Cell Chemical Biology
Review

# Selenium: Tracing Another Essential Element of Ferroptotic Cell Death

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https://doi.org/10.1016/j.chembiol.2020.03.012

The trace elements iron and selenium play decisive roles in a distinct form of necrotic cell death, known as ferroptosis. While iron promotes ferroptosis by contributing to Fenton-type reactions and uncontrolled lipid autoxidation, the hallmark of ferroptosis, selenium in the form of glutathione peroxidase 4 (GPX4), subdues phospholipid peroxidation and associated cell death. Beyond the canonical cystine/glutamate antiporter system $x_c^-$/glutathione/GPX4 nexus, recent studies unveiled the second mainstay in ferroptosis entailing extra-mitochondrial ubiquinone, ferroptosis suppressor protein 1, and NAD(P)H as electron donor. Unlike GPX4, this selenium- and thiol-independent system acts on the level of peroxyl radicals in membranes, thereby restraining lipid peroxidation. Therefore, ferroptosis is a multifaceted cell-death paradigm characterized by several metabolic networks, whereby metabolic dyshomeostasis may cause ferroptotic cell death and organ failure. Here, we discuss the basic features of ferroptosis with a focus on selenium, offering exciting opportunities to control diseases linked to ferroptosis, including transient ischemia/reperfusion and neurodegeneration.

## Introduction

The term ferroptosis was coined in 2012 to describe a regulated cell-death pathway that is marked by deleterious iron-dependent lipid peroxidation and is induced upon administration of small molecules such as erastin, sulfasalazine, and (*1S*, *3R*)-RSL3 (RSL3) (Dixon et al., 2012). At the same time, iron chelators have been successfully used to revert this kind of cell death, emphasizing the role of iron in ferroptosis execution (Dixon et al., 2012). Soon thereafter, a link to the trace element and essential micronutrient selenium was reported with the recognition that the selenium-dependent enzyme glutathione peroxidase 4 (GPX4), one of 25 cognate selenoproteins in human, regulates ferroptosis through its robust suppressive function (Friedmann Angeli et al., 2014; Yang et al., 2014). Selenoproteins are rare proteins present in all three domains of life that contain at least one selenium-containing amino acid in the form of selenocysteine (Sec) (Labunskyy et al., 2014). Although Sec differs from its functional homolog cysteine (Cys) only by a replacement of selenium with sulfur, it requires a highly complex and energetically demanding translation-decoding machinery that functions by recoding of the opal stop codon UGA and through synthesis of its own tRNA, known as *Trsp* (*TRU-TCA1-1*) (Hatfield et al., 2014).

Initial evidence for the tissue-protective function of selenium supplementation was already provided in the 1950s by Schwarz and Foltz (1958) using a rat model of diet-induced liver necrosis based on vitamin E deficiency. Later, the biological activity of selenium could be explained by the discovery of the first selenoprotein in mammals, GPX1 (Flohe et al., 1973; Rotruck et al., 1973). This was further corroborated by the identification of GPX4 (Ursini et al., 1982), which harbors the unique ability to detoxify membrane-bound phospholipid and cholesterol hydroperoxides, an absolute requirement to keep ferroptosis in check (Friedmann Angeli et al., 2014; Yang et al., 2014). In addition, Carlson et al. (2016) showed that liver-specific *Gpx4* deletion in mice does not lead to liver necrosis under vitamin E supplementation, providing further evidence that the liver degenerative phenotype discovered in 1958 was indeed due to ferroptotic cell death.

The advantage of selenolate-based versus thiolate-based catalysis at the active site of GPX4 has long remained unknown, partially owing to the fact that many selenoproteins are also present as Cys-containing homologs, at least in some species (Flohe et al., 2011). This is best illustrated for GPX4, as it is a selenoprotein in vertebrates but a Cys-containing enzyme in most organisms including plants, nematodes, fungi, and bacteria. Early work on heterologously expressed GPX4 with a site-directed mutation of Sec to Cys showed that mostly the regenerative steps of GPX4 by its main substrate glutathione (GSH) are strongly impaired, favoring overoxidation of the mutant enzyme (Maiorino et al., 1995). Recently, a study by Ingold et al. (2018) provided unequivocal evidence that Sec utilization in lieu of Cys by GPX4 is dispensable for embryogenesis in mice but essential for the development of a distinctive neuronal subpopulation through a mechanism that involves suppression of peroxide-induced ferroptosis. Therefore, it has been postulated that ferroptosis may represent an ancient vulnerability that emerged by the evolutionary incorporation of polyunsaturated fatty acids (PUFAs) into cellular membranes, thereby enabling the development of complex organisms (Ingold et al., 2018). However, as oxidation of PUFAs may lead to lipid peroxidation, subsequent membrane damage, and cell death, this requirement of PUFA utilization, as observed mainly in vertebrates, comes with an enormous liability (Ingold et al., 2018). In this review, we discuss recent developments in the ferroptosis field with a special focus on the biological role of selenium in the main ferroptosis regulator GPX4 and its putative exploitation in the prevention of disease.

