineage differentiation. In addition, Atoh1 is repressed by Hes1. In the present study, GPx2 KO mice maintained on -Se diets showed decreased mRNA expression levels of Hes1, whereas the Atoh1 mRNA levels were upregulated (Figure 7A and 7B) thereby suggesting that the enhanced goblet cell numbers observed in this group might at least to some extent be attributed to an aberrant activity of the Notch signaling pathway. To determine whether the enhanced mRNA expression of Atoh1 directly reflect its activity the mRNA expression levels of several Atoh1 downstream targets were analyzed. As shown in Figure 7C–7E, the mRNA expression levels of SAM pointed domain-containing Ets transcription factor (Spdef), Protein CBFA2T3 and Rap guanine nucleotide exchange factor 3 (Rapgef3), which are described to be upregulated by Atoh1 [28], show the same expression pattern like Atoh1. Thus, these results indicate an enhanced Atoh1 activity under GPX2 KO conditions combined with Se deficiency.

Not only Atoh1, but also the TF paired box protein Pax-4 (Pax4) is involved in the differentiation

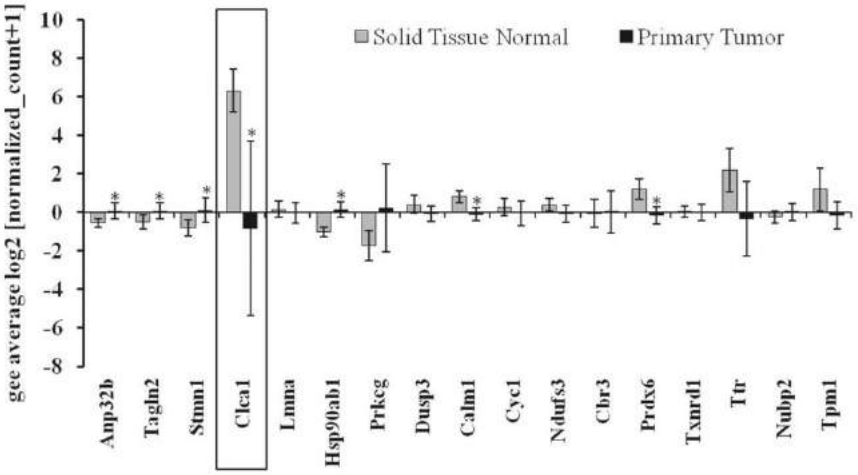

Figure 2: Gene expression pattern of potential targets defined via the murine proteomic profiling experiments in CRC patients. Analysis of the expression pattern of proteins, which were found to be differentially regulated by selenium and/or the GPx2 status in the murine model system (see also Table 1) in regard to their potential role in CRC patients (TCGA-COADREAD; $n = 434$). Expression data were Log2 transformed, normalized TCGA-COADREAD data were grouped by the sample types and expressed as means ± S.D. (solid tissue normal $n = 54$, primary tumor $n = 380$). *$p < 0.05$, student´s t-test.

of enteroendocrine cells in the intestine, which has been recently demonstrated using Pax4 deficient mice [29, 30]. The expression of Pax4 was strongly reduced in the GPx2 KO groups when compared to their respective WT groups (Figure 7F). Moreover, Pax4 expression levels positively correlated with the expression levels of Clca1 (Pearson Correlation Coefficients (PCC): 0.702, $p < 0.01$) and Chga (PCC: 8.03, $p < 0.01$) (Figure 7G and 7H).

DISCUSSION

To gain new insights into the functions of the selenoprotein GPx2, which is mainly located in the epithelium of the GIT, comparative proteomic analyses of colon tissues of mice lacking GPx2 expression and of WT counterparts were performed. In addition to the total loss of GPx2 expression, the selenium status was modulated in order to define if the resulting effects can be solemnly attributed towards the loss of GPx2 or if other selenoproteins such as GPx1 might also be involved. Being well aware that different selenium species in nutrition can cause different effects including Gpx1 expression [31, 32], we concentrated on selenite as the most common controllable selenium source. It has been previously reported that under +Se conditions other selenoproteins are up-regulated, which is merely the case under -Se conditions [24]. Thus, effects only seen in -SeKO mice might have been compensated by other selenoproteins under +SeKO conditions.

Based on the performed proteomic profiling analysis of colonic tissues CLCA1 was identified as a potentially downregulated target in both GPx2 KO

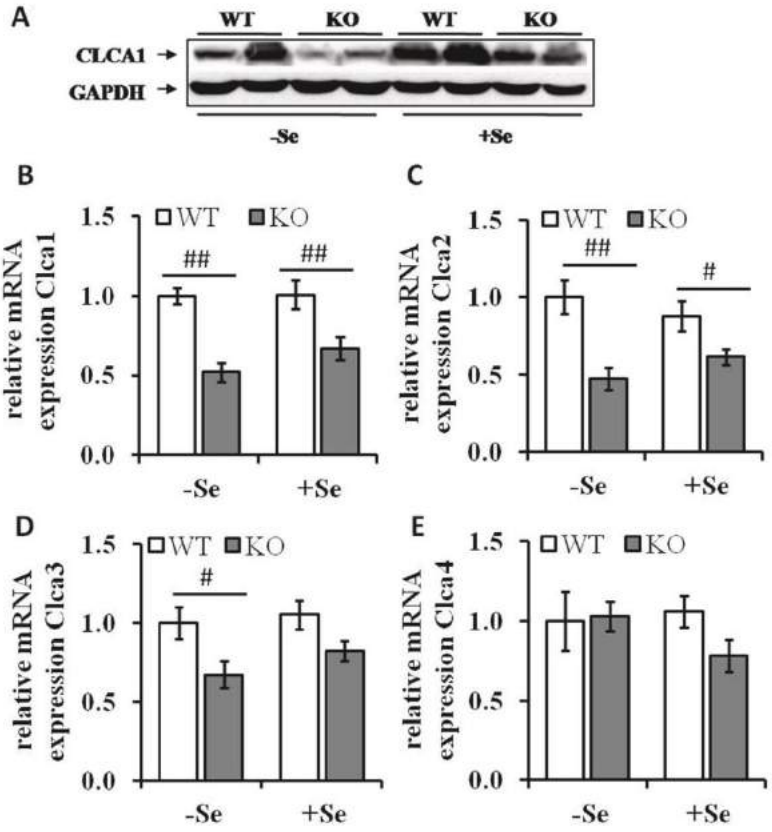

Figure 3: Expression levels of Clca isoforms are decreased by GPx2 knockout. (**A**) Protein levels of CLCA1 were analyzed by Western blotting; mRNA expression levels of (**B**) Clca1, (**C**) Clca2, (**D**) Clca3 and (**E**) Clca4 were analyzed by qRT-PCR, normalized to the amplification data of GAPDH, ß-actin and RPL13a and expressed in relation to the –SeWT group. Values are given as means ± S.E.M ($n = 9$ per group). #$p < 0.05$, ##$p < 0.01$ (one-way ANOVA).

groups. In addition, Clca1 mRNA was also strongly down-regulated in CRC lesions in comparison to normal tissue samples (TCGA-COADREAD). Furthermore, the Clca1 expression level seems to be of prognostic value since the OS of CRC patients was significantly improved in patients with high tumor-resident Clca1 expression. Similar results as described in this murine model system were recently reported in the context of studies focusing on CRC patients and thus Clca1 is currently discussed to have tumor-suppressive properties [33–35].

CLCA1 represents a multifunctional protein and has been linked to various diseases with mucus overproduction, including cystic fibrosis, asthma and chronic obstructive disease [34, 36–38]. Common features of these diseases are goblet cell hyperplasia, enhanced mucus production and increased expression levels of Clca1. As CLCA1 is secreted by goblet cells together with Muc2 in the GIT [39, 40], it might be involved in the regulation of mucus production and/or goblet cell formation. However, the histological analysis of colon tissues of -SeKO mice showed a marked increase in goblet cell numbers and an induced Muc2 mRNA expression level, but surprisingly low levels of Clca1 expression. Furthermore, in the GPx2 knockout group fed with the +Se diet the reduced Clca1 expression had no impact at all on the goblet cell formation process. Thus, Clca1 expression levels obviously neither correlate with the total goblet cell number nor with the Muc2 expression level in GPx2 KO mice. In addition, CLCA1 expression was rather restricted to goblet cells located in the upper part of the crypts, whereas the high number of goblet cells located at the crypt base observed in the GPx2 KO group maintained on -Se conditions fully lack CLCA1 expression. The authors thus hypothesize that under -SeKO conditions the observed aberrant localization of goblet cells, defined as being mostly stacked at the crypt base, obviously also suppressed their capability to express CLCA1. These results are moreover in line with a recent study using Clca1$^{-/-}$ mice thereby demonstrating neither the presence of Clca1 positive cells at the crypt bases, nor an effect of

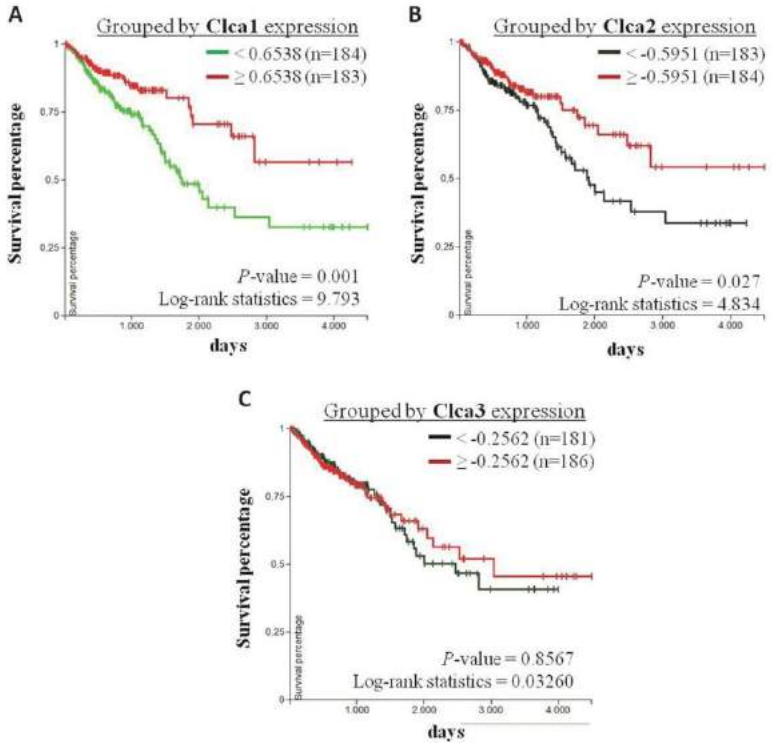

Figure 4: Correlation analysis of Clca isoform expression levels with overall survival of CRC patients. Kaplan-Meier curves of CRC patients with primary tumors (TCGA-COADREAD, n = 380) were analyzed using the UCSC Xena browser (https://xenabrowser.net). Patients were grouped by the 50 percentiles of (**A**) Clca1, (**B**) Clca2 and (**C**) Clca3 expression levels. The differences between the curves were analyzed with the log-rank test and considered to be statistically significant when $p < 0.05$.

Clca1 expression on mucus production [41]. To determine whether GPx2 is involved in the transcriptional regulation of Clca1 or the deregulation of CLCA1 occurs due to an aberrant arrangement of the different colonic cell types needs further investigation.

Cell fate decisions between the absorptive and secretory lineage are under control of the Notch signaling pathway. Depletion of Hes1 a direct target of Notch was associated with goblet cell hyperplasia [42], while Atoh1, which is repressed by Hes1, is required for the differentiation into the secretory lineage [43]. As GPx2 KO mice maintained under Se-deficient conditions showed enhanced expression of Atoh1 along with repressed Hes1 expression levels, the enhanced goblet cell formation and localization might be rather attributed to alterations in the Notch signaling pathway than to the aberrant Clca1 expression. Nevertheless, Clca1 is discussed to exhibit anti-proliferative activities and to be required for the spontaneous differentiation of CaCo-2 cells [44]. This suggests that Clca1 might be involved in the proliferation to differentiation transition (PDT) of CRC, which however could be different in the healthy intestine [44]. In normal intestinal epithelium, PDT is a critical step in self-renewal and alterations of the proliferation and differentiation processes lead to the development of diseases, including cancer. Thus, stem cells within the GIT must be constantly ready to respond to external stimuli to maintain normal homeostasis of the epithelium [45]. In the present murine study, the lack of GPx2 expression was associated with a strong down-regulation of the stem cell marker Lgr5. Usually, GPx2 is located at the crypt base, where stem cells are resident and lack of GPx2 is associated with enhanced apoptotic cell death in this area [24]. Therefore, it can be postulated that the reduced Lgr5 expression is attributed to a diminished number of stem cells. However, the whole intestinal integrity is not severely impaired in GPx2 KO mice arguing against this hypothesis. Vice versa, low levels of Lgr5 could also indicate that the Wnt

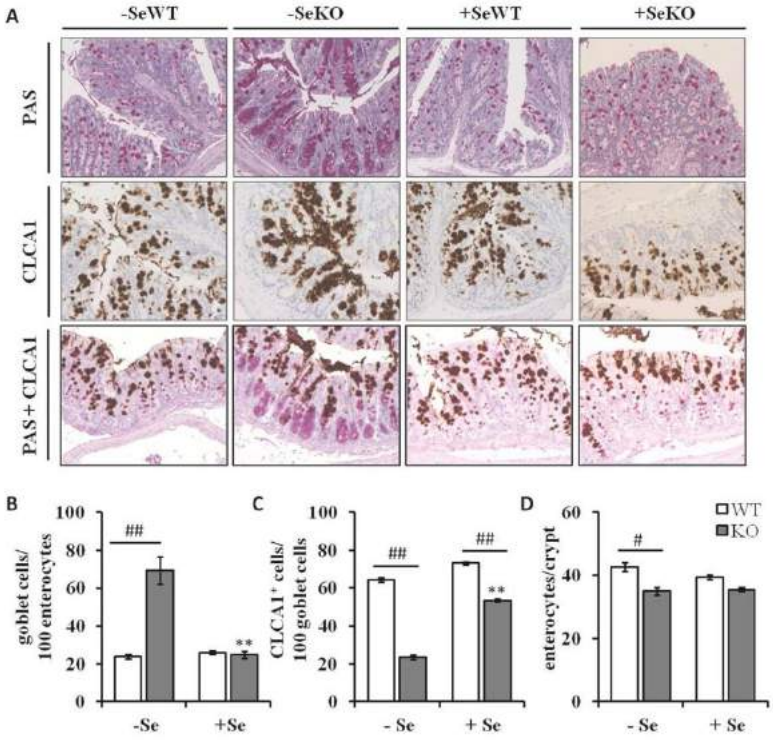

Figure 5: Histological and immunohistochemical analyses of colon sections. (**A**) Representative microscopy pictures of colon sections stained with PAS for counting goblet cells (upper row), an anti-CLCA1 antibody for visualizing CLCA1 positive cells (middle row), and co-staining of goblet cells and CLCA1 positive cells (bottom row) (**B**) number of goblet cells per 100 enterocytes, (**C**) number of CLCA1 positive cells per 100 goblet cells and (**D**) number of enterocytes per colon crypt are expressed as means ± S.E.M ($n = 4$ per group). *$p < 0.05$, **$p < 0.01$ vs. the respective -Se group; #$p < 0.05$, ##$p < 0.01$ WT vs. KO within the same Se supply (one-way ANOVA). HPF, high power field, CLCA1, Calcium-activated chloride channel regulator 1; PAS, periodic acid-Schiff.

signaling activity of the crypt niche is partially repressed by loss of GPx2 [8]. Using colonosphere cultures of tumor cells recently revealed that loss of GPx2 is associated with enhanced stem cell formation, highlighting differences between functions of GPx2 in tumor models and non-tumor systems. However, in this study overexpression of GPx2 was associated with enhanced transcription levels of the enteroendocrine cell marker Chga [46], which is in line with the observation reported in this study that lack of GPx2 leads to decreased expression levels of Chga. Furthermore, GPx2 KO mice exhibited decreased expression levels of Pax-4, a transcription factor discussed to be involved in the differentiation of enteroendocrine cells in the intestine [29, 30]. In the present study the GPx2 KO was also associated with decreased expression levels of several intestinal hormones, including, Ghrl, Tph1 and Sst. Yet, in Pax-4 deficient mice, a decreased Sst, Tph1, and Chga expression pattern was found, whereas Ghrl was rather upregulated [30]. Thus, Pax-4 might be partially responsible for the observed GPx2-mediated changes in the expression pattern of the respective hormones, but could be also the result of a general decrease in enteroendocrine cells. As intestinal hormones produced by enteroendocrine cells link the neuroendocrine system and various hormone regulators of the digestive function [9, 10] the trend for reduced weight gains of GPx2 KO mice might be a result of deregulated intestine functions.