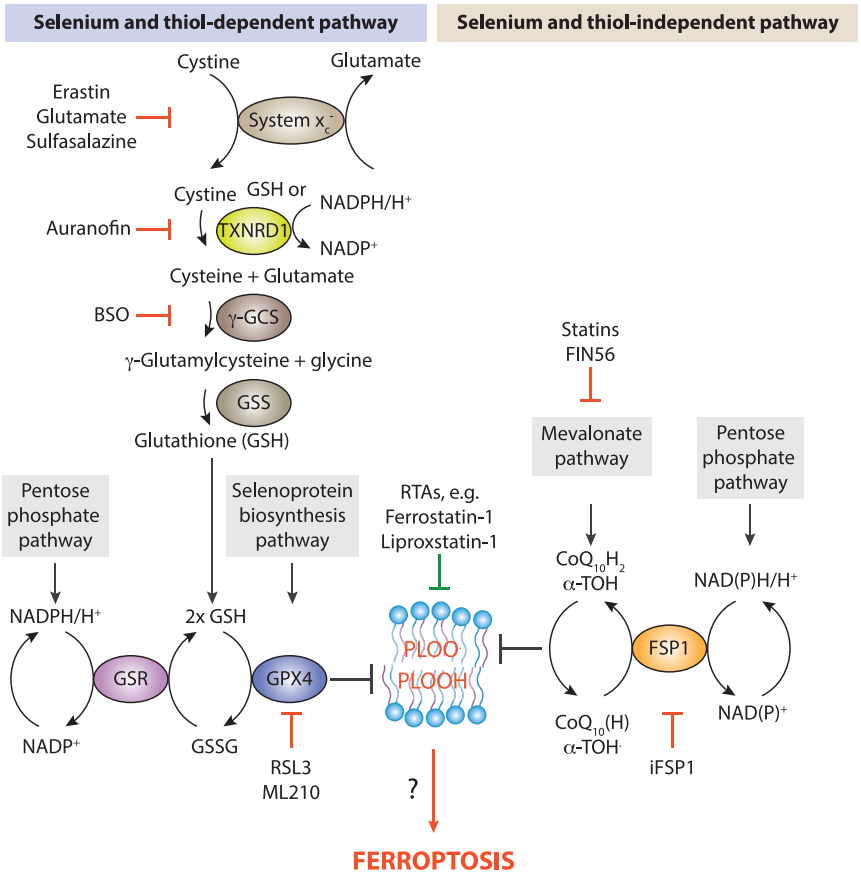


**Figure 1. The Key Mechanisms Controlling Ferroptosis**

Two metabolic pathways, a selenium-/thiol-dependent and a selenium-/thiol-independent pathway, are at play to control lipid peroxidation and associated ferroptosis. (Left) The canonical pathway controlling lipid peroxidation on the level of phospholipid hydroperoxides (PLOOH). Key members of this pathway are cystine uptake via system $x_c^-$, reduction of cystine to cysteine, glutathione biosynthesis, and glutathione peroxidase 4-mediated reduction of PLOOH to the corresponding alcohols. (Right) Two independent studies identified the NAD(P)H/FSP1/ubiquinone axis as the second mainstay in ferroptosis prevention on the level of one-electron reduction of peroxyl radicals in phospholipids ($PLOO^·$), thereby preventing autoxidation of cellular lipid bilayers. Ferroptosis inducers and inhibitors interfering at distinct steps of ferroptosis are indicated with red and green arrows, respectively. α-TOH, α-tocopherol; α-$TOH^·$, α-tocopheryl radical; BSO, L-buthionine sulfoximine; $CoQ_{10}$ $H_2$, ubiquinol; $CoQ_{10}$ (H), ubiquinone/semi-ubiquinone; FSP1, ferroptosis suppressor protein 1; γ-GCS, γ-glutamylcysteine synthetase; GPX4, glutathione peroxidase 4; GSR, glutathione reductase; GSH, glutathione; GSS, glutathione synthetase; GSSG, oxidized diglutathione; RTA, radical trapping antioxidant; TXNRD1, cytosolic thioredoxin reductase.

## The Ferroptosis Pathway

Ferroptosis is a form of regulated necrotic cell death that is marked by unique metabolic constraints and differs from other known forms of cell death such as accidental necrosis, apoptosis, pyroptosis, netosis, or autophagy by biochemical, morphological, and genetic means (Conrad et al., 2016; Dixon et al., 2012). Oxidative modifications of phospholipids in cellular membranes via iron-dependent lipid peroxidation are hallmarks of ferroptosis, which trigger associated membrane rupture and ultimately cell death (Conrad and Pratt, 2019). Genome-wide genetic screens led to the identification of the enzymes acyl-coenzyme A (CoA)-synthetase long-chain family member 4 (ACSL4) and lysophosphatidylcholine acyltransferase 3 (LPCAT3), which function through incorporation of PUFAs into cellular membranes (Dixon et al., 2015; Doll et al., 2017; Kagan et al., 2017). While cells expressing ACSL4 are particularly prone to undergo lipid peroxidation and subsequent cell death by ferroptosis when induced by pharmacological or genetic means, the respective knockout cells are especially resistant (Doll et al., 2017). Oxidation processes of PUFAs in cells can occur either by enzyme mediation, e.g., via lipoxygenases (LOX), or by spontaneous enzyme-independent autoxidation mechanisms catalyzed by iron or other redox-active metals (Conrad and Pratt, 2019). So far, several conflicting reports have appeared in the literature regarding the source of PUFA oxidation in terms of ferroptosis execution, which are reviewed in detail elsewhere (Conrad and Pratt, 2019). However, as a consensus, it may be hypothesized that LOX activities potentially contribute to the cellular pool of lipid hydroperoxides that initiate ferroptosis, while lipid autoxidation remains the main driver of ferroptotic cell death (Shah et al., 2018).

To date, two major mechanisms have been identified that cooperatively regulate uncontrolled lipid peroxidation and associated ferroptotic cell death in cells, namely, the selenium-dependent GPX4-GSH-cysteine axis and the ferroptosis suppressor protein 1 (FSP1)-ubiquinone ($CoQ_{10}$)-NAD(P)H pathway (Conrad and Pratt, 2019) (Figure 1). It was shown that inhibition of the cystine/glutamate antiporter system $x_c^-$ through small molecules, such as erastin and sulfasalazine, specifically triggers this cell-death modality by depleting cysteine for GSH synthesis followed by the generation of massive amounts of peroxidized phospholipids (Dixon et al., 2012, 2014). The early observation that the GSH-dependent selenoenzyme GPX4 functions by detoxifying membrane-bound phospholipid hydroperoxides (Ursini et al., 1982) underlines the importance of the GPX4-GSH-cysteine axis in ferroptosis suppression. Two independent discoveries facilitated the identification of GPX4 as a key ferroptosis player (Friedmann Angeli et al., 2014; Yang et al., 2014); (1) the ferroptosis-inducing agent (FIN) RSL3 exerts its mechanism of action as irreversible GPX4 inhibitor by covalently modifying its active-site Sec residue, thereby inducing cell death in certain tumor cell lines (Yang et al., 2014); and (2) tamoxifen-inducible conditional knockout mice with whole-body deletion of *Gpx4* (except brain) die from acute renal failure, triggered by ferroptotic cell death of kidney tubular cells (Friedmann Angeli et al., 2014). Of note, it was already appreciated even earlier that

conditional deletion of *Gpx4* in mouse embryonic fibroblasts and cortical and hippocampal neurons in the brain resulted in a form of non-apoptotic cell death associated with lipid peroxidation, now identified as ferroptosis (Seiler et al., 2008).

The mevalonate pathway, also known as the isoprenoid or HMG-CoA (3-hydroxy-3-methyl-glutaryl-CoA) reductase pathway, has also been linked to ferroptosis (Stockwell et al., 2017). This pathway generates isopentenyl pyrophosphate (IPP) and dimethylallyl pyrophosphate (DMAPP), which are essential precursors for the synthesis of a myriad of biomolecules such as cholesterol, vitamin K, or ubiquinone ($CoQ_{10}$). Within this context, statins, the classical HMG-CoA reductase inhibitors and cholesterol-lowering drugs, have been shown to sensitize cells to ferroptosis induced by FIN56 (Shimada et al., 2016). Since Sec-tRNA$^{[Ser]Sec}$ can be isopentenylated by tRNA isopentenyltransferase 1 (TRIT1) (Fradejas et al., 2013), it was suggested that interference with this reaction could result in decreased GPX4 expression as observed with FIN56 treatment (Shimada et al., 2016). However, it remains to be shown whether a lack of isopentenylation of Sec-tRNA$^{[Ser]Sec}$ indeed reduces GPX4 expression to disease-relevant levels in patients. While several patients could be identified as suffering from a *TRIT1*-related microcephaly, developmental delay, and epilepsy (Kernohan et al., 2017), fibroblasts isolated from another patient with an inactivating missense mutation in *TRIT1* did not undergo cell death, although ferroptosis susceptibility was not investigated in the study (Yarham et al., 2014). Additionally, FIN56 treatment was also associated with reduced $CoQ_{10}$ levels, providing an alternative potential mechanism of action for this compound (Shimada et al., 2016).

Two independent studies reported a second cell-autonomous and selenium-independent system that complements the GPX4-GSH-cysteine axis to suppress phospholipid peroxidation and ferroptosis, namely, the FSP1-$CoQ_{10}$-NAD(P)H pathway (Bersuker et al., 2019; Doll et al., 2019). By taking advantage of genome-wide screening approaches, apoptosis-inducing factor, mitochondrion-associated 2 (AIFM2) was identified, which fully protects against ferroptosis elicited by GPX4 deletion. Subsequently, AIFM2 was renamed to ferroptosis suppressor protein 1 (FSP1), which functions by catalyzing the regeneration of ubiquinone from ubiquinol by consuming NAD(P)H. Ubiquinol, the reduced form of $CoQ_{10}$, acts as a potent lipophilic antioxidant capable of trapping lipid peroxyl radicals that mediate lipid peroxidation (Bersuker et al., 2019; Doll et al., 2019). Recently, a genome-wide CRISPR/Cas-mediated activator screen allowed the identification of guanosine triphosphate cyclohydrolase 1 (GCH1), the rate-limiting enzyme for the synthesis of the antioxidant tetrahydrobiopterin ($BH_4$), as an alternative pathway that suppresses ferroptosis independently of the GPX4-GSH-cysteine axis (Kraft et al., 2019). The mechanism by which it protects against lipid peroxidation is likely based on the direct antioxidant function of $BH_4$ by selectively preventing oxidation of phospholipids specifically containing two unsaturated acyl chains. Additionally, $BH_4$ may also act on the level of $CoQ_{10}$ by preventing its exhaustion through alleviating oxidative stress and/or by fostering its synthesis by converting phenylalanine into tyrosine, which can be further converted to 4-OH-benzoate, a precursor to $CoQ_{10}$. Since the discovery of ferroptosis, several other important molecular determinants and key mechanisms that impinge on a cell's sensitivity or resistance toward ferroptosis, such as the role of iron, either in free or protein-bound form, have been reported and detailed in review articles elsewhere (Conrad and Pratt, 2019; Hassannia et al., 2019).

## Disease Implications and Pharmacological Modulation

Ferroptosis modulation by pharmacological means holds great promise for the treatment of a myriad of illnesses including degenerative diseases and cancer (Conrad et al., 2016; Seibt et al., 2019). The bona fide ferroptosis inhibitors ferrostatin-1 (Dixon et al., 2012) and liproxstatin-1 (Friedmann Angeli et al., 2014) exert their function as efficient radical trapping antioxidants (RTA), thereby preventing cellular autoxidation (Zilka et al., 2017). The protective effects of these compounds in mouse disease models include ischemia/reperfusion injuries of the kidney, liver, brain, heart, and intestine (Fang et al., 2019; Friedmann Angeli et al., 2014; Li et al., 2019a, 2019b; Linkermann et al., 2014; Tuo et al., 2017), acute renal failure (Friedmann Angeli et al., 2014), intracerebral hemorrhage (Li et al., 2017), neurodegeneration (Hambright et al., 2017), hemochromatosis (Wang et al., 2017), non-alcoholic steatohepatitis (Qi et al., 2020), and morphine tolerance (Chen et al., 2019), underlining the pathological role of lipid peroxidation and associated ferroptosis in numerous disease contexts. Vitamin E is perhaps the most efficient natural RTA found in organisms (Zilka et al., 2017). Therefore, it should be noted that vitamin E can mask the phenotype following *Gpx4* deletion in certain tissues and cells by protecting from ferroptosis in liver, vascular endothelium, T cells, and reticulocytes (Altamura et al., 2019; Carlson et al., 2016; Matsushita et al., 2015; Schwarz and Foltz, 1958; Wortmann et al., 2013).

From a selenium perspective, it is interesting to note that ebselen prevents ferroptosis induced by either erastin or *Gpx4* knockout in cells (Dixon et al., 2012; Matsushita et al., 2015). Ebselen is an organoselenium compound that functions as GPX mimetic through its hydroperoxide reducing action (Muller et al., 1984). The GPX-like catalytic activity was additionally confirmed by the observation that ebselen does not release or provide selenium for incorporation into GPX4 in mice *in vivo* (Wendel et al., 1984). Ebselen has been assessed in several clinical trials for its neuroprotective function in brain ischemia and stroke, but failed to be approved due to its insufficient efficacy compared with the placebo group in the study (Parnham and Sies, 2013).