MATERIALS AND METHODS

Animals and diets

C57BL6/J wild-type (WT) and GPx2 KO mice, generated as C57BL/6J;129SV/J hybrid have been backcrossed to the C57BL/6J background before entering the study [23]. Animals were housed under SPF-conditions with a 12 h dark/light cycle and had free access to food and water. At the age of 4 weeks, the mice were randomly assigned to four groups of 9 animals/group (-SeKO, +SeWT, +SeKO) and received diets containing either adequate levels of Se in form of selenite (+Se, 150 µg/kg diet) or a Se-deficient (-Se) diet for eight weeks. Se contents of the diets were confirmed by ICP-MS (-Se, < 20 µg Se/kg diet; +Se, 165 ± 1.9 µg Se/kg diet) as previously described [31]. The diets were based on torula yeast and Se-deficient wheat [31]. After an eight-week feeding period, mice were decapitated under CO_2 narcosis; blood was collected via heart punction in heparinized tubes and centrifuged for 15 min at 4°C and 2000 x g. Plasma was stored at -80°C until further analysis. Colon and liver tissue samples were excised, snap frozen in liquid nitrogen, and stored at -80°C until further use.

Animal experiments were performed in compliance with the German animal protection law. The mice were housed and handled in accordance with good animal practice as defined by the Federation of Laboratory Animal Science Associations (FELASA, www.felasa.eu/) and the national animal welfare body (GV-SOLAS, www.gv-solas.de/). The animal welfare committees of the German Institute of Human Nutrition (DIfE) as well as the local authorities (Landesamt für Umwelt, Gesundheit und Verbraucherschutz, Brandenburg, Germany) approved all animal experiments.

Determination of enzyme activities

For enzymatic assays, lysates of colonic and hepatic tissues were prepared in Tris buffer (100 mM Tris, 300

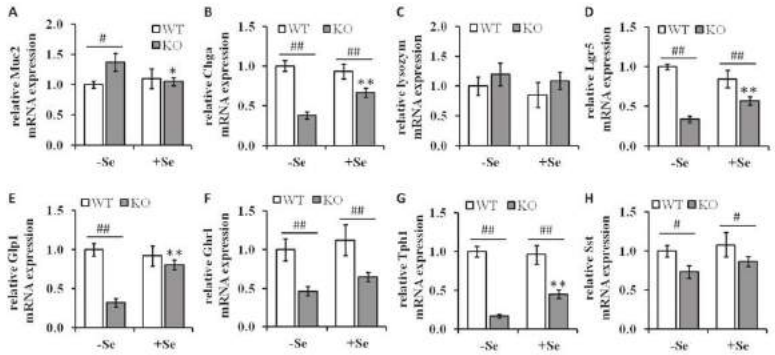

Figure 6: Analysis of differentiation markers in colon lysates of GPx2 KO mice. mRNA expression levels of differentiation markers of (**A**) Muc2 (goblet cells), (**B**) Chga (enteroendocrine cells), (**C**) lysozyme (paneth like cells) and (**D**) Lgr5 (stem cells) and the mRNA expression pattern of the intestinal hormones, (**E**) Glp1, (**F**) Ghrl, (**G**) Tph1 and (**H**) Sst were determined by qPCR, normalized to the amplification data of GAPDH, ß-actin and RPL13a and expressed in relation to the -SeWT group. Data are given as means ± S.E.M. (n = 9 per group). *$p < 0.05$, **$p < 0.01$ vs. the respective -Se group; #$p < 0.05$, ##$p < 0.01$ WT vs KO within the same Se supply (one-way ANOVA). Muc2, mucin-2; Chga, chromogranin A; Lgr5, Leucine-rich repeat-containing G-protein coupled receptor 5. Glp1, glucagon-like peptide 1; Ghrl, ghrelin; Tph1, tryptophan 5-hydroxylase 1; Sst, somatostatin.

mM KCl, 0.1 % Triton X-100, pH 7.0, Calbiochem® protease inhibitor cocktail II (Merck Millipore, Darmstadt, Germany)) using a TissueLyzer (2 × 30 sec; 30 Hz; Qiagen, Hilden, Germany), centrifuged (14.000 x g, 30 min, 4°C) and stored at -80°C until further analysis. Total GPx and total TrxR activities were determined as previously described [47]. GPx4 activities were measured according to the measurement of total GPx activity, but by applying the specific substrate phosphatidylcholine hydroperoxide (PCOOH, 1.25 mM) instead of 0.00375 % H_2O_2 [24].

Western blot analysis

For Western blot analysis, 30 µg total protein/lane were separated on 10 % SDS-polyacrylamide gels and transferred onto nitrocellulose membranes as described previously [48]. For immune detection the rabbit anti-GPx2 [49] (dilution 1:2000, incubation at 4°C overnight), rabbit anti-CLCA1 (Abcam #180851) and rabbit anti-GAPDH (Cell signaling technology, #2118, New England Biolabs GmbH, Frankfurt, Germany) were used as primary antibodies. HRP-linked anti-rabbit/anti-mouse antibodies (Cell Signaling Technology, #7074, #7076) were used as secondary antibodies.

Real-time RT-PCR

RNA was isolated and then subjected to PCR analysis as recently described [31]. Target- specific primers were designed using the program Primer3 [50] and are shown in Supplementary Table 1. Amplification data were analyzed according to the method of Pfaffl [51]. For normalization amplicons of GAPDH, ß-actin and RPL13a were employed.

Analysis of the protein expression pattern by difference gel electrophoresis (DIGE) and mass spectrometry

Four independent biological replicates per group were used for 2D-DIGE analysis based on the minimal labeling approach according to the manufacturer's instructions (SERVA Lightning SciDyes, SERVA Electrophoresis, Heidelberg, Germany). 25 µg total protein of each sample was labeled with 200 pmol of either SciDye3- or SciDye5 and the internal protein standard was labeled with 200 pmol of the SciDye2. 2DE separation was performed as recently described [52]. For gel image analyses the Delta2D software package (Decodon GmbH, Greifswald, Germany) was used. Whereas differentially expressed proteins were classified by a fold change ratio of 1.5 (increased or decreased) and a *p-value* of < 0.05, mass spectrometry analyses were performed on an ultrafleXtreme™ matrix-assisted laser desorption/ ionization time of flight mass (MALDI-TOF) mass spectrometer (Bruker Daltonics Inc., Bremen, Germany). The resulting peptide mass fingerprinting datasets were analyzed using the MASCOT software package (Matrix Science, Dauheim, USA) [53].

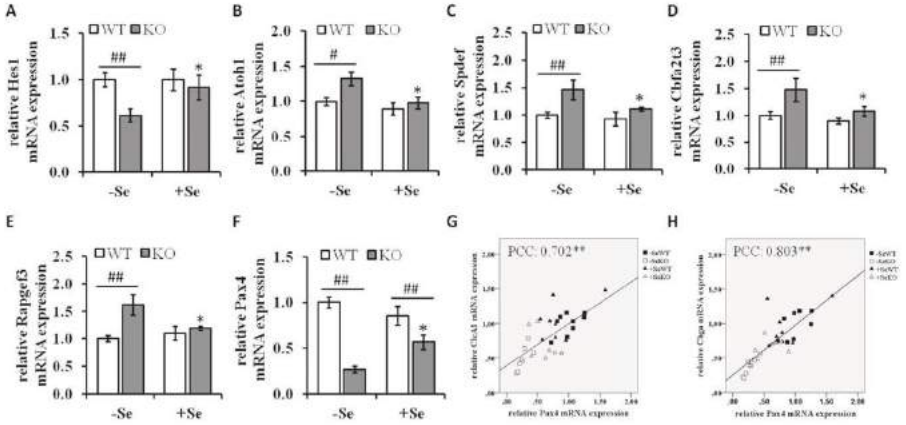

Figure 7: mRNA expression of components involved in differentiation processes. mRNA expression levels of the Notch signaling components (**A**) Hes1, (**B**) Atoh1, the Atoh1 downstream targets (**C**) Spdef, (**D**) Cbfa2t3, (**E**) Rapgef3 as well as of the transcription factor (**F**) Pax4 were determined by qRT-PCR normalized to the amplification data of GAPDH, ß-actin and RPL13a and expressed in relation to the –SeWT group. Data are expressed as means ± S.E.M. ($n = 9$ per group). $^*p < 0.05$, $^{**}p < 0.01$ vs. the respective -Se group; $\#p < 0.05$, $\#\#p < 0.01$ WT vs KO within the same Se supply (one-way ANOVA). Correlation analysis of the expression pattern of Pax4, (**G**) Clca1 and (**H**) Chga were conducted by determining the Pearson Correlation Coefficients (PCC). **indicate significant correlations with a *p*-value < 0.01. Atoh1, protein atonal homolog 1; CLCA1, calcium-activated chloride channel regulator 1; Hes1, hairy and enhancer of split-1; Pax4, paired box protein Pax-4.

Histological staining

Colon tissues of mice (n = 4/group) were washed with 4% formaldehyde and then fixed overnight in 4 % formaldehyde. Fixed colon samples were embedded in paraffin, cut into 4 µm slices and prepared for periodic acid-Schiff (PAS)/Hematoxylin-eosin (HE) staining according to standard protocols. For immunostaining the rabbit anti-CLCA1 antibody (Abcam #180851) was used as primary antibody followed by HRP-linked polymer system detection. Evaluation of PAS positive goblet cells and evaluation of CLCA1 positive cells were performed by counting the cells in a 0.5 mm² high power field (HPF) [54]. Additionally, the number of PAS-positive cells were normalized to 100 enterocytes.

In silico analysis of TCGA data of CRC patients

The UCSC Xena Cancer Genomics Browser (https://xenabrowser.net/) [55, 56] was used to visualize TCGA genomic data (cohort TCGA Colon and Rectal Cancer (COADREAD), 434 patients; poly-A+illimuina hiSeq). The gene expression data was sorted according to the sample types: solid tissue normal, n = 54; primary tumor, n = 380. In addition, Kaplan-Meier curves of CRC patients with primary tumors were generated using the same tool by analyzing the 50% percentiles of RNAseq data of the respective genes. The analysis was conducted using the UCSC Xena tool (log Rank test) [55, 56]. Data were considered as significantly different if $p < 0.05$.

Statistical analysis

Mean values were calculated from 9 animals per group or 4 animals per group in the case of the proteomics and Western blot analysis, respectively, and given as means ± their standard error of the mean (S.E.M). SPSS 20 was used to analyze significant differences within the groups. One-way ANOVA (LSD post hoc test) was applied if the normality of distribution (Shapiro-Wilk test) and the homogeneity of variance (Levene test) were defined. Otherwise, the Games-Howell test was employed. If only two groups were compared the student´s *t-test* was applied. Differences between the groups were considered to be significant at $p < 0.05$.

CONCLUSIONS

In summary, the current study indicates that loss of GPx2 modulates the expression levels of cell-type specific markers, which might reflect a dysbalance between absorptive and secretory cell types. In addition, not only the number, but also the localization of differentiated cell types was changed upon loss of GPx2. The exact underlying mechanisms still need to be resolved, but might involve the Notch signaling pathway as well as the transcription factor Pax-4. Some of the effects found in the current study only became obvious when the GPx2 KO was combined with Se deficiency conditions. The results indicate that other selenoproteins, most probably GPx1, can compensate at least to some extent for the loss of GPx2 during intestinal differentiation, but only in the presence of sufficient Se. As both GPx1 and GPx2 are major antioxidant enzymes, redox modification of signaling pathways such as Notch and Wnt might be the cause for aberrant cell fate decisions as defined within this report.

Abbreviations

AOM, azoxymethane; Atoh1, protein atonal homolog 1; Chga, chromogranin A; CLCA, Clca, Calcium-activated chloride channel regulator; CRC, colorectal carcinoma; DIGE, difference gel electrophoresis; DSS, dextran sodium sulfate; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; Ghrl, ghrelin; GIT, gastrointestinal tract; Glp1, glucagon-like peptide 1; GPx, glutathione peroxidase; Hes1, hairy and enhancer of split-1; HPF, high power field; KO, knockout; Lgr5, leucine-rich-repeat-containing G-protein coupled receptor 5; MALDI-TOF MS, Matrix-assisted laser desorption/ionization time of flight mass spectrometry; Muc2, mucin-2; Nrf2, nuclear factor (erythroid-derived 2)-like 2; OS, overall survival; PAS, periodic acid-Schiff; PAX-4, paired box protein; PCC, Pearson Correlation Coefficients; PDT, proliferation to differentiation transition; Se, selenium; Sst, somatostatin; TCGA, The Cancer Genome Atlas; TF, transcription factor; Tph1, tryptophan 5-hydroxylase 1; TrxR, thioredoxin reductases; WT, wild type.

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CONFLICTS OF INTEREST

The authors would like to state that they have no potential conflicts of interest to report.

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4.4 Article IV - Modulation of MHC class I surface expression in B16F10 melanoma cells by methylseleninic acid

Modulation of MHC class I surface expression in B16F10 melanoma cells by methylseleninic acid

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ORIGINAL RESEARCH

Modulation of MHC class I surface expression in B16F10 melanoma cells by methylseleninic acid

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ABSTRACT

The essential trace element selenium (Se) might play a role in cancer prevention as well as for cancer therapy. Its metabolite methylselenol is able to kill cells through distinct mechanisms including induction of reactive oxygen species, DNA damage and apoptosis. Since methylselenol affects innate immune responses by modulating the expression of NKG2D ligands, the aim of this study was to determine whether the methylselenol generating compound methylseleninic acid (MSA) influences the expression of the MHC class I surface antigens and growth properties thereby reverting immune escape.

Treatment of B16F10 melanoma cells expressing low basal MHC class I surface antigens with dimethyldiselenide (DMDSe) and MSA, but not with selenomethionine and selenite resulted in a dose-dependent upregulation of MHC class I cell surface antigens. This was due to a transcriptional upregulation of some major components of the antigen processing machinery (APM) and the interferon (IFN) signaling pathway and accompanied by a reduced migration of B16F10 melanoma cells in the presence of MSA. Comparative "ome"-based profilings of untreated and MSA-treated melanoma cells linked the anti-oxidative response system with MHC class I antigen processing. Since MSA treatment enhanced MHC class I surface expression also on different human tumors cell lines, MSA might affect the malignant phenotype of various tumor cells by restoring MHC class I APM component expression due to an altered redox status and by partially mimicking IFN-gamma signaling thereby providing a novel mechanism for the chemotherapeutic potential of methylselenol generating Se compounds.

Abbreviations: 2D-DIGE, two-dimensional differential gel electrophoresis; APM, antigen processing machinery; CALR, calreticulin; CMV, cytomegalovirus; CTL, cytotoxic T lymphocytes; DMDSe, dimethyldiselenide; DNTB, 5,5′-dithiobis 2-nitrobenzoic acid; ER, endoplasmic reticulum; EstD/estD, esterase D; G6PD/G6pd, glucose 6-phosphate dehydrogenase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GO, gene ontology; GSH, glutathione; Gsta4, glutathione S-transferase a4; HDAC, histone deacetylase; hnRNPA2/B1/Hnrnpa2b1, heterogeneous ribonucleoprotein family A2/B1; IFN, interferon; IRF, interferon-regulated factor; JAK-1/Jak1, janus kinase 1; LMP, low molecular weight protein; mAb, monoclonal antibody; MHC, major histocompatibility complex; MIC, MHC class I polypeptide-related sequences; MSC, methylselenocysteine; NKG2D, natural killer group 2 member D; Nqo1, NAD(P)H dehydrogenase (quinone) 1; Nrf2, nuclear factor (erythroid-derived 2) like 2; PDI, protein disulfide isomerase; PD-L1, programmed death ligand 1; PRXD1/Prdx1, peroxiredoxin 1; ROS, reactive oxygen species; Se, Selenium; SeMet, selenomethionine; STAT1/Stat1, signal transducer and activator of transcription 1; TNBC, triple negative breast cancer cells; TAP, transporter-associated with antigen processing; TPN/Tapbp, tapasin; ULBP, UL-16 binding protein

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2D-DIGE; anti-tumoral immune response; APM components; methylseleninic acid; MHC class I; selenium

Introduction

Selenium (Se), an essential trace element, is metabolized to a variety of low molecular weight compounds and selenoproteins. While inorganic Se is mainly represented by selenate and selenite, members of the organic Se forms are selenomethionine (SeMet) and methylselenocysteine (MSC).[1] In contrast, methylseleninic acid (MSA) is a synthetic Se compound, which has been implemented in a number of *in vitro* and *in vivo* experimental studies.[2] The metabolism of the different Se compounds is complex and closely regulated. The two key metabolites selenide and methylselenol have been shown to be crucial for the biological function of these substances. In addition, the cellular thioredoxin (TRX) and glutaredoxin systems known to play an important role in the maintenance of the intracellular redox balance are involved in the reduction of various Se compounds.