Besides its relevance for a number of degenerative diseases, it has become evident that ferroptosis offers new strategies for the treatment of therapy-resistant tumor entities. Several recent studies provided independent evidence that treatment of tumors with standard chemotherapy leaves a residual population of cancer cells, so-called persister cells. These persister cells are characterized by a high PUFA-enriched state and consequently display a high dependency on the GPX4 axis, making them particularly vulnerable to ferroptosis induction (Hangauer et al., 2017; Tsoi et al., 2018; Viswanathan et al., 2017). Expression of ACSL4 in triple-negative breast cancer cells unequivocally predicted their sensitivity toward ferroptosis induction, due to their high propensity of incorporating PUFAs into cellular membranes (Doll et al., 2017). Notably, PUFA enrichment in tumor cells was linked to changes in cellular plasticity and the potential

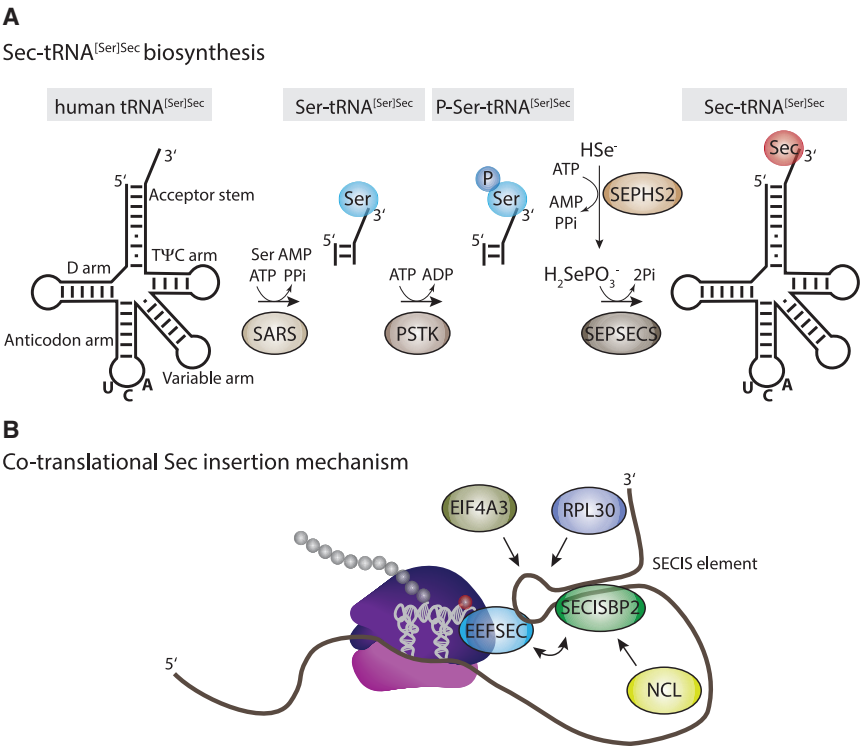


**Figure 2. Selenoprotein Biosynthesis**

(A) Selenocysteine-specific tRNA, $tRNA^{[Ser]Sec}$, is first aminoacylated with serine (Ser) yielding Ser-$tRNA^{[Ser]Sec}$. Ser-$tRNA^{[Ser]Sec}$ is then phosphorylated and converted to Sec-$tRNA^{[Ser]Sec}$ by the use of $H_2SePO_3^-$. PSTK, phosphoseryl-tRNA kinase; SARS, seryl-aminoacyl-tRNA synthetase; Sec, selenocysteine; SEPHS2, selenophosphate synthetase 2; SEPSECS, Sep (O-phosphoserine) tRNA:Sec (selenocysteine) tRNA synthase; Ser, serine.

(B) Co-translational incorporation of Sec into the nascent polypeptide chain requires the concerted action of a number of factors that either bind to the stem-loop-like structure in the 3′ untranslated region of the mRNA, called selenocysteine insertion sequence (SECIS) element, or to the Sec-$tRNA^{[Ser]Sec}$, thereby allowing the decoding of the UGA opal codon. EEFSEC, eukaryotic elongation factor, selenocysteine-tRNA-specific; EIF4A3, eukaryotic translation initiation factor 4A3; NCL, nucleolin; RPL30, ribosomal protein L30; SECISBP2, SECIS binding protein 2.

to undergo epithelial-mesenchymal transition (EMT) as well as associated metastasis (Viswanathan et al., 2017). Given the need for GPX4 expression in these relatively difficult-to-treat tumor entities, inhibitors of this selenoenzyme represent promising new drug candidates (Eaton et al., 2019; Sakamoto et al., 2017). To date, a class of compounds consisting of RSL3 and ML162 has been discovered that exploit a chloroacetamide moiety to covalently acylate the catalytic Sec residue, leading to GPX4 inactivation and subsequent cell death by ferroptosis (Eaton et al., 2019; Weiwer et al., 2012; Yang et al., 2014). An analogous mechanism has been reported for ML210 containing a diacylfuroxan substituent that acts as masked nitrile oxide prodrug for the irreversible inhibition of GPX4 (Eaton et al., 2019; Weiwer et al., 2012). Despite these encouraging discoveries, there are still several drawbacks in the use of the aforementioned covalent GPX4 inhibitors. First, this class of compounds is inherently metabolically unstable, and therefore its use *in vivo* is limited (Viswanathan et al., 2017). Second, by using a chemical-proteomics approach investigating potential selenoprotein-based targets of RSL3, Gao et al. (2018) showed that this compound can covalently modify the active-site Sec of not only GPX4 but also almost all selenoproteins in a non-specific manner. Concomitantly, the identification of thioredoxin as the primary drug target of the ferroptosis-inducing drug ferroptocide, which harbors a chloroacetamide-reactive group, clearly suggests that not only Sec-containing enzymes but also those with highly reactive cysteines, as found in thioredoxin, can also be targeted by chloroacetamide (Llabani et al., 2019). Third, as the resistance to ferroptosis inducers and GPX4 inhibitors RSL3, ML162, and ML210 directly correlates with FSP1 expression in certain cancer cell lines, co-treatment options with FSP1 inhibitors such as iFSP1 need to be considered in the design of future efforts in anticancer drug discovery (Doll et al., 2019).