Furthermore, Se has been suggested to be chemopreventive and to exert anti-carcinogenic effects on many solid and haematopoietic tumors such as bladder, prostate and breast cancer as well as lymphoma.[3,4] The functional activity of

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selenoproteins might be due to their anti-oxidative effects or to an altered expression pattern of genes involved in cancer initiation and progression.[5,6] This was further strengthened by epidemiologic studies revealing an inverse correlation of the Se status and cancer incidence as well as cancer mortality.[7] Furthermore, Se is one of the few trace elements, for which anti-carcinogenic effects have been proven in a randomized, double blinded, placebo controlled study.[8] Clinical interventional studies demonstrated that a supra-nutritive intake of Se has positive effects in the prevention of several tumor types, which were also confirmed by *in vivo* and *in vitro* experiments.[9] In contrast, the SELECT study studying the chemopreventive potential of Se within a heterogeneous group of healthy man failed to reduce the risk for prostate cancer.[10] These controversial data might be due to the different Se compounds used in these trials. Several lines of evidence have implicated that the Se metabolite methylselenol is the active Se compound for anti-carcinogenic effects.[11,12] Se compounds directly entering the monomethylated pool of Se compounds are more effective in reducing tumorigenesis than those preferentially metabolized to hydrogen selenide.[13,14] Methylselenol induces in combination with intracellular thiols reactive oxygen species (ROS).[15] MSA, a proximal precursor of the redox active Se metabolite methylselenol, is intracellularly reduced to methylselenol by several non-enzymatic steps [14] and exhibits cytotoxic potential in different tumor entities, e.g., colon,[16] melanoma[17] and mammary carcinoma.[4] Furthermore, MSA has both anti-carcinogenic and anti-metastatic potential *in vivo* without affecting the general behavior of animals. The combined treatment of triple negative breast cancer cells (TNBC) with MSA and paclitaxel upon xenoengraftment in mice reduced tumor growth more than paclitaxel alone,[18] which might be due to induction of apoptosis and/or cell cycle arrest. The chemopreventive/chemotherapeutic effects of MSA might be associated with an inhibition of histone deacetylases (HDACs). HDACs are involved in the regulation of the gene expression pattern and often overexpressed in tumor cells. In B cell lymphoma and esophageal cancer, MSA reduces HDAC activity[19,20] by modulation of conserved cysteine residues in the catalytic center of HDACs.[21]

Different models of tumor-initiation showed that natural killer (NK) cells and the interferon (IFN) system are involved in the immunity of tumors. The NK-mediated anti-tumoral responses require the expression of the activating receptor NKG2D (natural killer group 2 member D).[22] NKG2D recognizes infected or abnormal cells by binding to ligands, which are induced on stressed or transformed cells thereby leading to their elimination.[22] Recently, Hagemann-Jensen and coworkers showed that the selenocompound MSA affects the immunogenicity of tumors by modulating different ligands of the NKG2D receptor, like the MHC class I polypeptide-related molecules (MICA, B) leading to enhanced NK cell responses.[23] Since NKG2D expression is not only limited to NK cells, but also found on human $CD8^+$ T cells[24,25] it plays a key role in both NK and T cell-mediated tumor elimination.[26]

The human major histocompatibility complex (MHC) class I molecules are subdivided into classical HLA-A, -B and -C (MHC class Ia) and non-classical HLA-E, -F and -G (MHC class Ib) antigens and are expressed on the cell surface in association with $ß_2$-microglobulin. While the expression of HLA-A, -B and -C antigens are frequently lost or downregulated on tumor cells, HLA-G and/or HLA-E antigens are often overexpressed in tumors of distinct origin, e.g., ovarian carcinoma, colon carcinoma and melanoma.[27-29] Since HLA-E can bind to the inhibitory CD94/NKG2A receptors expressed on NK cells and a subset of T cells, HLA-E expression could lead to an escape of tumor cells from immune surveillance.[30] Interestingly, the selenocompound selenite is able to reduce HLA-E antigen expression in different tumor cells, which was accompanied by an enhanced NK cell-mediated killing[31] suggesting that selenite might be able to potentiate the antitumor cytotoxicity in settings of NK cell-based immunotherapies.

In contrast, for proper T-cell responses MHC class Ia surface expression is required, which is controlled by different components of the MHC class I antigen processing machinery (APM). MHC class I molecules present antigenic peptides on the surface of cells, which are predominantly generated by proteasomal degradation of intracellular proteins. The generated peptides are translocated by the transporter-associated with antigen processing (TAP) from the cytosol into the lumen of the endoplasmic reticulum (ER) and there loaded onto MHC class I molecules, which is facilitated by different chaperones, e.g., tapasin (TPN), ERp58, protein disulfide isomerase (PDI). Subsequently, the loaded MHC class Ia molecules are transported via the trans-Golgi apparatus to the cell surface and there presented to $CD8^+$ cytotoxic T lymphocytes (CTL). However, total, partial loss or downregulation of MHC class I surface expression or APM components were found in many tumors of distinct origin thereby limiting the respective antitumor immune responses,[32,33] which were often associated with disease progression and reduced patients' survival.[34-36] The underlying molecular mechanisms of MHC class I abnormalities are diverse and include either irreversible structural alterations or reversible deregulatory processes of APM components. While mutations, deletions and/or loss of heterozygosity rather occur rarely, the expression of APM components in tumors is frequently regulated by transcriptional, epigenetic or post-transcriptional control mechanisms.[32,37,38] Moreover, the expression pattern of MHC class I APM components seems to be linked to changes in the cellular or environmental metabolism. Recently, oxidative stress has been shown to alter cellular glutathione levels, which is associated with changes in the MHC class Ia dimer formation on the cell surface.[39] The impact of changes in the cellular redox status on the modulation of the MHC class Ia expression level is further supported by the role of the oxidizing enzyme ER oxidoreductase α (ERO-1a), which is controlled by oxidative folding.[40]

Since one major goal in the field of tumor immunology is to re-establish and to maintain/bolster MHC class Ia surface expression in tumors thereby increasing their immunogenicity the identification of key molecules or substances overriding tumor intrinsic escape routes, such as the downregulation of MHC class Ia surface expression used by tumor cells will help to improve durable tumor rejection. In order to get better insights into the role of redox processes on the immunogenicity of tumor cells, B16F10 melanoma cells were treated with various concentrations of selenocompounds including MSA and their influence on the regulation of MHC class I surface expression level and/or the expression pattern of APM components

were determined. The MSA-mediated altered redox metabolism caused a transcriptional upregulation of APM components leading to increased levels of MHC class I cell surface antigens. In addition, MSA mimics interferon-gamma (IFNγ) signaling by also upregulating members of IFNγ responsive genes.

Results

Effects of MSA treatment on cell viability

A prerequisite for the analyses of the immunogenic phenotype of tumors and of their proteome expression profiles in the presence of MSA is to determine the effects of MSA treatment on cell viability. Therefore, B16F10 melanoma cells were treated with different concentrations of MSA for 24 and 48 h, respectively. While lactate dehydrogenase (LDH) activity in the supernatants of B16F10 cells did not differ between the untreated and MSA-treated groups indicating that the viability of these cells was not compromised, XTT assays revealed more metabolic active cells under MSA treatment in comparison to the control group indicating that other enzymes catalyzing the formation of formazan might be upregulated. As formazan is also metabolized by NAD(P)H, the transcription of the NAD(P)H producing enzymes was determined. As shown in Fig. S1, the expression level of both glucose-6-phosphate dehydrogenase and 6-phospho-gluconate dehydrogenase were upregulated in response to MSA treatment. Furthermore, wound healing (Fig. 1A) as well as migration assays (Fig. 1B) support a decreased migration rate of B16F10 cells in the presence of MSA.

Association of the MSA-induced decreased migration of melanoma cells with reduced AKT phosphorylation

Since protein kinase B (AKT) is one of the key regulators of proliferation, cell survival and migration,[41] the phosphorylation status of AKT as well of its negative regulator PTEN were investigated in untreated (control) and MSA-treated B16F10 cells by Western blot analysis. When compare with the control cells MSA treatment resulted in a decreased phosphorylation rate of AKT at serine residue 473, whereas the phosphorylation status at position threonine 308 as well as of the total AKT protein expression level remained unaffected by this treatment (Figs. 2A and B). In contrast, the phosphorylation rate as well as the protein expression level of PTEN increased in response to MSA treatment. Since AKT phosphorylation might also regulate the glucose metabolism, the glucose uptake rates were determined in untreated and MSA-treated B16F10 cells by flow cytometry. As shown in Figs. 2C and D, the glucose uptake rate of MSA-treated cells was slightly reduced, which is in accordance with the reduced AKT phosphorylation rate.

MSA-mediated changes in the protein expression pattern of B16F10 melanoma cells

To gain further insights into the anti-carcinogenic potential of MSA, 2D-DIGE analysis of untreated (control) and MSA-treated B16F10 cells was performed in an untargeted approach (Fig. 3, Table 1). The consensus map across all experimental samples (three biological replicates/group, n = 6) was comprised of 872 distinct spots. In total, 33 differentially expressed protein spots could be identified, by subjecting them to MALDI-TOF-MS analyses resulting in the identification of 14 unique protein species. The identified proteins were grouped according to their biological function(s) (Fig. 3C). The expression pattern of three of the upregulated proteins are controlled by the transcription factor Nrf2.[42] These include glucose 6-phosphate dehydrogenase (G6PD), peroxiredoxin 1 (PRXD1) and S-formylglutathione hydrolase (ESTD), all involved in mounting/maintaining the anti-oxidative defense. In addition, two ER-resident enzymes named protein disulfide isomerase (PDI) and calreticulin (CALR) were upregulated in response to MSA treatment. PDI exhibits oxidoreductase functions and together with CALR is involved in the MHC class I assembly pathway.[43,44] In contrast, the heterogeneous ribonucleoprotein A2B1 (hnRNPA2B1), which is involved in splicing processes, was downregulated in the presence of MSA.

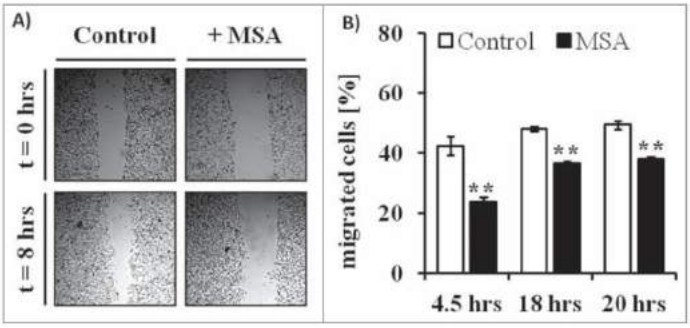

Figure 1. Altered wound healing and migration of B16F10 melanoma cells in response to MSA treatment. (A) To investigate wound-healing processes scratch assays were performed. Therefore, confluent monolayers of B16F10 cells cultured in complete media supplemented with 10% FCS were mechanically disrupted with a sterile pipette tip, left untreated (control) or treated with 2.5 μM MSA and then photographed immediately (0 h) and 8 h after scratching. The assays were performed in triplicates. (B) For migration assays B16F10 cells either left untreated or treated with MSA for 24 h were seeded into trans-well chambers. All assays were independently performed at least three times as described in the Material and Method section. $^{**}p < 0.01$ (Student's *t*-test).

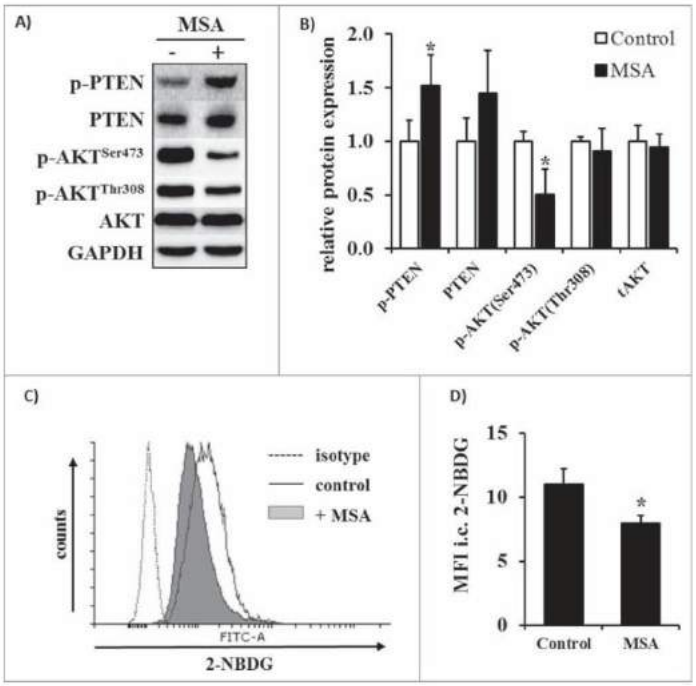

Figure 2. Changes in the phosphorylation pattern of AKT and glucose uptake after MSA exposure of B16F10 melanoma cells. (A) Representative phosphorylation states and expression levels of AKT and PTEN in B16F10 cells following treatment with 2.5 μM MSA for 24 h were determined by Western blot analyses as described in the method section. (B) Relative quantification of p-AKTSer473, p-AKTThr308, total AKT, p-PTENSer380/Thr382/383 and total PTEN expression levels normalized to GAPDH and thereafter set in relation to the control group. (C) Glucose uptake of B16F10 cells after treatment with 2.5 μM MSA was measured using the fluorescent glucose analog 2-NBDG, which was determined by flow cytometry as described in the Material and methods section. (D) 2-NBDG fluorescent intensities were normalized to the respective unstained control. All assays were independently performed at least three times. $^{*}p < 0.05$ (Student's *t*-test). 2-NBDG, 2-(N-(7-Nitrobenz-2-oxa-1,3-diazol-4-yl) Amino)-2-Deoxyglucose.

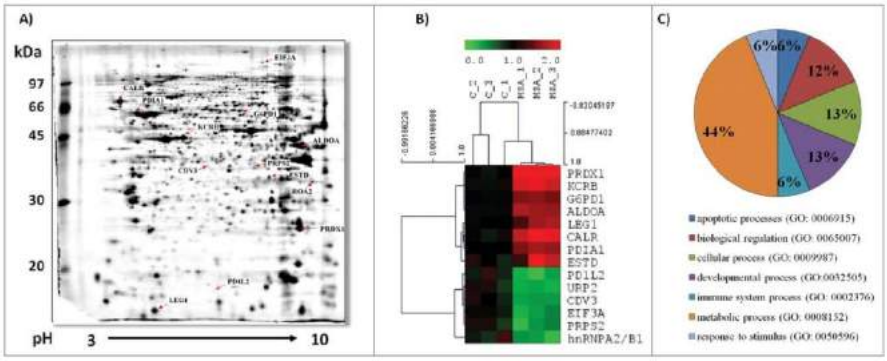

Figure 3. Differential protein expression pattern of untreated and MSA-treated B16F10 melanoma cells. (A) A representative preparative protein expression pattern of B16F10 melanoma cells following two-dimensional gel electrophoresis (2-DE) is shown as described in Materials and methods sections. Differentially expressed proteins as defined by 2D-DIGE were identified by MALDI-TOF-MS analyses as described in the material section and are indicated by their protein IDs (see also Table 1). (B) Representative heat map and cluster analysis for the panel of identified differentially expressed proteins was done using the software TM4 MeV4.9 (http://www.tm4.org/). (C) Classification analysis of the differentially expressed proteins according to their biological processes was performed using the Panther gene ontology (GO) software (http://pantherdb.org/).

Table 1. Differentially expressed proteins in response to MSA treatment

Protein	Uniprot ID	Score MS	Sequence Coverage (%)	No. of matched peptides	Theoretical Mass (kDa)	Theoretical pI	Ratio MSA/C
Peroxiredoxin-1 (PRDX1)	P35700	268	78	25	22.39	8.26	2.57 ↑
Calreticulin (CALR)	P14211	158	44	21	48.136	4.33	1.96 ↑
Creatine kinase B-type (KCRB)	Q04447	235	75	30	42.971	5.40	1.88 ↑
S-formylglutathione hydrolase (ESTD)	Q9R0P3	116	47	14	31.87	6.7	1.69 ↑
Fructose-bisphosphate aldolase A (ALDOA)	P05064	266	85	27	39.787	8.31	1.64 ↑
Protein disulfide-isomerase (PDIA1)	P09103	262	48	33	57.422	4.77	1.58 ↑
Glucose-6-phosphate 1-dehydrogenase X (G6PD1)	Q00612	407	66	49	59.681	6.06	1.52 ↑
Galectin-1 (LEG1)	P16045	118	61	11	15.198	5.32	1.52 ↑
Eukaryotic translation initiation factor 3 subunit A (EIF3A)	P23116	100	21	28	162.237	6.38	0.38 ↓
Protein CDV3 (CDV3)	Q4VAA2	70	54	9	29.711	5.84	0.38 ↓
Ribose-phosphate pyrophosphokinase 2 (PRPS2)	Q9CS42	57	37	9	35.163	6.15	0.37 ↓
Heterogeneous nuclear ribonucleoproteins A2/B1 (hnRNPA2/B1)	O88569	179	52	25	37.437	8.97	0.33 ↓
Fermitin family homolog 3 (URP2)	Q8K1B8	59	24	11	76.157	6.60	0.32 ↓
Programmed cell death 1 ligand 2 (PD1L2)	Q9WUL5	56	19	5	28.201	8.46	0.28 ↓

Values are given as means ($n = 3$) in relation to the untreated control group. ↑ indicate significantly upregulated proteins, ↓ indicate significantly downregulated proteins ($p < 0.05$, Student's *t*-test).