## Selenoprotein Biosynthesis

To incorporate Sec into proteins, organisms have evolved a highly complex and energetically demanding Sec insertion system that allows for the incorporation of this rare amino acid at specific UGA codons via a *cis*-acting Sec insertion sequence (SECIS) element, a stem-loop-like structure found in the 3′ untranslated region of selenoproteins in vertebrates (Hatfield et al., 2014) (Figure 2A). The biosynthesis of Sec takes place on its own tRNA, designated Sec-$tRNA^{[Ser]Sec}$. Thereby, aminoacylation of $tRNA^{[Ser]Sec}$ with serine is the first step in the synthesis process, which is catalyzed by seryl-tRNA synthetase to yield seryl-$tRNA^{[Ser]Sec}$. Next, seryl-$tRNA^{[Ser]Sec}$ is phosphorylated by phosphoseryl-tRNA kinase in order to obtain P-Ser-$tRNA^{[Ser]Sec}$, providing the backbone for selenium loading. Concomitantly, selenide ($HSe^-$) is converted to selenophosphate ($H_2SePO_3^-$) catalyzed by SEPHS2 (selenophosphate synthetase 2). $H_2SePO_3^-$ is then incorporated into P-Ser-$tRNA^{[Ser]Sec}$ by SEPSECS (Sep(O-phosphoserine) tRNA:Sec [selenocysteine] tRNA synthase) by replacing phosphate in a nucleophilic substitution. Subsequently, $H_2SePO_3^-$ is hydrolyzed, resulting in Sec-$tRNA^{[Ser]Sec}$. The inherent advantage of Sec biosynthesis on its own tRNA is to avoid accumulation of free Sec, which may be too reactive within cells by itself. Thus, virtually no free Sec can be detected, and it has been proposed that selenoprotein GPX1 may function as a safe storage device for Sec. In turn, free Sec can be degraded by selenocysteine lyase.

Not only the synthesis of the Sec-loaded $tRNA^{[Ser]Sec}$ is energetically costly (Hatfield et al., 2014), but also the decoding of the

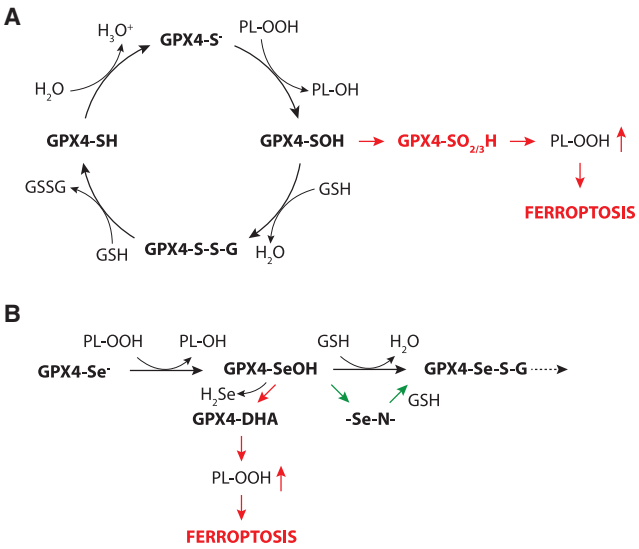


**Figure 3. Sec Utilization Confers High Resistance to Peroxide-Induced GPX4 Inactivation**

(A) Due to the impaired regenerative steps of Cys-containing GPX4 using glutathione (GSH), the sulfenic form of GPX4 (GPX-SOH) becomes prone to peroxide-induced overoxidation, yielding sulfinic and sulfonic forms (GPX4-$SO_{2/3}$H). Since these forms are irreversibly overoxidized, intracellular PLOOH accumulate, causing ferroptosis.

(B) Under normal cellular conditions, the selenenic form of wild-type GPX4 (GPX-SeOH) becomes readily recycled by two molecules of GSH (in analogy to the catalytic cycle shown in A). Under conditions of low GSH concentrations and high steady-state peroxide levels, GPX4-SeOH may undergo β-cleavage, forming dehydroalanine (DHA), an enzymatically dead form of GPX4. As a protective mechanism, GPX4-SeOH may also form an intermediate, selenylamide (-Se-N-), with the amino group of the glycine adjacent to Sec, which can be resolved when cellular redox conditions are restored.

opal codon UGA requires the orchestrated action of several factors acting in *cis* and in *trans*, as otherwise eukaryotic translation termination factor 1 would cause disassembly of ribosomes at the UGA codon (Figure 2B). As such, binding of SECIS binding protein 2 (SECISBP2) and some other protein factors to the SECIS element allow for the co-translational incorporation of Sec into the nascent polypeptide chain. Incorporation of Sec compared with that of the canonical amino acids is, however, a very inefficient process. Comparing the efficiencies of Sec and Cys incorporation, it was initially reported that Sec incorporation ranges somewhere between 4% and 5%, again highlighting the need to understand the importance of selenium utilization in a select set of selenoproteins mainly in higher organisms (Suppmann et al., 1999). Yet efficiencies of Sec incorporation in mammalian selenoproteins can be higher and can reach even up to 60% depending on the selenoprotein (Fradejas-Villar et al., 2017).

## Role of Selenolate-Based versus Thiolate-Based Enzyme Catalysis of GPX4 *In Vivo*

To decipher the long-lasting mystery of selenolate-based versus thiolate-based catalysis *in vivo*, Ingold et al. (2018) developed a novel mouse model in which Sec was replaced by Cys in the active site of GPX4, referred to as $Gpx4^{Cys/Cys}$ mice. $Gpx4^{Cys/Cys}$ mice on a mixed genetic background were surprisingly born at the Mendelian ratio and viable until about 2 weeks after birth, whereupon mice had to be sacrificed due to spontaneous seizures. This is in stark contrast to systemic *Gpx4* knockout mice, which were repeatedly shown to already have an embryonic lethal phenotype at the gastrulation stage (Imai et al., 2003; Seiler et al., 2008; Yant et al., 2003). The occurrence of seizures could be explained by the selective loss of parvalbumin-positive interneurons. This is in accordance with a previous report of conditional deletion of *Trsp*, wherein the same type of neurons was affected (Wirth et al., 2010). To investigate this phenomenon on a biochemical level, it was further demonstrated that Sec-containing GPX4 is intrinsically resistant to overoxidation while the Cys mutant is irreversibly oxidized upon increasing concentrations of peroxides, resulting in inactivation of the protein and, ultimately, cell death via ferroptosis (Ingold et al., 2018) (Figure 3A). In addition, cells deficient in the gene encoding $tRNA^{[Ser]Sec}$, *Trsp*, in the GPX4-Cys background showed that selenium is essential for the full antiferroptotic role of GPX4, and that other selenoproteins are not required for cell survival, at least in proliferating cells (Ingold et al., 2018). These findings are highly reminiscent of what was earlier reported for another essential selenoprotein in mammals called mitochondrial thioredoxin reductase (TXNRD2) (Conrad et al., 2004). By comparing purified wild-type murine TXNRD2 with the Cys ortholog from *Drosophila melanogaster* (DmTR), Hondal's group showed that Sec in the active site of mouse TXNRD2 confers strong resistance toward a number of pro-oxidant conditions including $H_2O_2$, whereas DmTR was by far more sensitive to overoxidation. Generating a chimeric enzyme by replacing the C-terminal active site of *D. melanogaster* enzyme with the C-terminal active site of mouse TXNRD2 ("SCUG") conferred full resistance to overoxidation as seen in wild-type mouse TXNRD2 (Maroney and Hondal, 2018; Snider et al., 2013), confirming that Sec confers key protection from irreversible enzyme overoxidation.