Validation of representative differentially expressed proteins in response to MSA treatment

The upregulation of the PRXD1, G6PDH, ESTD, PDI and the downregulation of hnRNPA2/B1 were validated by RT-PCR in untreated (control) and MSA-treated B16F10 cells (Fig. 4A). The mRNA and protein expression levels of PDI were enhanced in the presence of MSA (Fig. 4B). Since PRDX1 and G6PDH are both regulated by Nrf2,[42] the expression level of the latter was determined by Western blot analysis. As shown in Fig. 4C, no nuclear Nrf2 accumulation was detected in untreated B16F10 melanoma cells, whereas MSA treatment strongly enhanced Nrf2 accumulation in the nucleus. Furthermore, the transcriptomic profiling of additional Nrf2 targets revealed an enhanced Nrf2 activity in response to MSA treatment. Since Nrf2 activation in combination with the transcriptional induction of Nrf2 target genes could be mediated by enhanced ROS levels, intracellular ROS levels were analyzed using a ROS-sensitive reporter dye. As shown in Figs. 5A and B, increased ROS levels were detected within 4 h after MSA treatment. Furthermore, total glutathione (GSH) levels were approximately 2-fold increased in B16F10 cells after 24 h MSA treatment (Fig. 5C) suggesting that MSA might interrupt/interfere with the cellular redox homeostasis.

Identification of MSA-induced alterations in the redox status of proteins and their correlation with MHC class I APM components

In order to determine the MSA-mediated changes in the redox status, redox proteome analysis using iodoTMT tags, which target free cysteine residues, were employed. This led to the identification of proteins with an altered redox status including several ER-resident proteins, which are directly involved in the MHC class I assembly, such as e.g., ERO1a, PDIA1, PDIA3, PDIA4 and CALR. In addition, TRIM28 showed an altered

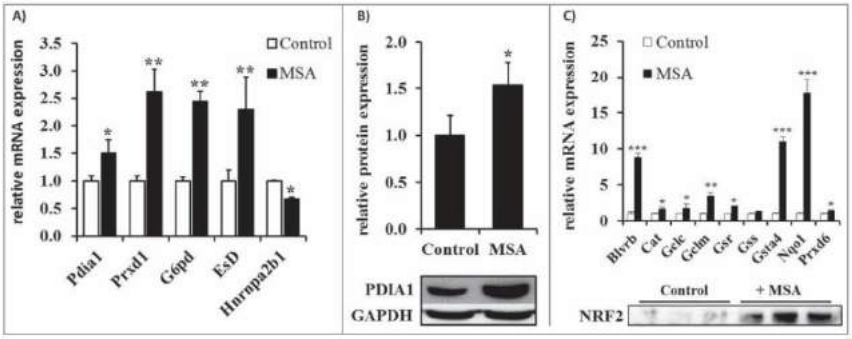

Figure 4. Validation of selected MSA targets obtained by proteomic analysis. (A) mRNA expression levels of differentially expressed proteins were determined by qPCR analyses as described in the Materials and methods section. The relative mRNA expression levels were normalized to the reference genes RPL13a, GAPDH and ß-actin and subsequently defined in relation to the control group. (B) Densitometric analysis of the relative PDI1A expression levels based on the Western blot analysis as representatively shown in the lower panel. (C) mRNA analysis of different Nrf2 target genes determined by qPCR (upper panel) and nuclear Nrf2 content of B16F10 cells left untreated or treated with 2.5 μM MSA was determined by Western blot analysis (lower panel). All experiments were independently performed at least three times. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ (Student's *t*-test).

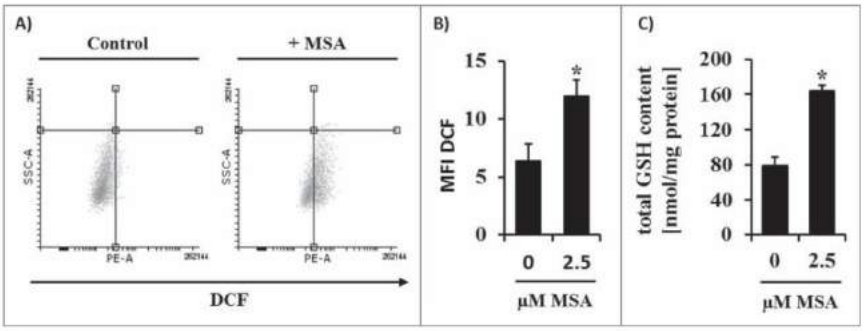

Figure 5. Changes of the intracellular ROS and GSH levels upon MSA treatment of B16F10 cells. B16F10 cells were left untreated or treated with MSA for 4 h. Intracellular ROS concentrations in these cells were determined using the fluorescent reporter dye DCF by flow cytometry as described in the material and method section. (A) Representative dot blots and (B) MFI of DCF normalized to unstained cells. (C) Intracellular levels of total GSH were measured spectrophotometrically as described in the method section. All experiments were performed at least three times. $^{*}p < 0.05$ (Student's *t*-test). DCF, 2',7'-Dichlorfluorescein.

redox-status in response to MSA treatment (personal communications).

By employing the R2: Genomics Analysis and Visualization Platform (http://hgserver1.amc.nl/cgi-bin/r2/main.cgi) correlations between the expression rates of hnRNPa2/b1, TRIM28 and selected components of the MHC class I APM in melanoma were determined (Fig. 6). With this approach, inverse associations of hnRNPa2/b1 to the MHC class I APM component TPN (PC: −0.222, $p = 1.1 \times 10^{-6}$) and the interferon regulatory factors IRF5 (PC: −0.282, $p = 4.7 \times 10^{-10}$) and IRF9 (PC: −0.221, $p = 1.3 \times 10^{-6}$) were found. In addition, inverse correlations of TRIM28 with STAT1 (PC: −0.405, $p = 5.8 \times 10^{-20}$), STAT2 (PC: −0.232; $p = 3.5 \times 10^{-7}$), STAT3 (PC: −0.243; $p = 9.5 \times 10^{-8}$) and with NLRC5 (PC: −2.89; $p = 1.7 \times 10^{-9}$) were detected, whereas the Nrf2 target NQO1 positively correlated with HLA-A (PC: 0.230; $p = 2.4 \times 10^{-6}$).

Enhanced MHC class I surface antigens of B16F10 cells after MSA treatment

Since Nrf2 has been shown to induce the expression of proteasomal subunits necessary for the generation of peptides[45] and MSA mediated an altered expression or redox status of CALR, PDI, ERO1a and TRIM28, respectively, it was postulated that MSA upregulates the expression of MHC class I antigen molecules in B16F10 cells, which are per se characterized by low MHC class I cell surface expression levels due to the impaired expression of several APM components. This immune escape phenotype of B16F10 could be

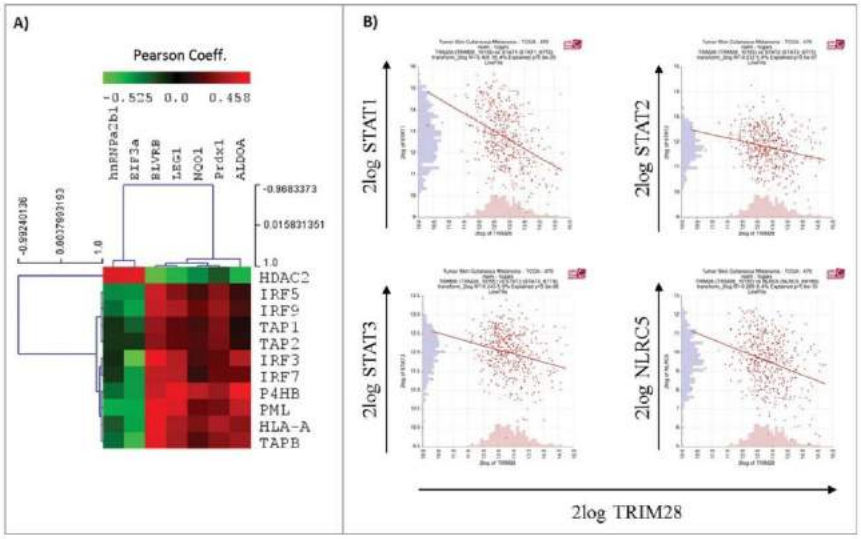

Figure 6. Correlation analysis. Correlation analysis of selected differentially expressed (A) or redox-modified (B) proteins were performed by employing the R2: Genomics Analysis and Visualization Platform (http://hgserver1.amc.nl/cgi-bin/r2/main.cgi).

restored by IFNγ treatment.[46] As shown in Fig. 7, a dose-dependent approximately 2- to 4-fold increase of the MHC class I surface expression level in B16F10 melanoma cells treated for 24 h with MSA was detected, which was stable for at least 48 h (Figs. 7A and B). In addition, treatment with DMDSe also resulted in an upregulation of the MHC class I surface expression level comparable to that of MSA. In contrast, treatment with selenite showed no effect, whereas SeMet treatment at least for the higher doses even resulted in a downregulation of the MHC class I surface expression level (Figs. 7C–E).

General effects of MSA treatment on the MHC class I expression level of tumor cells

To study whether the effects observed in B16F10 cells in response to treatment with the compound MSA could be also extended to other tumor cells, three human melanoma cell lines (WM1552c, UKRV, Colo875), two human mammary carcinoma cell lines (SK-BR-3 and BT-474) and the human colon carcinoma cell line SW480 were left untreated and treated with MSA as described for B16F10 cells, before their MHC class I expression levels were determined by flow cytometry. As shown in Figs. 8A–F, treatment with MSA increased the MHC class I surface expression levels in all the tested tumor cell lines.

Transcriptional upregulation of MHC class I APM components by MSA

In order to define the underlying molecular mechanism which leads to the observed upregulation of the MHC class I surface expression levels in response to treatment with MSA, the mRNA and protein expression pattern of selected APM components were determined. As shown in Fig. 9A MSA increased the transcript levels of a panel of APM components including the IFNγ-inducible proteasomal subunits low molecular weight protein (LMP)2 and LMP7, TAP1, TAP2, TPN, β_2-m, H-2D and H-2K, yet for LMP10 rather a downregulation of the corresponding transcript level was observed. In addition, the mRNA levels of

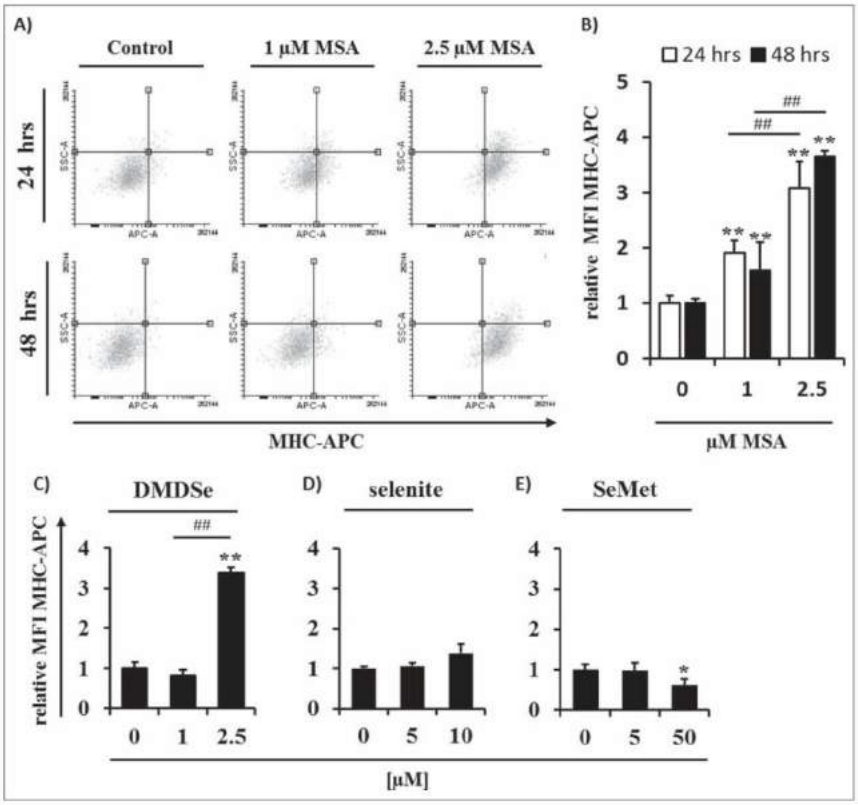

Figure 7. Effects of MSA on the MHC class I surface expression of B16F10 melanoma cells. (A) Dot blots of SSC versus anti-MHC class I staining of B16F10 cells treated with different concentrations of MSA (0–2.5 μM) for 24 and 48 h, respectively, are shown. Analyses were performed by flow cytometry as described in Materials and methods. The fluorescence intensity of (B) MSA, (C) DMDSe, (D) selenite and (E) selenomethionine (SeMet) treated cells were normalized to the respective isotype controls. Data represent means ± SD and are expressed in relation to the untreated (0 μM MSA) group. All experiments were performed at least three times. ** indicate significant differences ($p < 0.01$, Student's *t*-test) of the treated vs. control group. ## indicate significant differences ($p < 0.01$, Student's *t*-test) within the MSA-treated groups. SSC, sideward scatter.

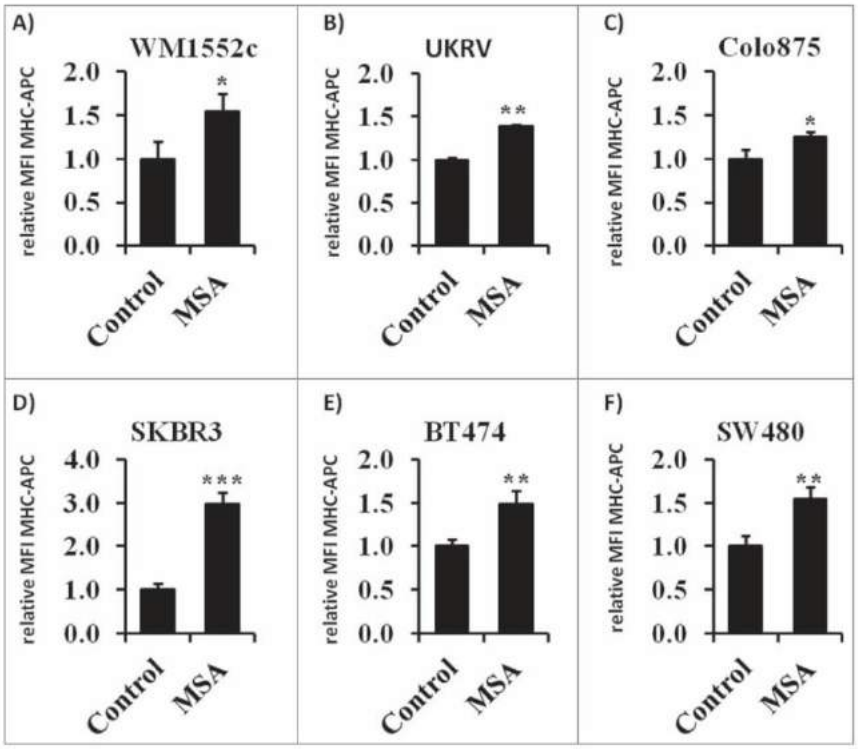

Figure 8. MHC class I surface expression of tumor cell lines following treatment with 2.5 μM MSA for 24 h as analyzed by flow cytometry. One representative experiment of three independent experiments is shown. Bars represent mean fluorescence intensity (MFI) of MHC-APC normalized to the isotype control within the given independent experiments. ** indicate significant differences ($p < 0.01$, Student's *t*-test).

NLRC5, a critical co-activator of genes of the MHC class I presentation pathway,[47] were upregulated. To understand the molecular mechanisms of the MSA-mediated regulation mechanism, also the promoter activities of TAP1, TAP2, TPN and LMP2 were determined in response to treatment with MSA and revealed enhanced activation states (Fig. 9B). Furthermore, the protein expression levels of LMP2 and LMP7 were analyzed by Western blot supporting the coordinated expression both at the mRNA and protein levels for these APM components. Moreover, MSA treatment resulted in an enhanced transcript level of signal transducer (STAT)1 and in a shift of STAT1ß toward STAT1α expression at the protein level. In addition, the phosphorylation states of STAT1 and JAK1 were increased following MSA treatment suggesting that MSA enhances the expression of MHC class I surface antigens by inducing APM components and by mimicking IFNγ signaling (Figs. 9C and D).

Effect of MSA on the IFNγ signal transduction in B16F10 cells

In addition to components of the MHC class I APM the transcript levels of components of the IFNγ signaling pathway were analyzed in response to treatment with either MSA or IFNγ alone as well as to treatment with a combination of both substances (Fig. 10). MSA and IFNγ treatment alone increased the transcript levels of IRF1, IRF5, IRF7 and IRF9, whereas IRF4 was downregulated by MSA and upregulated by IFNγ. Combination of both substances resulted in a further increase of IRF5, STAT1 and STAT2 transcript levels.

Discussion

The trace element Se has been discussed to exhibit chemopreventive potential. Furthermore, newer studies reveal also chemo-therapeutic effects of Se.[3,4] However, the mechanisms, by which Se exerts those beneficial effects are largely unknown, but include their anti-oxidative properties, the induction of apoptosis and inhibition of cell proliferation. There exist two key metabolites of Se, which are critical for chemoprevention/chemotherapy.[48] Hydrogen selenide necessary for the formation of selenoproteins results mainly from inorganic selenocompounds such as selenate or selenite, respectively, and methylselenol, which is generated from organic selenocompounds. In this study, the pro-oxidant MSA, a synthetic selenocompound and precursor of methylselenol, induced MHC class

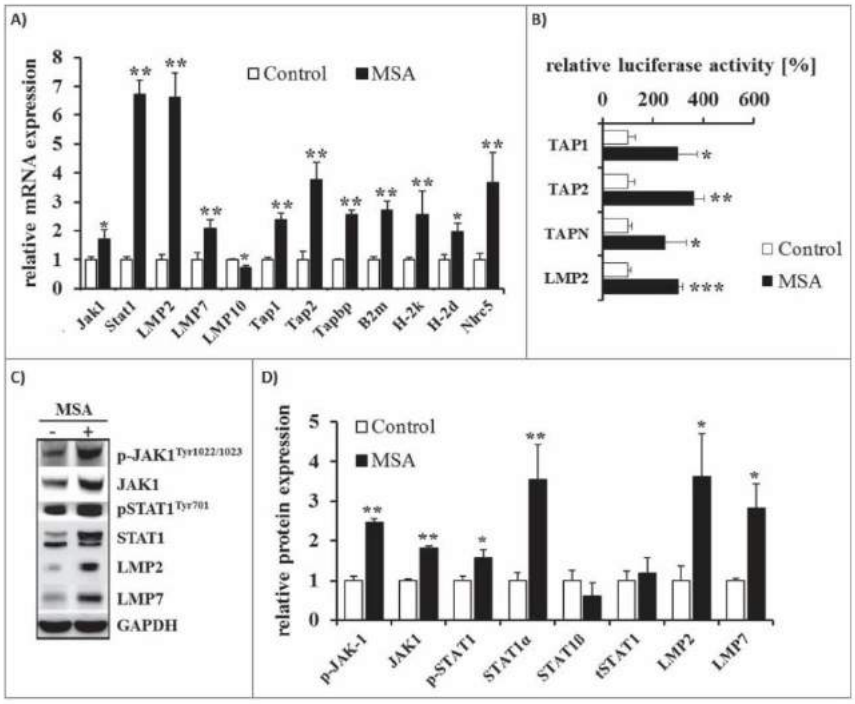

Figure 9. Analysis of APM component expression in B16F10 cells after treatment with MSA. (A) mRNA expression levels of APM components were determined by qPCR analyses normalized to the set of reference genes RPL13a, GAPDH and ß-actin as well as in relation to the control group. (B) Different wild-type (WT) APM-luc promoters and the ß-gal promoter were transiently co-transfected into B16F10 cells 24 h prior treatment with MSA for 24 h. Luc activity was determined as described in Materials and methods and normalized to ß-gal activity. Data are expressed in relation to untreated controls. (C) Representative Western blots of APM components and (D) their quantification by measuring the OD normalized to GAPDH in relation to the control group are shown. Data represent means ± SD of at least three independent experiments. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ (Student's *t*-test).

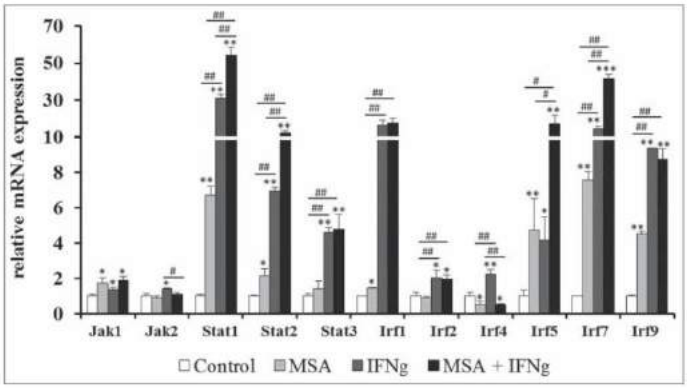

Figure 10. Changes in the mRNA expression levels of the IFNγ signaling pathway in response to MSA and IFNγ treatment. B16F10 cells were treated with 2.5 μM MSA and 200 U/mL IFNγ alone or in combination for 24 h. The mRNA expression levels were determined by qPCR analyses, normalized to the reference genes RPL13a, GAPDH and ß-actin and subsequently defined in relation to untreated control group. Data represent the mean of at least three independent experiments. * indicate significant differences between the treated and control groups ($^{*}p < 0.05$, $^{**}p < 0.01$, Student's *t*-test). # indicate significant differences within the different treatment groups ($^{\#}p < 0.05$, $^{\#\#}p < 0.01$, Student's *t*-test).

I surface expression not only in B16F10 melanoma cells, but also on several other tumor cell lines, including human cancer cells. These data were in line with the effects of the pro-oxidative selenocompound DMDSe, which also induces MHC class I surface expression. In contrast, the selenocompounds selenite and SeMet known to produce less amounts of ROS and methylselenol during their metabolic processing/turnover failed to increase MHC class I surface expression. These results indicate that the effects on the MHC class I expression pattern are rather mediated by the formation of ROS or methylated selenocompounds, such as methylselenol, as by Se itself. This hypothesis is in accordance to results by Hagemann-Jensen and coworkers demonstrating a MSA- and DMDSe-mediated upregulation of NKG2D receptor ligands, whereas selenite, selenate, SeMet and SeCys did not influence MICA and MICB expression.[23] In accordance to our study, Enqvist and co-authors[31] described no effect of selenite on the expression of MHC class Ia molecules, but detected a strong post-transcriptional downregulation of HLA-E antigens, which was associated with an enhanced NK cell-based tumor killing. HLA-E antigens act as both inhibitory and activating ligands for CD94/NKG2 receptors depending on the antigen load: Upon loading with a peptide derived from an HLA-Ia leader sequence, HLA-E can interact with the inhibitory receptor CD94/NKG2A, while upon loading with a HLA-G leader sequence HLA-E can interact with the activating receptor NKG2C.[49] In contrast to this important role of HLA-E in the innate immunity, only few experiments revealed that MHC class Ib molecules could interact with T cells.[30,50] It could be shown that HLA-E$^+$ targets loaded with peptides derived from splice variants of the stress inducible anti-oxidant enzyme peroxiredoxin 5 (Prdx5) are recognized by HLA-E-restricted CD8$^+$ T cells.[51] In contrast, complexes composed of HLA-E and Prdx5 did not interact with NKG2A and therefore did not downregulate NK cell function.[51] HLA-E might be upregulated in response to cytokines like IFNγ[52] suggesting that an upregulation of HLA-E in response to MSA cannot be excluded. Thus, further analyses are required to determine the impact of MSA treatment on the expression of MHC class Ib molecules.

In our study, the induction of MHC class Ia was caused by a transcriptional upregulation of components of the APM, including proteasomal subunits and components of the peptide loading complex. Furthermore, MSA partially mimics IFNγ signaling, such as the upregulation of STAT1, JAK1, IRF1, IRF5, IRF7 and IRF9 on the mRNA and/or protein expression levels. In addition, MSA treatment leads to activation of the transcription factor Nrf2, thus resulting in the initiation of the upregulation of genes involved in the anti-oxidant defense. The observed enhanced Nrf2 accumulation in the nucleus could be mediated by the formation of ROS in response to treatment with MSA. Under normal conditions, Nrf2 is bound to its inhibitor protein Keap1 and marked for proteasomal degradation. ROS or electrophilic agents modify redox-sensitive cysteine residues of Keap1, leading to conformational changes and the release of Nrf2, which in turn could translocate into the nucleus and initiate the transcriptional induction of its target proteins.[42] In our study, a strong upregulation of Nrf2 targets was observed, such as NQO1. Next to its role in the anti-oxidative defense, NQO1 is described to stabilize p53 and thus prevents its proteasomal degradation.[53] Concerning the peptide transporter subunit TAP1, it has been described that p53 regulates its expression and enhances the peptide transport as well as the expression of MHC class I peptides on the cell surface.[54] This might be a link, given that MSA enhances p53 protein levels as well as its activity.[55,56]

It is noteworthy that IFNγ is an important inducer of ROS, via NOX enzymes, that causes oxidative stress and an enhanced formation of oxidative damaged proteins.[57,58] Furthermore, it could be shown that the upregulation of subunits of the so-called immuno-proteasome (LMP2, LMP7, LMP10) might rather be an mechanism to degrade oxidative-damaged proteins.[59] Also, in our study an upregulation of proteasomal subunits after treatment with MSA could be shown, which might be attributed to the enhanced activity of the transcription factor Nrf2 mediated in response to MSA treatment, which has been found to induce the transcriptional upregulation of LMP2 and LMP7.[45]

Despite the clear association between the observed induction of ROS along with the increased expression of Nrf2 (oxidative stress response) and the elevated MHC class I surface expression levels, which are associated with an upregulation of APM components, the exact molecular mechanisms of the observed effects in response to treatment with MSA have not yet been identified. One explanation might be that MSA has been suggested to inhibit HDACs.[20] In line with this hypothesis, HDAC inhibitors have been described to upregulate both MHC class I and class II molecules at the cell surface.[60] In addition, MSA has been shown to induce the expression of MICA and MICB in Jurkat cells to the same extent as HDAC inhibitors.[23] Therefore, further analysis are required to test whether the modulation of the expression pattern of MHC class I molecules by MSA is indeed mediated at least in part by decreased HDAC activities.

In conclusion, our data provide a new molecular mechanism which might explain the potential anti-carcinogenic properties of Se. As MHC class I molecules are often downregulated in tumor cells and thus regarded as one of the key mechanism by which tumor cells might escape from immune surveillance, treatment with MSA might be in particular beneficial for the treatment of tumors exhibiting low MHC class I surface expression levels, thereby improving and likely reshaping the mounting of an immune response.

Material and methods

Cell culture

The murine melanoma cell line B16F10 and human cell lines WM1552c, UKRV, Colo875, BT474 and SK-BR-3 were routinely cultured in RPMI media supplemented with 10% FCS, 100 U/mL penicillin and 100 μg/mL streptomycin, whereas the human colon carcinoma cell line SW480 was maintained in complete DMEM media. All cells were cultured at 37°C in 5% (v/v) CO_2 humidified air.

Reagents and treatment of cells

MSA, dimethyldiselenide, sodium selenite and SeMet were all purchased from Sigma-Aldrich. IFNγ was purchased from

Immunotools (#12343534). Stock solutions were prepared in either water (MSA, selenite, SeMet) or DMSO (DMDSe). Cells were seeded and incubated to reach adherence. After 24 h, cells were supplemented with the indicated concentrations of Se compounds and/or 200 U/mL IFNγ and incubated as indicated prior to the respective analyses. Non-supplemented and DMSO treated cells served as controls.

Cytotoxicity assay

Cytotoxicity was determined by measurement of LDH release using the CytoTox 96® Non-Radioactive Cytotoxicity Assay (#G1781, Promega) according to the manufactures instructions. In brief, 5×10^3 cells were seeded per 96-well. After 24 h, total media was removed and replaced by media containing different concentrations of MSA for additional 24 h. LDH release was measured at 490 nm using the infinite M200 pro plate reader (Tecan, Germany).

Cell viability assay

To asses cell viability in response to MSA treatment the CellTiter-Glo® Luminescent Cell Viability Assay (#G7571, Promega) was used according to the manufactures instructions. In brief, 5×10^3 cells were seeded per 96-well. After 24 h, total media was removed and replaced by media containing different concentrations of MSA (0–10 μM) for additional 24 h. Luminescence measurement was done using the luminometer MicroLumatPlus LB96V (EG&G Berthold Technology, Germany).

Wound healing and migration

For monolayer wound-healing assay, cells were seeded in 12-well plates. At a 100% confluence wounds were made using a sterile pipette tip. Wound size was measured after 8 h as described previously.[61] For determination of the cell migration, 1×10^5 of 24 h treated or untreated cells, respectively, cells were seeded into the upper well of the Transwell chamber system (Corning), with medium containing 0.5% FBS. At the lower chamber, 10% FKS was added as an attractant. After incubation at 37°C for 4.5, 18 and 20 h, respectively, the non-migrated cells of the top insert were completely removed, and the cells on the bottom insert surface were lysed with CellTiter-Glo (Promega) before the ATP content was measured in the luminometer (EG&G Berthold Technology, Germany).

2D DIGE analysis

Three biological replicates per sample representing either the control group or the MSA-treated group were subjected to undergo proteomic profiling. DIGE analysis was performed using the minimal labeling approach according to the manufacturer's instructions (NH DyeAGNOSTICS, Halle, Germany) with the exception that 25 μg of the respective protein lysates as well as the internal protein standard were labeled with 100 pmol of the given G-dye. 2D gel electrophoresis and subsequent MALDI-TOF MS analysis were performed as described previously.[62] Differentially expressed proteins were clustered according to their functions using the Panther gene ontology (GO) software (http://pantherdb.org/).[63]

Flow cytometry

Flow cytometric analysis was performed as previously described.[64] Briefly, cells were stained with following monoclonal antibodies (mAb): APC-conjugated anti-mouse MHC class I (H-2D^b) (#17–5999, ebioscience), APC-conjugated mouse IgG2a (#400222, Biolegend), APC-conjugated anti-human HLA-ABC (#555555, BD biosciences), APC-conjugated mouse IgG2B (# IC00417, RD systems), before fluorescence was determined on a BD LSRFortessa cytometer (BD Biosciences). Data were analyzed on a free tool "Flowing software" (http://www.uskonaskel.fi/flowingsoftware/).

Determination of intracellular ROS and GSH levels

For determination of ROS levels, 2×10^5 cells were transferred into a FACS analysis tube, washed with PBS and re-suspended in 1 mL PBS containing 1 μM DCFH-DA (AAT Bioquest®, Inc., CA). After 30 min of incubation at 37°C, cells were centrifuged, supernatant discarded and the cell pellet re-suspended in RPMI media supplemented with or without MSA followed by an incubation of 4 h. After washing the cells with PBS analysis was performed in FL channel of the BD LSRFortessa™ cytometer.

Total GSH (reduced and oxidized) concentrations were determined in plasma and liver homogenates according to a standard procedure coupled to GSH reductase and DTNB.[65] The concentrations were calculated using a GSSG standard curve ranging from 1 to 10 nM GSH equivalent/mL.

Determination of glucose uptake

5×10^5 untreated or MSA-treated cells were re-suspended in 2-NBDG solution (0.1 mM in PBS) and incubated for 20 min at RT prior to analysis by flow cytometry (BD LSRFortessa cytometer).

RT-PCR

Total RNA was extracted using the NucleoSpin RNA extraction kit (MACHEREY-NAGEL) according to the manufactures instructions and converted to cDNA using the cDNA synthesis kit and Oligo dT primer from Thermo Scientific. Amplification of the cDNA was performed using the GoTaq® Real-Time qPCR system (Promega) and SybrGreen as a fluorescent reporter. Target specific primers were conducted using the program Primer3[66] and listed in Table S1. Analysis of amplification data were performed with the Rotor-Gene 6000TM series software (Qiagen, Hilden, Germany) using the method according to Pfaffl.[67] Amplifications of GAPDH, RPL13a and ß-Actin were used for normalization. The data are represented as relative mRNA expression levels as x-fold of the control group of at least three separate experiments.

Determination of promoter activity of TAP1, TAP2, TPN and LMP2

Constructs and procedure are described elsewhere.[68] In brief, 5×10^3 cells were seeded into 96-well plates. After 24 h, cells were co-transfected with 0.3 μg pAPM-luc and pSV-galactosidase vector (Promega), which served as transfection control, using Lipofectamine in OptiMEM (Invitrogen) according to the manufacturers' instructions. After 6 h, cells were washed and cultured in complete media for further 24 h, before treatment with 2.5 μM MSA. Untreated cells served as the control. After 24 h, cells were lysed and Luc activity was determined using the Luc-assay system (Promega) according to the manufactures instructions. The transfection efficiency was determined by measuring ß-gal activity. Relative luc activity was calculated as (luc activity/ß-gal activity)—(mean pGL3 – mean b-gal activity).