All these studies established a unique role for Sec providing a marked resistance to peroxide-induced irreversible overoxidation of these two selenoproteins; but what are the mechanisms that may protect or irreversibly inactivate the wild-type enzyme? Early mass spectrometry studies by Burk's group indicated that purified selenoprotein P (SELENOP) and GPX1 from rat plasma contained a fraction of peptides that lost selenium but instead contained dehydroalanine (DHA) (Ma et al., 2003). The mechanism by which this occurs is through β-cleavage of selenenic acid yielding redox-inactive, enzymatically dead DHA, which was later confirmed for GPX1 in red blood cells (Cho et al., 2010; Wang et al., 2011). This mechanism seems to be shared with GPX4, as high steady-state lipid hydroperoxide levels along with low GSH concentrations may cause DHA formation (Orian et al., 2015). Beyond its inherently high resistance toward overoxidation, selenenic acid of GPX4 was additionally reported to form a transient selenylamide bond with the amino group of the adjacent glycine (Figure 3B). Whether any of these mechanisms are at play at the observed rapid loss of GPX4 upon transient ischemia/reperfusion injury remains to be formally shown (Li et al., 2019b), but seems likely given the high pro-oxidative load occurring during reverse electron flow at mitochondrial complex I in response to an ischemic event (Chouchani et al., 2014).

## Evolutionary Impact of PUFA Utilization

The great oxygenation event marked the accumulation of oxygen on Earth and enabled the beginning of complex eukaryotic

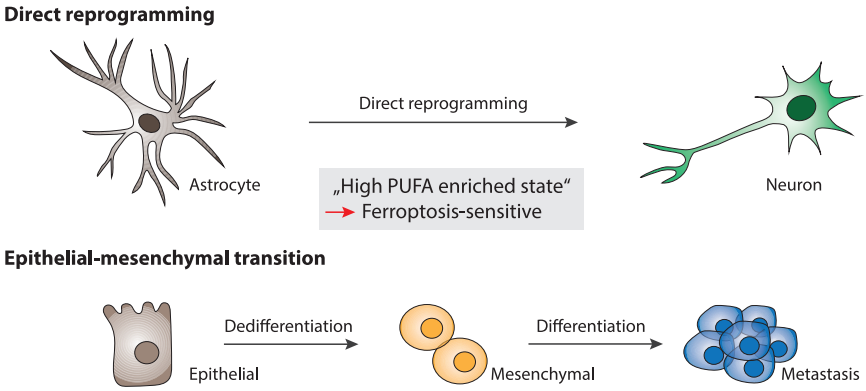


**Figure 4. High PUFA-Enriched State Facilitates Cellular Plasticity and Differentiation**

Direct reprogramming of astrocytes into neurons requires a PUFA-enriched state, rendering cells extremely sensitive to ferroptosis that can be overcome by the use of ferroptosis inhibitors. Cancer cells exploit epithelial-mesenchymal transition in terms of therapy resistance as well as for the formation of distant metastases. The switch in cellular plasticity is accompanied by a high dependence on cellular PUFA content and the expression of lipid peroxidation-regulating enzymes, thus leaving a potential vulnerability for anticancer strategies.

and multicellular forms of life. PUFA-enriched phospholipids are a prerequisite to allow the development of complex organisms with sophisticated cellular networks as well as the development of intricate nervous systems (Conrad et al., 2018). A drawback is that PUFAs are easily oxidizable, and oxidation of PUFAs in cellular membranes can lead to aberrant lipid peroxidation, subsequent membrane damage, and cell death via ferroptosis (Stockwell et al., 2017). Yet the selenoprotein GPX4 is the only enzyme currently known that efficiently and directly reduces peroxidized phospholipids to their corresponding alcohols, thereby keeping ferroptosis in check (Friedmann Angeli et al., 2014; Yang et al., 2014), with the exception of FSP1, which can halt lipid autoxidation via ubiquinone (Doll et al., 2019). Thus, one may speculate whether an evolutionary pressure exists to retain Sec-containing GPX4 to enable and maintain plasticity of cellular membranes, which correlates with an increased PUFA requirement and in turn renders complex biological life possible. It is worth mentioning here that high levels of lipid peroxidation prevented successful direct neuronal reprogramming by inducing cell death, whereas neuronal cell-fate conversion could be promoted by the use of ferroptosis inhibitors such as liproxstatin-1 (Gascon et al., 2016) (Figure 4). Conversely, cancer cells evolve by exploiting cellular plasticity to drive resistance mechanisms toward several classes of antitumor therapies or to dedifferentiate and form distant metastases (Figure 4). A study by Viswanathan et al. (2017) indeed showed that increased expression of PUFAs as well as dependency on GPX4 was found across various therapy-resistant states, including EMT in epithelial-derived carcinomas, transforming growth factor β-mediated therapy resistance in melanoma, and during treatment-induced neuroendocrine transdifferentiation in prostate cancer.