Western blot analysis

For Western Blot analysis, cells were washed in 0.1 M sodium phosphate buffer containing 5 mM EDTA and protease as well as phosphatase inhibitors (Halt™, Thermo Scientific) and lysed using a TissueLyser (Qiagen, Germany). After centrifugation (13.000 x g, 30 min, 4°C) the supernatants were collected and stored at −80°C until further analysis. Determination of protein content was performed according to the method described by Bradford[69] modified for a 96-well plate. Western blot analysis was performed as described previously.[70] The following primary antibodies were purchases from Cell Signaling Technology and used for immune detection: anti-JAK1 (CST #3332), anti-phospho-JAK1 (CST #3331), anti-phospho-STAT1 (CST #9167), anti-STAT1 (CST #9172), anti-LMP2 (biotrend #PW8205), anti-LMP7 (biotrend #PW8200), anti-PDI1A (CST #3501), anti-phospho-PTEN (CST #9549), anti-PTEN (CST #9556), anti-AKT (CST #9916), anti-phopsho-AKT^{Ser473} (CST #9271), anti-phospho-AKT^{Thr308} (CST #2965), anti-GAPDH (CST #2218). The anti-Nrf2 antibody (Santa Cruz Biotechnology, sc-722) was kindly provided by the group of Prof. Lillig (University of Greifswald).

The secondary HRP-coupled anti-rabbit/anti-mouse Abs were purchased from cell signaling technology and DakoAgilent, respectively.

Statistical analysis

Mean values were calculated from results of at least three independent experiments and given as means ± their standard deprivation (SD). SPSS 20 software was used to analyze significant differences within the groups, therefore Student's *t*-test was implemented after analyzing variance homogeneity. Differences between the groups were considered to be significantly different at $p < 0.05$.

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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5 Discussion

Based on the anti-oxidant functions of selenoproteins, Se is discussed to play roles in the prevention of diseases, such as cancer and diabetes[249]. Furthermore, evidence exists that Se compounds exhibit anti-tumor properties and thus might be used in the therapy of cancerous diseases, but the current data situation is controversial. Se is biologically active in a wide range of natural and synthetic derivatives that significantly differ in their intermediary metabolism, resulting in metabolites with distinct characteristics. Analyzing the current literature regarding Se and its association with disease prevention and/or promotion revealed that causes of inconsistencies might be a result of the distinct sources and doses of Se used in the different studies. For instance, increased risk to develop type 2 diabetes (T2D) was found as a secondary outcome of the Nutritional Prevention of Cancer (NPC) Trial, in which healthy probands were daily supplemented with 200 µg Se in form of Se yeast[191]. In contrast, the Selenium and Vitamin E Cancer Prevention Trial (SELECT), conducted to assess the efficacy of SeMet (200 µg/day) on the incidence of prostate cancer, was prematurely terminated as Se supplementation did not show beneficial effects, but as a secondary outcome, a statistically non-significant effect of Se on T2D risk (RR 1.07; 99 % CI 0.94 to 1.22; $P = 0.16$) was reported[193,195]. In addition, other epidemiological studies failed to find a positive correlation between Se intake and T2D[182] or reported lower baseline Se levels to be associated with a higher incidence of diabetes[186,189]. Regarding the latter, insulin-like properties were also found in animal studies, in which Se displayed anti-diabetic effects[250–252]. It is noteworthy that in these studies doses of selenate were used that would be toxic to humans.

Insulin and insulin receptor interaction initiates the insulin signaling cascade accompanied by the generation of H_2O_2 that deactivates key phosphatases and thereby potentiates kinase activities[253]. Interestingly, transgenic mice over-expressing the selenoprotein GPX1 develop obesity, hyperglycemia, hyperinsulinemia, and furthermore insulin resistance [254]. Vice versa, lack of GPX1 activity improved insulin sensitivity and diminished the development of high-fat-diet-induced obesity[255]. Thus, it is conceivable that Se mediated alterations of the cellular redox status might contribute to either development or improvement of metabolic disorders. Next to islets of the pancreas, the gastrointestinal tract (GIT) plays a crucial role in the control of energy homeostasis by fulfilling tasks in digestion, absorption of nutrients and their assimilation[256]. Moreover, enteroendocrine cells are involved in the control of energy intake and glucose homeostasis[256]. Since GPX2 is highly enriched in the GIT, it could be speculated that this enzyme fulfills decisive functions next to the detoxification of H_2O_2. This hypothesis

is further supported by the observation that within the GIT GPX2 is located in the crypt bases that is not the primary location for absorption processes, but hosts stem cells that are able to produce daughter cells that rapidly differentiate into distinct cell lineages[257]. The perception that next to Se availability GPX2 expression is regulated via transcription factors, e.g. β-catenin and STAT1, known to be involved in the control of proliferation and differentiation processes indicates a role of GPX2 in maintaining mucosal homeostasis[154,258]. Together, the cellular redox state might influence **(i)** the insulin target tissues thereby altering their insulin sensitivity and **(ii)** the GIT, where next to digestion and absorption the production of hormones regulating the energy intake and glucose homeostasis takes place. Furthermore, as mentioned above, different Se compounds might exhibit distinct effects on the cellular redox status. The conflicting results reported by several studies regarding harmful and/or beneficial effects of Se supplementation on the development of metabolic disorders provided the basis for this dissertation and led to the direct comparison of Se compounds most frequently used as supplements.

5.1 Impacts of distinct Se compounds on the cellular redox status

Reactive oxygen species (ROS) are produced in cells in a variety of organelles, including the endoplasmic reticulum (ER), peroxisomes, and lysosomes, whereas mitochondria are thought to be the major producers of ROS[259]. Excessive amounts of ROS can damage macromolecules, e.g. DNA, proteins, and lipids, whereas moderate ROS levels, in particular H_2O_2, exhibit second messenger-like functions and are involved in cellular signaling processes[162]. Therefore, maintaining the cellular redox homeostasis achieved by balancing ROS production and ROS detoxification is essential for normal cell function[162]. Se, as part of anti-oxidant selenoproteins, is involved in maintaining the cellular redox homeostasis. Furthermore, Se influences the activity of Nrf2 resulting in altered activities of enzymes involved in the antioxidant response.

In the **articles I, II,** and **III**, the influence of distinct Se compounds that commonly used in supplements for humans on the expression and activity levels of different anti-oxidant selenoproteins and non-selenoproteins were determined in selected tissues of mice.

Furthermore, the respective Se oversupply doses were chosen to mimic Se doses used in human trials (**article I and II**), while also Se doses at the recommended level (**article I, II and III**), as well as Se deficient conditions, were applied (**article I, II and III**). More recent literature revealed that Se compounds interrupting the cellular redox-homeostasis by the generation of methylselenol are more potent in reducing tumor growth compared to those

mainly yielding hydrogen selenide. Based on these results, the influence of MSA, a methylselenol precursor, on the cellular redox status was analyzed in an *in vitro* model (**article IV**).

In line with previous reports, severe Se deficiency led to a marked decrease of GPX activities in liver and colon tissues of mice (**articles I – III**). However, the activities of selenoproteins did not differ within the groups supplemented with Se. Moreover, Se oversupply only slightly increased GPX activities compared to the recommended level. This indicates that already under uptake of adequate Se doses GPXs are fully active, an observation that is in concert with previous reports[140,157]. Furthermore, all Se compounds used in the current study were able to generate sufficient levels of hydrogen selenide that is necessary for selenoprotein synthesis. Recently, an *in vitro* experiment was conducted to compare different Se compounds with respect to nutritional value vs. toxicity in murine and human liver cells[260]. Herein, the authors found that selenite was superior over selenate and SeMet to induce GPX1 activity. Notably, loss of GPX2 (**article III**) resulted in a compensatory up-regulation of total GPX activity in colon tissues, an effect that might be attributed to an enhanced activity of GPX1[261]. Similar effects have not been observed in other tissues than those belonging to the GIT.

While hepatic Se levels were already saturated in liver tissues under recommended intake of selenite and selenate, high SeMet intake resulted in an accumulation of Se (**article I**). In contrast to the inorganic Se-compounds selenite and selenate, SeMet exerts special properties as it can substitute for methionine (Met) leading to its non-specific incorporation into body proteins. Thus, SeMet is stored in the organism until the respective proteins are degraded[21,262]. Interestingly, SeMet can be oxidized to selenoxides by peroxides, but can be unlike to sulfoxides easily and non-enzymatically reduced under consumption of organic thiols such as GSH[263]. Hence, SeMet acts as a peroxidase mimic to achieve rapid removal of peroxides that are formed on most amino acids after exposure to ROS[264,265]. Thus it is conceivable that protein-bound SeMet confers to enhanced protection of neighboring amino acids against over-oxidation[21], but this, however, has not been shown *in vivo.* Hence, a previous study failed to detect a protective effect of SeMet residues against tyrosine nitration[266]. Notably, in the current study (**article I**) high intake of SeMet not only resulted in Se accumulation in liver tissues, but also enhanced the presence of free (non-oxidized) thiols. Moreover, high SeMet supply resulted in comparable low levels of GSH in hepatic tissues as observed in the Se-deficient group. This phenomenon is hard to explain by an enhanced excretion rate of oxidized GSH (GSSG) into the plasma as assumed to take place under Se-deficient conditions (**article I**). Thus, it could be speculated that SeMet directly removed peroxides leading to a

reduced H_2O_2 status and further a decreased GSH biosynthesis. Notably, a trend of decreased mRNA expression levels of enzymes involved in GSH biosynthesis were observed in the group fed with high doses of SeMet (**article I**). Furthermore, Met is necessary for the synthesis of GSH. Whether high SeMet levels interfere with GSH biosynthesis in mammals needs to be clarified. In general, it is thought that Met utilizing enzymes are not able to discriminate between Met and SeMet[267]. Together it is conceivable that decreased hepatic GSH levels are contributed to an altered GSH biosynthesis. To clarify this point more comprehensive analysis including specifying the ratio of reduced vs. oxidized GSH need be performed. Furthermore, the formation of GSeH, the Se containing GSH analog, in response to high doses of SeMet needs to be investigated. In addition, it is questionable whether non-specifically incorporated SeMet into proteins alters the specific functions and/or activities of the respective proteins. It could be shown that substitution of Met with SeMet in amyloid proteins modulated their aggregation and neurotoxicity[268]. The authors concluded that modulation of fibrillogenesis by SeMet incorporation affects the amyloid diseases. However, whether fibrillinogenesis is decreased, increased or unchanged was dependent on the location where Met was replaced by SeMet[268]. According to the *trans*-sulfuration pathway, SeMet can be transformed to Sec[21,43]. Very recently, it has been reported that exposure to SeMet not only affects non-specific Met substitution, but also Sec-misincorporation into proteins leading to protein aggregation[269]. In line, Lazard et al. reported that SeMet toxicity is mediated by the *trans*-sulfuration pathway amino acids seleno-homocysteine and/or selenocysteine[267]. In general, the substitution of Met by SeMet or Cys by Sec occurs randomly, which in turn means that these effects are hard to control.

It is well known that the cellular redox status affects the activity of enzymes that are targets of Nrf2[162]. Several studies revealed that the expression of these non-selenoproteins can be manipulated by Se availability[163,270]. Already in the 1970s, it could be shown that Se deficiency leads to an upregulation of the Nrf2 target gene heme oxygenase 1 (Hmox1)[271]. Further investigations revealed enhanced activities of glutathione *S*-transferases (GSTs) that exhibit peroxidase-like functions when selenium-dependent GPXs are lacking[272]. In an experiment using mice fed with Se deficient diets, it could be shown that this resulted in an enhanced activity of NQO1, GSTs, and Hmox1[211]. This is in accordance with data presented in the **article I** showing in liver tissues of mice maintained on Se deficient diets upregulated mRNA expression levels of NQO1 and several other Nrf2 targets resulting in enhanced activity of the respective enzymes.

However, the up-regulation of Nrf2 targets were solemnly observed in hepatic tissues and not in the lysates obtained from colons, where the mRNA expression patterns as well as activity levels of Nrf2 targets were not altered under Se deficiency (**article II**). This is in contrast to the results of a study conducted by Müller et al.[163]. Herein, marginal Se levels (86 µg Se per kg diet) did not affect the expression pattern of Nrf2 target genes in hepatic tissues of rats. The conflicting results might be explained either by species-specific differences or by different Se doses chosen in the studies. Whereas 86 µg Se per kg diet results in slight Se deficiency[163], < 20 µg Se per diet (**article I and II**) induces severe Se deficiency. Given that 50 µg Se per kg diet resulted in a strong upregulation of GPX activity[273], a different oxidative status could be responsible for the conflicting results. Besides the induction of Nrf2 target genes under Se deficient conditions also high doses of Se might stimulate the activity of GSTs and NQO1 as previously shown using selenocysteine Se-conjugates or dimethyldiselenides[274,275]. In the current study, oversupply of distinct Se compounds did not result in changes in the activity levels of the enzyme NQO1, neither in hepatic nor in colonic tissues of mice. As the induction of Nrf2 activity is mediated by the cellular levels of H_2O_2 the given doses within the animal experiments might be too low to induce sufficient levels of ROS. For instance, daily treatment of mice with 2 mg Se in form of selenite induced the activity of Nrf2[276], whereas in the current study only 0.75 mg Se per kg diet were given. However, treatment of murine melanoma cells with the methylselenol precursor methylseleninic acid (MSA) induced intracellular accumulation of ROS accompanied by Nrf2 translocation into the nucleus and induction of Nrf2 target genes (**article IV**). MSA might interrupt the cellular redox status in different ways: (**i**) Depletion of cellular GSH levels, which are necessary for its intermediary reduction to methylselenol[217,277], (**ii**) superoxide anion radical formation in the presence of oxygen [278], and (**iii**) oxidative modification of redox-sensitive Cys residues resulting in the formation of selenotrisulfides (S-Se-S), selenosulfides (Se-S), protein disulfides (S-S), and diselenides (Se-Se) [31]. It is obvious that the observed upregulation of GSTP1 under Se deficiency (**article I**) and in response to MSA treatment (**article IV**, data not shown) is mediated by Nrf2 activation as under both conditions also the prototype of Nrf2 targets, NQO1, was strongly induced. In contrast, high SeMet treatment resulted in enhanced hepatic mRNA expression as well as activity levels of this GST isoform even without affecting other Nrf2 targets (**article I**). Thus, it is questionable if an altered Nrf2 activity contributes to induction of GSTP1 mediated by high doses of SeMet. A previous study has shown an increased GST activity in response to high doses of selenocysteine Se conjugates, which was specifically mediated by the isoforms GSTA2 and

GSTP1, whereas other GST isoforms were unaffected[274]. Again it is noteworthy that SeMet can function as a selenocysteine source[21,43]. GST enzymes catalyze the conjunction of GSH with a wide range of electrophilic xenobiotics and thereby facilitate their excretion[279]. In comparison to other GST isoforms, the p-class is more effective in eliminating α,β-unsaturated carbonyl compounds which are derived from the metabolism of natural cellular constituents and foreign compounds as well as from the environment[279]. The GST-mediated detoxification of electrophilic groups is important for the survival of cells, as most of them are geno- and/or cytotoxic[279]. Interestingly, it could be shown that GSTP1 gene expression is up-regulated in response to protein malnutrition an effect that could be diminished by replenishing the diets with Met and Cys[280]. Furthermore, the same authors later showed that limiting the amount of Met and Cys in rat hepatocytes upregulates GSTP1, whereas similar effects were not observed by depletion of other amino acids. Thus, the authors concluded that a sulfur amino acid specific mechanism is responsible for the upregulation of GSTP1[281]. This hypothesis is in line with a study in which GSH biosynthesis was inhibited with buthionine sulphoximine (BSO). Herein, GSTP1 induction mediated by Met restriction was similar to that resulting from BSO treatment[282]. Next to Nrf2, several other factors are responsible for the induction of GSTP1 expression and activity, generally attributed to the existence of a strong enhancer, named GSTP enhancer I (GPE1), which has two 12-*O*-tetradecanoylphorbol-13-acetate response elements (TRE)[282]. A number of factors activate activator protein-1 (AP-1), that in turn binds to TRE and induces the upregulation of GSTP1[283]. GSTP1 upregulation by Met restriction was found to be due to decreased GSH levels and induced activity of the AP-1 signaling pathway [282]. Notably, *in vitro* experiments showed an activation of AP-1 as a consequence of SeMet treatment[284]. Thus, the observed enhanced GST activity in response to high SeMet treatment might be a result of the low GSH levels within this group and an enhanced activity of AP-1. However, this needs to be clarified in future.