Sec utilization by GPX4 is predominantly preserved in vertebrates including mammals, fish, birds, and amphibians (Ingold et al., 2018), which have the ability to develop complex nervous systems and mainly esterify long-chain PUFAs, such as arachidonic acid, adrenic acid, and docosahexaenoic acid into their lipid bilayers (Conrad et al., 2018; Wallis et al., 2002). Such an enrichment with PUFAs is pivotal for synapse formation and development of complex brains (Bazinet and Laye, 2014). Besides brain, PUFAs are particularly abundant in organs such as liver, kidney, and testis—tissues that are highly dependent on a functioning GPX4 enzyme (Conrad et al., 2018). In contrast, invertebrates mainly utilize monounsaturated and saturated fatty acids in their lipid bilayers, and organisms such as higher plants and fungi use the readily available sulfur to express corresponding Cys homologs (Ingold et al., 2018). ACSL4 was found to be downregulated in Cys-containing GPX4 cells (Ingold et al., 2018), providing circumstantial evidence for a potential compensatory mechanism to reduce the cellular PUFA content in membranes, thus lowering the risk of lipid peroxidation and subsequent generation of lethal ferroptosis signals (Doll et al., 2017). Consequently, one may wonder whether protection from ferroptosis through Sec-containing GPX4 arises from an evolutionary requirement caused by the incorporation of PUFAs into cellular membranes, particularly in neuronal networks (Ingold et al., 2018).

## Physiological Implications of Selenium Metabolism with Focus on GPX4

Selenium, discovered in 1817 by a Swedish scientist in Gripsholm, was initially regarded as a toxin, as horses and livestock feeding on plants that grew on soil with high amounts of selenium suffered from necrotic hoof malady and hair loss in the tail and mane (Franke, 1934). Since the discovery by Schwarz and Foltz in 1958 that the selenium-containing compound "Factor 3" prevented liver necrosis in vitamin E-deficient rats, it is now widely accepted that selenium is a toxin at high levels but an essential micronutrient at low levels (Hatfield et al., 2014). In humans, the functions of selenium depend on 25 dedicated selenoproteins that incorporate Sec into their active sites (Gladyshev et al., 2016; Kryukov et al., 2003). Therefore, the effects of nutritional selenium may be considered as pleiotropic, and selenium deficiency has been associated with several pathological conditions ranging from heart disease, myopathy, cancer, inflammation, mammalian development, and immune function to male infertility, atypical resistance to thyroid hormones, familial glucocorticoid deficiency, epilepsy, pontocerebellar hypoplasia type 2D, and neurodegenerative disorders (Cardoso et al., 2017; Labunskyy et al., 2014; Schweizer and Fradejas-Villar, 2016). Interestingly, it has been shown in mice that during selenium depletion, brain selenium levels are maintained at the expense of other tissues (Burk and Hill, 2009). Evidence from studies in humans has suggested a role for selenium in seizures, coordination, Parkinson's disease, Alzheimer's disease, dementia, and cognitive decline (Rayman, 2012). In addition, inactivating mutations of the *GPX4* gene in humans have been associated with

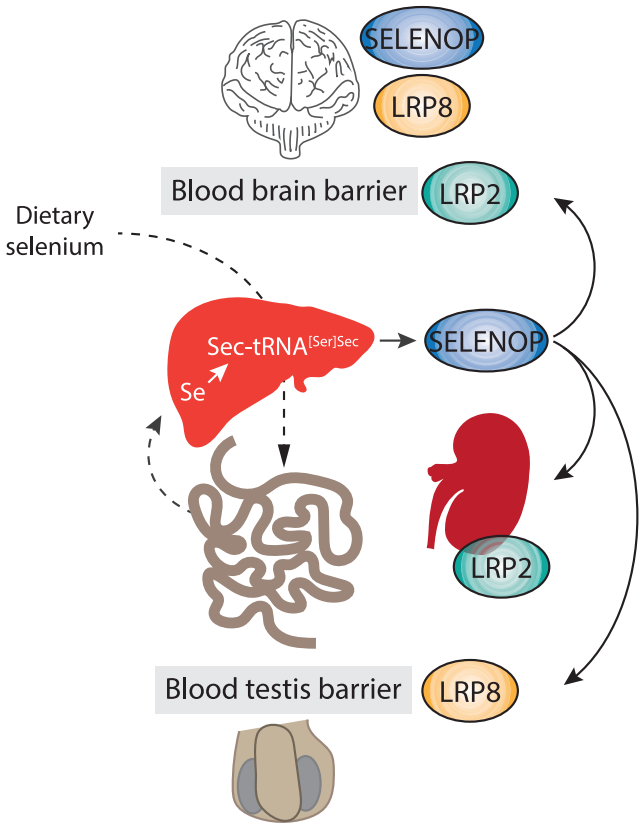


**Figure 5. Selenoprotein P-Mediated Redistribution of Selenium in the Body**

Selenium is taken up from the diet mainly in organic form usually in selenoproteins in the gastrointestinal tract, from which they enter the liver via the portal vein. Here they are degraded, and selenium is used to synthesize selenoprotein P (SELENOP), which contains up to ten Sec residues. Upon secretion, SELENOP is transported via the blood to target organs that strongly depend on selenium such as brain, testis, and kidney. Two receptors, low-density lipoprotein receptor-related protein 2 (LRP2, also known as megalin) and low-density lipoprotein receptor-related protein 8, apolipoprotein E LRP8 (alias APOER2), are responsible for receptor-mediated uptake/and retention in the target tissues, ensuring sufficient selenium supply particularly under selenium-depleted conditions.

Sedaghatian-type spondylometaphyseal dysplasia (Smith et al., 2014). Several clinical trials investigated the role of selenium in cancer prevention (Clark et al., 1996; Hatfield and Gladyshev, 2009; Lippman et al., 2009; Nicastro and Dunn, 2013; Rayman, 2012). However, most of the results have proven contradictory or insufficient, presumably due to the unknown selenium status of enrolled patients or dual functions of certain selenoproteins to either prevent or promote cancer (Hatfield et al., 2014). In addition, it was postulated that selenium supplementation may only be beneficial in the presence of inadequate nutrient availability (Rayman, 2012).