Taken together, the cellular redox status is differentially affected by distinct Se compounds. Most strikingly, SeMet treatment resulted in effects also observed under Met restriction. In general, Met metabolizing enzymes cannot distinguish between Met and its Se analog SeMet. This results in non-specific incorporation of SeMet into body proteins instead of Met. As Met is necessary for GSH biosynthesis, it cannot be excluded that the alteration in total GSH levels within liver tissues are mediated by an interference of SeMet with Met metabolism.

5.2 Impact of distinct Se compounds on metabolic characteristics in mice

Both, long-term Se supplementation and a permanently high activity of the selenoprotein GPX1 have been positively correlated with the early development of insulin resistance (IR) and T2D[285–287]. In the current study oversupply of different Se derivatives resulted in distinct patterns of metabolic parameters (**article I**). Whereas selenite treatment did not result in any changes, high doses of selenate enhanced fasting plasma insulin levels and the HOMA-IR score but plasma glucose levels were not affected. Together, these data suggest an impaired sensitivity to insulin in the high selenate-treated group. Due to the elevated plasma insulin levels by high doses of selenate the abundance of phosphorylated AKT, a kinase mediating insulin signals was increased. A key action of insulin is to stimulate glucose uptake into cells via translocation of GLUT4 to the cell membrane, an AKT dependent mechanism[288]. Furthermore, activated AKT phosphorylates and thereby inactivates glycogen synthase kinase 3 (GSK3), a pathway that promotes glucose storage in form of glycogen. Moreover, insulin inhibits the *de novo* synthesis of glucose by blocking key enzymes involved in gluconeogenesis and glycogenolysis[289]. The forkhead box (FOXO) proteins play important roles in integrating insulin signaling to the glucose and lipid metabolism[290,291]. FOXO proteins undergo AKT-mediated phosphorylation, resulting in exclusion of these proteins from the nucleus and decreased expression of FOXO target genes[290]. Thus, insulin-induced AKT activation counteracts FOXO activity leading to reduced glucose production during the state of feeding[292]. Using transgenic mice where AKT phosphorylation sites of FOXO1 were replaced by alanine, Zhang et al. demonstrated that constitutive active FOXO1 does not impair TG levels but suppresses several proteins required for lipid synthesis, including sterol regulatory element-binding protein 1 (SREBP1c), Acetyl-CoA carboxylase (ACC), and fatty acid synthase (FAS)[293]. Mice treated with high doses of selenate displayed elevated mRNA expression levels of ACC and FAS indicating a decreased FOXO1 activity that might be mediated by the high activity status of AKT in this group (**article I**). Vice versa, mice fed with high doses of SeMet exhibited low AKT activities but decreased expression levels of ACC and FAS (**article I**). Thus, an altered activity of AKT known to regulate the activity of FOXO proteins might contribute to the different metabolic phenotypes developed with distinct Se compounds. It is noteworthy that translocation of FOXO proteins in response to treatment with distinct Se compounds needs to be analyzed in the future.

In GPX1 overexpressing mice, an altered AKT phosphorylation status indicated the insulin resistance of the animals[285]. In contrast, selective overexpression (OE) of GPX1 in the pancreas of db/db mice resulted in a significantly enhanced β-cell mass, a ~40 % increased

insulin synthesis, and reversion of diabetes[254,294]. Thus, these data indicate that general OE of GPX1 exhibits distinct effects from selective OE in the pancreas.

An enzyme playing a role in the development of early insulin resistance is mediated by the insulin antagonistic protein-tyrosine phosphatase 1B (PTP1B). This enzyme functions as a negative regulator of the insulin signaling pathway via the dephosphorylation of insulin-signaling components[295]. PTP1B KO mice are characterized by decreased levels of circulating insulin accompanied by increased phosphorylation of the insulin receptor in liver and muscle tissues. Further, these mice are more sensitive to endogenous or injected insulin when compared to their WT counterparts[296–298]. Additionally, PTP1B is involved in regulating hepatic lipid metabolism[299,300]. PTP1B contains a redox-sensitive Cys residue within its catalytic domain[301,302] that can be oxidized in the presence of H_2O_2 ultimately leading to diminished enzymatic activity [303,304]. Thus, PTP1B is an example of a protein that connects the cellular redox status with the metabolic homeostasis. Se is involved in modulating the cellular H_2O_2 content via the activity of H_2O_2-detoxifying selenoproteins, the regulation of Nrf2 targets involved in the anti-oxidant response, and by superoxide anion radical formation. As presented in **article I** the hepatic activity of PTPs differs remarkably depending on the given Se compound. In contrast to a previous report[305], Se deficiency did not result in enhanced inactivation of PTP even though the activities of anti-oxidant selenoproteins (GPX, GPX4, TXNRD) were markedly decreased. It is noteworthy that in the very same group the overall thiol-redox-status was not affected. Thus, compensatory effects might play a role in this setting, for instance, enhanced activity of GSTs that also exhibit peroxidase properties[306]. Therefore, the low body weights, as well as the low TG contents in plasma observed under Se deficient conditions could rather be explained by the diminished food intake, a phenomenon that had also been reported previously[307]. In contrast to the 20 weeks feeding period of mice described in **article I and II,** in **article III** mice were maintained under Se deficiency for only 8 weeks. Herein, no significant differences in final body weight gains were observed within Se-fed and Se-deficient mice. Further, it was obvious that mice lacking GPX2 activity exhibited decreased growth properties (**article III**). Interestingly, GPX2 KO mice, independent of the Se status, displayed an altered expression pattern of several intestinal hormones that fulfill functions in the regulation of food intake, energy expenditure and lipid metabolism[308,309] (**article III**). For instance, Grehlin, produced by X/A-like cells, that stimulates appetite and enhances food intake, was found to be markedly decreased in mice lacking GPX2 enzyme activity. In the current study the decreased mRNA levels of several intestinal hormones was found in the groups lacking GPX2 activity. However, as these

hormones are released into the blood, ELISA assays should be carried out to test if the levels of the respective hormones are decreased also in the serum of the respective mice.

The importance of intestinal enteroendocrine cells/hormones for the regulation of energy homeostasis was recently demonstrated by Mellitze et al.[310]. In mice, the lack of all intestinal enteroendocrine cells led to an altered intestinal architecture, impaired lipid absorption and glucose homeostasis, as well as growth retardation[310]. At this point, it cannot be excluded that long-term Se deficiency (20 weeks, **article I and II**) might lead to similar interruption of mucosal homeostasis. For instance, the protein abundance of CLCA1 was not altered after eight weeks of Se deficiency (**article III**), whereas long-term Se deficiency decreased its protein abundance (**article II**). Further, colonic GPX activity was with 140 mU/mg protein ~ 1.7-fold higher after 20 weeks of Se feeding compared to the eight weeks experiment. In addition, mice of the eight weeks experiment had slightly higher total GPX activities under Se deficient conditions. Thus, the growth retardation observed in the Se deficient group described in **article I** might be a result of diminished food intake mediated by interrupted mucosal homeostasis, but further analyses are required to investigate this issue.

Surprisingly, long-term supplementation with high doses of SeMet significantly reduced body weight gains in mice (**article I**). Total body weights adjusted to food intake shows that these mice were hyperphagic, and therefore, the high SeMet mediated reduction in body weights is not a response to decreased caloric intake as observed in the group maintained under Se deficiency. Furthermore, comparing high versus adequate Se levels it is conspicuous, that PTP activity increased with SeMet supply, a possible explanation for the decreased abundance of hepatic phosphorylated AKT in these mice (**article I**). This effect might be a result of the H_2O_2 detoxifying properties of SeMet resulting in less inactivation of PTPs. In contrast to previous reports [300], in the current study enhanced activity of PTP did not result in increased lipid synthesis, but rather was accompanied by decreased TG levels in plasma of mice. Remarkably, within this group also expression levels of enzymes involved in lipid synthesis were decreased. Thus, the altered activity status of AKT, influencing the activity of FOXOs, rather contributes to decreased TG levels **(article I**). Contradictory, a recently published study showed that SeMet supply enhances TG levels in plasma of mice. This is in line with our report showing increased TG levels from –Se to 150 µg/kg diet. However, the authors observed a further increase in TG levels under diets of 600 µg/kg diet whereas in our experiment 750 µg /kg diet markedly decreased TG levels[311].

As described above, high SeMet treatment resulted in marked changes of the cellular redox status when compared to the other treatment regimes. In particular, the free thiol content was

enhanced, GSH levels decreased and GSTP activity upregulated; characteristics, also observed in mice maintained under Met restriction[282,312]. Besides participating in *S*-glutathionylation reactions, GSTP regulates several signaling pathways, including cellular metabolism, and has been described to exhibit diverse catalytic and non-catalytic functions related to metabolic diseases[313,314]. As early as 1989, Thomas et al. reported decreased hepatic GST activities in diabetic rats[315]. Insulin and glucagon regulate GSTs expression, e.g. in primary hepatocytes GSTP expression was completely inhibited by administration of glucagon indicating that GSTP might play a role in the development of diabetes [316]. Within the GSTP promoter, an insulin response element (IRE) could be detected that is essential for insulin-mediated suppression of GSTP expression[317]. An enzyme regulated by GSTP that is involved in potentiating insulin resistance is the c-Jun *N*-terminal kinase (JNK), also known as a stress-activated protein kinase. JNK reduces glucose metabolism and antagonizes insulin signaling via serine phosphorylation of the insulin receptor substrates as well as of AKT[318]. Opposed to tyrosine phosphorylation, serine phosphorylation of molecules involved in insulin signaling contributes to their inactivation[318]. Thus, enhanced JNK activity would inhibit AKT activity resulting in FOXO activation and its nuclear translocation. Under physiological conditions, a portion of GSTP is bound to JNK. Depending on the cellular redox status GSTP dissociates from the complex and releases JNK. Furthermore, upregulation of GSTP would result in an increase in inactive JNK, which has been recently shown in tumors[319]. Thus, the observed upregulated GSTP expression and GST activity under SeMet supplementation might exhibit dual roles regarding the development of insulin resistance on the one hand and proliferation of cancer cells on the other hand.

Besides the importance of the AKT pathway for proper insulin signaling, high AKT activity is also associated with the high proliferation rates of tumor cells. In **article IV** it is shown that treatment of melanoma cells with the pro-oxidant Se compound MSA diminished AKT phosphorylation that was accompanied by decreased proliferation as well as migration rates of these cells. Furthermore, MSA treatment impaired glucose uptake into these cells. Thus, it is conceivable that interference of the AKT pathway via Se supplementation contributes to a decreased tumor growth, while it mediates the development of insulin resistance.

Taken together, metabolic parameters of mice fed with distinct Se compounds differed markedly each from another, a phenomenon that might be attributed to the specific nature exhibited by the different Se compounds. In this regard mice treated with high doses of SeMet exhibited a unique response pattern that might be attributed to the marked changes of the

cellular redox status observed within this group. Most of the alterations induced by high SeMet supply are also described in mice fed with a Met restricted diet. Thus, the data of the current study might indicate an interference of SeMet with Met metabolism. This needs to be considered when thinking about supplementing Se to prevent diseases such as cancer. Furthermore, an indiscriminate usage of Se supplements cannot be justified, and Se supplementation for disease prevention is only indicated in subjects with low Se status to reach optimal Se levels.

5.3 Impact of Se on anti-tumor immunity

Se compounds that generate high levels of methylselenol are described to be more potent in reducing the tumor growth than those predominantly generating hydrogen selenide. Methylseleninic acid (MSA) originates from the organic decomposition of methylselenocysteine (MSC)[21] and was tested in several xenograft tumor models, including prostate and colon cancer. In all these studies MSA showed promising effects as it reduced tumor growth without affecting the general behavior of the animals[57,219–221]. The underlying mechanisms of these findings are only insufficiently understood but include caspase 3 activation and modulation of proinflammatory cytokines[221]. Furthermore, MSA inhibits the activity of AKT, an enzyme often highly active in tumor cells and necessary for cell proliferation. MSA impacts the proteome (**article IV**) as well as the redox-proteome (data not shown) in B16F10 cells. Most strikingly, proteins involved in antigen presentation were affected by exposure to MSA leading to an enhanced abundance of MHC class I molecules on the cell surface of these cells, an effect also observed in other types of tumor cells (**article IV**). In contrast, the Se compound selenite mediated only a slight, whereas SeMet rather decreased the MHC class I abundance on the cell surface (**Figure 10**). This observation might be an explanation for results obtained from *in vivo* studies showing that MSA exhibits superior effects regarding the inhibition of tumor growth over selenite and SeMet[57].

For proper anti-tumor effects, a functional immune system is necessary as specific lymphocytes can recognize and eliminate transformed cells. MHC class I molecules predominantly present endogenous generated peptides on the cell surface to $CD8^+$ T cells. Taken into account that tumor cells frequently downregulate MHC class I molecules on their cell surface to escape from immune surveillance[320,321], substances that upregulate MHC class I molecules and shape tumor cells towards an immunogenic phenotype might be useful as additives to T cell-based immunotherapeutic approaches[322].

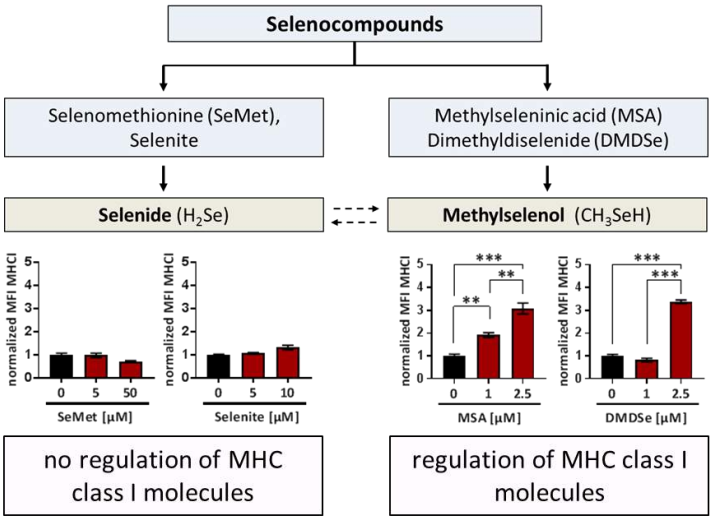

Figure 10: Regulation of MHC class I molecules by different Se compounds.
The metabolism of different Se compounds is complex and closely regulated, with two key metabolites: Selenide (H_2Se) and methylselenol (CH_3SeH). MHC class I molecules are only upregulated by CH_3SeH-generating compounds.
B16F10 cells were treated with different Se compounds for 24 hrs and the abundance of MHC class molecules was analyzed via Flow cytometry (** $p < 0.01$, *** $p < 0.001$, student´s t-test)
DMDSe, Dimethyldiselenide; MSA, Methylseleninic acid; SeMet, Selenommethionine.

Several studies investigated the effects of Se supplementation on the activity status of specific lymphocytes. For instance, Hoffmann et al. intended to study how different levels of dietary Se affect the development of T helper (Th)2-driven immune responses and found that Se intake was not related to the development of allergic airway inflammation in a simple dose-response manner[323]. Low doses of dietary Se (0.08 ppm) resulted in lowest levels of allergic responses, whereas diets containing adequate Se levels (0.25 ppm) led to the highest susceptibility to induced airway inflammation. In contrast, Se doses above the recommendations (2.7 ppm) did not lead to significant differences compared to the other two groups, suggesting that Se oversupply might differentially affect Th1 and Th2 responses[323]. Furthermore, *ex vivo* stimulation of $CD4^+$ T cells obtained from mice fed with different concentrations of Se (moderate – adequate – high) resulted in a dose-dependent increase of their proliferation capacity [324]. Interestingly, high Se intake enhanced the strength during activation of T cell receptor (TCR) signaling, increased Ca^{2+} mobilization, oxidative burst, and translocation of nuclear factor of activated T cells (NFAT), and finally to increased transcription of IFNγ[324]. In contrast, the expression of IL-4 in TCR-stimulated $CD4^+$ T cells isolated from mice maintained under Se deficient conditions was highest, whereas no differences regarding this cytokine could be observed comparing adequate and high Se levels. Taken together, these data suggest that high Se intake shifts the differentiation of $CD4^+$ T cells towards Th1 cells[324], a phenotype beneficial for anti-tumor responses.

Several studies suggested a role of the cellular redox status in modulating the activation of T cells into T effector cells. Interestingly, TCR stimulation leads to the generation of superoxide and hydrogen peroxide that induces T cell apoptosis or enhances T cell proliferation[325–328]. Therefore, it can be speculated that the Se intake affects T cell differentiation processes through selenoproteins that regulate the cellular redox status. In line with this hypothesis is a study showing that $CD4^+$ T cells isolated from mice maintained on diets with high Se intake exhibited an enhanced overall abundance of free cellular thiols [324]. Moreover, the differences regarding TCR induced Ca^{2+} flux and proliferative capacity of $CD4^+$ T cells fed with different Se doses were negated when cells were treated with *N*-acetylcysteine (NAC), an exogenous source of free thiols[324]. Furthermore, Shrimali et al. reported that T cells lacking all selenoproteins exhibited increased levels of ROS, a decreased proliferative capacity, reduced T cell receptor signaling and were functional defective. Also in this study, it was shown that the observed effects mediated by loss of all selenoproteins could be reversed by adding NAC[229,230]. In contrast, a study investigating the role of GPX1 in T cells revealed that the ablation of GPX1 in Th cells promoted their differentiation into Th1 cells rather than into Th2 cell[329]. This was associated with an increased oxidative burst during TCR induced activation of these GPX1 KO $CD4^+$ T cells. The authors assumed that TCR induced generation of ROS is essential for IFNγ and IL-2 production and that this increase in intracellular ROS levels is limited by GPX1 in $CD4^+$ T cells[329]. Furthermore in this model NAC treatment inhibited IL-2 production by the Th cells of both WT and KO mice, and consequently decreased their proliferative capacity[329]. In contrast to results obtained from mice maintained under Se deficiency or exhibiting a total lack of selenoproteins, the results of the latter study suggest that an enhanced oxidative tone favors the development of Th1 cells. These data might indicate that GPX1 plays a role different from other selenoproteins regarding T cell differentiation. A few studies have shown that GPX1 might also play a role as a potential pro-oxidant. For instance, overexpression of GPX1 sensitized mice to acetaminophen-induced hepatotoxicity by increasing cellular protein nitration[330]. In contrast, primary hepatocytes isolated from GPX1 knockout mice were more resistant to peroxynitrite-induced toxicity[331]. The underlying mechanisms are thought to be mediated by depleting or enhancing cellular GSH levels, which serves as a scavenger of peroxynitrite[332]. Given that GPX1 utilizes GSH during the catalytic cycle and that GSH modulates the Th1 versus Th2 development as well as the proliferative capacity of Th cells[333], higher GSH levels might be an explanation for the observed effects in mice lacking specifically this one selenoprotein.

Together, these results suggest that changes in the content of intracellular ROS levels and free thiols, respectively, are the key mechanism by which selenoproteins influence the differentiation and activation processes of T cells. Depending on the concentration, site of generation, and the source, ROS might lead to inhibition and/or activation of T cells[325–328].

Taken together, the data of the current study (**article IV**) show for the first time the reversion of a tumor escape mechanism mediated by the pro-oxidant Se compound MSA. Together with the described impacts of Se on immune functions, in particular, the activation and proliferation of lymphocytes with anti-tumor properties, Se is proposed as an additive useful in immunotherapeutic approaches as it **(i)** might reverse the escape of tumor cells from immune surveillance and **(ii)** might boost the immune system towards anti-tumor immunity (**Figure 11**).

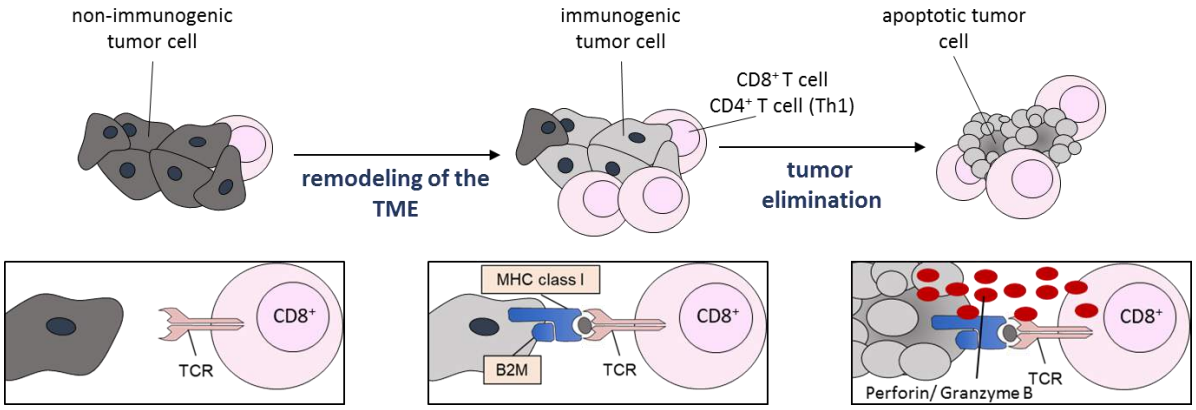

Figure 11: Proposed benefits of pro-oxidant Se compounds in enhancing anti-tumor immunity
Specific lymphocytes of the immune system, e.g. $CD8^+$ T cells or $CD4^+$ Th1 cell, are excellent in identifying and eliminating transformed cells. However, tumor cells developed several mechanisms to escape from this so-called immune surveillance. Besides the up-regulation of co-inhibitory molecules, such as PD-L1, the downregulation of MHC class I molecules is an established tumor immune escape mechanism. In **article IV** it could be shown that the pro-oxidant Se compound MSA leads to the up-regulation of MHC class I molecules on the cell surfaces of different tumor cells. Together, with data published by other authors showing an enhances proliferation and activity status of lymphocytes necessary for proper anti-tumor responses, it is hypothesized that therapeutic doses of pro-oxidant Se compounds might enhance anti-tumor immune responses leading to the destruction of tumor cells. TCR, T cell receptor; B2M, Beta 2 microglobulin; TME, Tumor microenvironment

6 Concluding remarks and perspectives

Regarding the relationship between Se and the development of metabolic disorders, e.g. insulin resistance and T2D, the results obtained from clinical trials as well as from animal experiments are rather heterogeneous. Herein, the choice of the Se form might be a reason for inconsistencies. As shown in the current study, depending on their specific nature distinct Se compounds affect the cellular redox status to a varying extent. Meanwhile, it is well accepted that specific types of ROS, in particular H_2O_2, function as second messengers and therefore can impact cell signaling processes. Thus, supplementing Se not only reduces ROS levels that can be harmful to cells but also have an impact on the regulation of redox signaling processes. Furthermore, it is important to consider that the generation of bioactive Se metabolites is in some cases dependent on the enzymatic set up of the cells, e.g. lyases. Se compounds are attractive substances in cancer therapeutic approaches. Most studies regarding tumor inhibition by Se were conducted with selenite. However, methylated Se compounds become more and more attractive. Methylated Se compounds inhibit AKT activity and therefore reduced proliferation processes. The molecular mechanisms behind these observations are still not well understood but include the modulation of calcium metabolism for instance. In contrast, proper AKT signaling is necessary for mediating insulin signaling cascades and therefore to prevent the development of insulin resistance. Thus, while the very same pathway affected by Se might lead to diminished tumor growth it also bears the risk to develop metabolic disorders. Given that tumor cells are more susceptible to Se toxicity compared to non-transformed cells, it is conceivable to use specific Se compounds as anti-cancer agents. Furthermore, specific Se compounds have been shown to shift the immunogenic phenotype of tumor cells that might lead to a reversal of immune escape mechanisms. As demonstrated in this thesis, the presence of MHC class I molecules on the cell surface of tumor cells can be modified only by specific Se compounds. Given that MHC class I molecules are essential for proper T cell-based tumor cell elimination, this observation indicates that Se in combination with immunotherapy might enhance the therapeutic outcome. However, this needs to be proven in proper *in vivo* models in future. Very recently, it was published that combination of SeMet and the co-stimulatory molecule anti-Ox40 reduces the tumor growth in mice. Herein, the authors developed a system combining a fusion protein composed of a tumor-targeting protein and a mutated variant of cystathionine gamma-lyase that can convert SeMet to methylselenol. Thus, the combination of methylselenol and anti-Ox40 antibodies not only reduced tumor growth, but also led to a significant increase in cytotoxic $CD8^+$ T cell in lymph

nodes of mice inoculated with metastatic ovarian cancer. Furthermore, the authors found that addition of immunostimulation with the prodrug system increased the ratio of cytotoxic to immunosuppressive regulatory T cells (Tregs)[334]. As the frequency of Tregs infiltrated into tumors correlates with poor patient´s prognosis[335], it would be interesting to explore if similar results can also be observed in the TME.

So far, most of the studies showing anti-carcinogenic effects in response to Se treatment indicate that methylselenol might be the key metabolite. However, for defining novel anti-tumor strategies in combination with specific Se compounds it is essential to investigate the Se metabolites that are generated during the metabolism of Se in more detail. Thus, an indiscriminate usage of Se compounds is not justified. In addition, the therapeutic window of the Se is rather narrow. Herein, the levels applicable to humans need to be investigated as high doses of Se are known to be toxic and therefore side effects in humans cannot yet be excluded.

7 Author contributions

I. **C. Lennicke**, J. Rahn, A.P. Kipp, B.P. Dojčinović, A.S. Müller, L.A. Wessjohann, R. Lichtenfels, B. Seliger, Individual effects of different selenocompounds on the hepatic proteome and energy metabolism of mice, Biochim. Biophys. Acta - Gen. Subj. 1861 (2017) 3323–3334. doi:10.1016/j.bbagen.2016.08.015.

CL, ASM, RL conceived and designed the study as well as the experiments. CL and JR performed the experiments. In particular, CL did the feeding experiment and performed all experiments with the exception of the proteomic analysis which was performed by JR and the measurements of Se levels in liver tissues were performed by BPD. CL performed statistical analysis, interpreted the results, and prepared the figures as well the tables. CL, JR, APK, LAW, RL, and BS wrote the paper.

II. J. Rahn, **C. Lennicke,** A.P. Kipp, A.S. Müller, L.A. Wessjohann, R. Lichtenfels, B. Seliger, Altered protein expression pattern in colon tissue of mice upon supplementation with distinct selenium compounds, Proteomics. 17 (2017). doi:10.1002/pmic.201600486.

CL, ASM, RL conceived and designed the study. JR and CL performed the experiments. In particular, CL did the feeding experiment and performed the analyses included in Figure 1 and Table 1. JR, RL, APK, CL, and BS wrote the manuscript.

III. **C. Lennicke**, J. Rahn, C. Wickenhauser, R. Lichtenfels, A.S. Müller, L.A. Wessjohann, A.P. Kipp, B. Seliger, Loss of epithelium-specific GPx2 results in aberrant cell fate decisions during intestinal differentiation, Oncotarget. (2017).

CL, ASM, RL, APK conceived and designed the study. APK performed the feeding experiment. CL performed all experiments with the following exceptions: proteomic analysis (JR), IHC (CW). CL performed statistical analysis, interpreted the results, and prepared the figures and tables. CL, JR, RL, LAW, APK, and SB wrote the manuscript.

IV. **C. Lennicke**, J. Rahn, J. Bukur, F. Hochgräfe, L.A. Wessjohann, R. Lichtenfels, B. Seliger, Modulation of MHC class I surface expression in B16F10 melanoma cells by methylseleninic acid., Oncoimmunology. 6 (2017) e1259049. doi:10.1080/2162402X.2016.1259049.

CL and SB conceived and designed the study. CL performed all experiments with the following exceptions: proteomic analysis (JR), generation of vectors for promotor assays (JB), redox-proteomic assay (FH). CL performed statistical analysis, interpreted the results, prepared figures and tables. CL, RL, LAW, and SB wrote the manuscript.

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9 Statutory declaration

I declare that I have authored this thesis independently, that I have not used other than the declared sources/resources, and that I have explicitly marked all material which has been quoted either literally or by content from the used sources.

I have not applied anywhere else for a doctoral degree nor I have obtained a doctoral degree based on my presented studies or failed a doctoral examination (this includes similar procedures and titles in countries other than Germany).

_____	_____
place and date	Claudia Lennicke

10 Curriculum Vitae

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Scientific career

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03/2012 – 02/2013 Research Assistant at the Leibniz Institute of Plant Biochemistry (IBP), Halle, Germany

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11 Scientific achievements

11.1 List of publications

C. Lennicke, J. Rahn, C. Wickenhauser, R. Lichtenfels, A.S. Müller, L.A. Wessjohann, A.P. Kipp, B. Seliger, Loss of epithelium-specific GPx2 results in aberrant cell fate decisions during intestinal differentiation, Oncotarget. 9 (**2018**). doi.org/10.18632/oncotarget.22640.

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J. Rahn, **C. Lennicke**, A.P. Kipp, A.S. Müller, L.A. Wessjohann, R. Lichtenfels, B. Seliger, Altered protein expression pattern in colon tissue of mice upon supplementation with distinct selenium compounds, Proteomics. 17 (**2017**). doi:10.1002/pmic.201600486.

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C. Lennicke, J. Rahn, N. Heimer, R. Lichtenfels, L.A. Wessjohann, B. Seliger, Redox proteomics: Methods for the identification and enrichment of redox-modified proteins and their applications, Proteomics. 16 (**2016**). doi:10.1002/pmic.201500268

C. Lennicke, J. Rahn, R. Lichtenfels, L.A. Wessjohann, B. Seliger, *Hydrogen peroxide Production, fate and role in redox signaling of tumor cells*, Cell Commun. Signal. 13 (**2015**). doi:10.1186/s12964-015-0118-6.

11.2 Published abstracts

C. Lennicke, S. Ahmad, R. Shrimali, B. Seliger, Y. Rustum, J.E.. Janik, M. Mkrtichyan**,** S. Gupta, S.N. Khleif. *Selenium, the element of the moon, improves immunotherapies on earth*. Journal for ImmunoTherapy of Cancer. 5 (**2017**) (Suppl 2):87. doi.org/10.1186/s40425-017-0288-4

11.3 Conference presentations

C. Lennicke, S. Ahmad, R. Shrimali, B. Seliger, Y. Rustum, J.E. Janik, M. Mkrtichyan**,** S. Gupta, S.N. Khleif. *Selenium, the element of the moon, improves immunotherapies on earth*. Poster presentation delivered at SITC 32nd annual meeting, National Harbor Washington, USA, November 2017

C. Lennicke, F. Hochgraefe, J. Rahn, R. Lichtenfels, S.N. Khleif, B. Seliger. *Reversing the tumor immune escape with Selenium*. Oral presentation delivered at the 2nd World Immunotherapy Council Young Investigator Symposium, National Harbor Washington, USA, November 2017

C. Lennicke, F. Hochgräfe, R. Rahn, R. Lichtenfels, B. Seliger. *Redox remodeling as a strategy to shape immunity.* Poster presentation delivered at the 13th Tumor Immunology Meets Oncology (TIMO) Meeting, Halle, Germany, May 2017

C. Lennicke, J. Rahn, J. Bukur, F. Hochgräfe, L.A. Wessjohann, R. Lichtenfels, B. Seliger. *Link of methylseleninic acid-mediated altered redox status with modulation of MHC class I surface antigen* expression in tumor cells. Oral presentation delivered at the Frontiers in Redox Biology and Medicine 5th Young Professionals Workshop on Plasma Medicine, Rostock, German, November 2016

C. Lennicke, J. Rahn, J. Bukur, F. Hochgräfe, L.A. Wessjohann, R. Lichtenfels, B. Seliger. *The selenocompound methylseleninic acid mimics interferon gamma signaling and restores MHC class I expression.* Oral and poster presentation delivered at the 2nd AEK Autumn School, Berlin, Germany, November 2016

C. Lennicke, J**.** Rahn, L.A. Wessjohann, R. Lichtenfels, B. Seliger. *Impact of methylseleninic acid treatment on the modulation of MHC class I in tumors.* Oral presentation delivered at the 32nd Annual Conference of the German Society of Minerals and Trace Elements, Berlin, Germany, October 2016

C. Lennicke, R**.** Rahn, F. Hochgräfe, M. Gellert, L.A. Wessjohann, R. Lichtenfels, B. Seliger. *New insights into the anti-carcinogenic potential of methylseleninic acid.* Poster presentation delivered at the 3rd meeting of the study group redox biology of the German Society for Biochemistry and Molecular Biology (GBM), Düsseldorf, Germany, July 2016

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C. Lennicke, R. Rahn, R. Lichtenfels, A.P. Kipp, A.S. Müller, L.A. Wessjohann, B. Seliger. *Selenium supplementation: Good or Bad?*. Poster presentation delivered at 3rd Doctoral Conference of the Medical Faculty, MLU Halle-Wittenberg, Halle, Germany, November 2015

C. Lennicke, R. Rahn, A.P. Kipp, R. Lichtenfels, A.S. Müller, L.A. Wessjohann, B. Seliger. *Impact of distinct Selenium compounds on molecular targets of energy metabolism in hepatic tissue of mice.* Oral presentation delivered at the 30th Annual Conference of the German Society of Minerals and Trace Elements, Freising, Germany, October 2014

C. Lennicke, J. Rahn, A.P. Kipp, R. Lichtenfels, A.S. Müller, B. Seliger. *Impact of Selenium on the Hepatic Glutathione Metabolism in Mice*. Poster presentation delivered at 3rd Research conference of the Medical Faculty, MLU Halle-Wittenberg, Halle, Germany, October 2014

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