The observation that selenium utilization is indispensable for life is supported by the early embryonic lethal phenotype of mice devoid of the Sec-specific tRNA gene *Trsp* (Bosl et al., 1997). Studies using mouse knockout models showed that GPX4 and selenoprotein T (and perhaps TXNRD1) are the only two selenoproteins whose deficiency leads to the same early embryonic lethality as that resulting from *Trsp* deficiency (Bondareva et al., 2007; Boukhzar et al., 2016; Jakupoglu et al., 2005; Yant et al., 2003). In addition, the use of tissue-specific animal models revealed that *Gpx4* deletion frequently mirrored the phenotype observed with the respective *Trsp* knockout, as was shown in the case of certain neurons (Wirth et al., 2010, 2014), highlighting the central role of GPX4 among selenoproteins for tissue protection. Moreover, some of the pathologies arising from mutations in the human *selenocysteine insertion sequence-binding protein 2* (*SECISBP2* alias *SBP2*) gene, such as male infertility and increased markers of lipid peroxidation in plasma, can be unequivocally traced back to insufficient expression of GPX4 (Saito et al., 2015; Schoenmakers et al., 2010).

The generation of conditional mouse models has greatly aided our understanding of the physiological functions of GPX4 (Conrad et al., 2018). GPX4 was shown to be essential in protecting neurons of several brain regions, such as hippocampus, cortex, cerebellum, and motor neurons (Chen et al., 2015; Hambright et al., 2017; Seiler et al., 2008; Wirth et al., 2010, 2014). In addition to its role in the brain, GPX4 is also crucial for the survival of endothelial cells, photoreceptor cells, kidney tubular cells, CD8-positive T cells, hepatocytes, and reticulocytes, as well as B1 and marginal zone B cells (Altamura et al., 2019; Carlson et al., 2016; Friedmann Angeli et al., 2014; Matsushita et al., 2015; Muri et al., 2019; Ueta et al., 2012; Wortmann et al., 2013).

SELENOP (Gladyshev et al., 2016) is considered the main selenium transport protein in mammals (Saijoh et al., 1995). SELENOP is predominantly synthesized in the liver and plays an important role in transporting selenium to organs, such as brain, testis, and kidney (Figure 5). When dietary selenium is limited, SELENOP aids in preserving brain selenium levels at the expense of other organs, ensuring proper neuronal function (Nakayama et al., 2007). A similar phenomenon was observed in the case of testis, whereas kidney selenium concentrations decrease comparably with levels of whole-body selenium. This mechanism can be explained by receptor-mediated uptake through ApoER2, also known as low-density lipoprotein receptor-related protein 8 (LRP8), which facilitates the uptake of SELENOP into testis and brain, whereas another receptor, megalin (low-density lipoprotein-related protein 2 [LRP2]), facilitates the uptake of SELENOP into proximal tubule of the kidney (Burk and Hill, 2009; Chiu-Ugalde et al., 2010) (Figure 5). Selenium supply and maintenance of the brain at the expense of other tissues may indicate that in the case of low selenium supply, the synthesis of certain selenoproteins such as GPX4 is prioritized. In fact, mice deficient in *Selenop* or *ApoER2* in combination with a selenium-deficient diet develop brain injury and neurodegeneration, reminiscent of *Trsp* and *Gpx4* knockout models (Hill et al., 2004; Valentine et al., 2008). SELENOP is also expressed inside the brain (Saijoh et al., 1995), primarily by glial cells and astrocytes (Yang et al., 2000; Zhang et al., 2008), and has been postulated to store and/or transport brain selenium (Renko et al., 2008; Schweizer et al., 2005), possibly providing additional means for the synthesis of GPX4 in neurons (Cardoso et al., 2017; Pitts et al., 2012).

Recently, it was confirmed that selenium supplementation leads to increased GPX4 expression and subsequent resistance toward ferroptosis induction (Alim et al., 2019; Belavgeni et al., 2019; Vande Voorde et al., 2019). As a potential therapeutic approach to prevent GPX4 loss and associated

neurodegeneration, Alim et al. (2019) developed a therapeutic peptide consisting of HIV-Tat, a cell-penetrating peptide that facilitates transport of cargo across the blood-brain barrier as well as into cells, and the C-terminal domain of SELENOP (Tat SelPep) to efficiently deliver selenium for GPX4 synthesis. Tat SelPep protected primary neurons from hemin-induced and homocysteic acid-induced ferroptotic cell death *in vitro* and significantly improved the outcome in mouse models of intracerebral hemorrhage and ischemic stroke *in vivo* (Alim et al., 2019). On the other hand, it has long been known that sodium selenite, normally not present in the diet, can be easily supplemented and even possesses high blood-brain barrier penetrance. The key advantage of delivering selenium via Tat SelPep over inorganic selenium remains to be fully demonstrated. Nonetheless, given the importance of GPX4 in neuroprotection and the initial findings that Alzheimer's disease brain samples show reduced GPX4 expression and iron dyshomeostasis (Lane et al., 2018; Yoo et al., 2010), novel therapeutic approaches to maintain GPX4 expression in the brain may provide hitherto unseen strategies to ameliorate neurodegenerative diseases linked to impaired GPX4 function and associated ferroptosis.

## Concluding Remarks

The selenoperoxidase GPX4 has emerged as the key regulator of ferroptosis. As such, maintaining its expression by supplying sufficient amounts of selenium in obligate selenium-dependent organs is a prerequisite for full ferroptosis resistance as a strategy to prevent pathological cell loss and degenerative diseases. In contrast, targeting GPX4 in the context of cancer might be a promising scenario to sensitize cancer cells toward ferroptosis or to eradicate tumors. Yet GPX4 is known to lack suitable binding pockets, which poses a major challenge for conventional medicinal chemistry approaches. Therefore, potent GPX4 inhibitors described so far are all strong electrophiles irreversibly modifying the active-site Sec. In addition to stability issues, this class of inhibitors suffers from a lack of specificity, exemplified by RSL3, which was shown to inhibit almost all human selenoproteins. Nonetheless, all of these important discoveries made in the last few years should guide us in the rational design of improved future therapies based on ferroptosis modulation.

## ACKNOWLEDGMENTS

Funding was received from the Deutsche Forschungsgemeinschaft (DFG) CO 291/5-2 and CO 291/7-1 to M.C. and PR 1752/3-1 to B.P., the German Federal Ministry of Education and Research (BMBF) through the Joint Project Modeling ALS Disease In Vitro (MAIV, 01EK1611B), the VIP+ program NEUROPROTEKT (03VP04260) to M.C., the Government of the Russian Federation (2019-220-07-7053), as well as the $m^4$ Award provided by the Bavarian Ministry of Economic Affairs, Regional Development and Energy (StMWi) to M.C. and B.P. The authors greatly appreciate the insightful and valuable comments of reviewers.

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