

Unresolved questions regarding cellular cysteine sources and their possible relationships to ferroptosis

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Unresolved questions regarding cellular cysteine sources and their possible relationships to ferroptosis

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Abstract

Cysteine is required for synthesis of glutathione (GSH), coenzyme A, other sulfur-containing metabolites, and most proteins. In most cells, cysteine comes from extracellular disulfide sources including cystine, glutathione-disulfide, and peptides. The thioredoxin reductase-1 (TrxR1)- or glutathione-disulfide reductase (GSR)-driven enzymatic systems can fuel cystine reduction via thioredoxins, glutaredoxins, or other thioredoxin-fold proteins. Free cystine enters cells thorough the cystine-glutamate antiporter, xCT, but systemically, plasma glutathione-disulfide might predominate as a cystine source. Erastin, inhibiting both xCT and voltage-dependent anion channels, induces ferroptotic cell death, so named because this type of cell death is antagonized by iron-chelators. Many cancer cells seem to be predisposed to ferroptosis, which has been proposed as a targetable cancer liability. Ferroptosis is associated with lipid peroxidation and loss of either glutathione peroxidase-4 (GPX4) or ferroptosis suppressor protein-1 (FSP1), which each prevent accumulation of lipid peroxides. It has been suggested that an xCT inhibition-induced cellular cysteine-deficiency lowers GSH levels, starving GPX4 for reducing power and allowing membrane lipid peroxides to accumulate, thereby causing ferroptosis. Aspects of ferroptosis are however not fully understood and need to be further scrutinized, for example that neither disruption of GSH synthesis, loss of GSH, nor disruption of glutathione disulfide reductase (GSR), triggers ferroptosis in animal models. Here we reevaluate the relationships between Erastin, xCT, GPX4, cellular cysteine and GSH, RSL3 or ML162, and ferroptosis. We conclude that, whereas both Cys and ferroptosis are potential liabilities in cancer, their relationship to each other remains insufficiently understood.

1. Roles and critical requirement of cellular cysteine

Although the abundance of cysteine (Cys) is 20th out of the 21 translated amino acids in the human proteome, with only the hyperreactive selenium (Se)-containing Cys-analog selenocysteine (Sec) having lower representation (Sec occurs in 25 human proteins, <0.1% of the proteome); 92% of human proteins contain at least one Cys and it represents 2.26% of the amino acids in the human proteome (Miseta & Csutora, 2000). Cys is also used for biosynthesis of glutathione (γ -L-glutamyl-L-cysteinyl glycine; GSH), coenzyme A (CoA), H₂S, taurine, iron-sulfur clusters, and other sulfur (S)-containing metabolites. GSH, the most abundant intracellular low molecular weight thiol (3–10 mM) (Hansen, Roth, & Winther, 2009), is noteworthy as its thiol-S comes exclusively from Cys (Stipanuk, Dominy,

Lee, & Coloso, 2006). No cell could likely survive without a reliable source of Cys. Cys itself, however, is unstable in the extracellular environment, wherein it effectively oxidizes into the disulfide-linked dimeric form of Cys, cystine (hereafter abbreviated CSSC; see below). Thus, cells typically acquire Cys by assimilating CSSC from extracellular sources and reducing this to 2 Cys in the cytosol, although cancer cells can often upregulate alternative sources of Cys, such as more *de novo* synthesis through transsulfuration due to activation of transcription factor “nuclear factor, erythroid-derived-2, like-2” (NRF2) in cancer cells (Bonifacio, Pereira, Serpa, & Vicente, 2021); the transsulfuration pathways is discussed at further detail below.

Cellular dependence upon Cys is considered a potentially targetable liability in cancer (Combs & DeNicola, 2019; Daher, Vucetic, & Pouyssegur, 2020; Jyotsana, Ta, & DelGiorno, 2022). As with any therapy, to be useful, cancer cells need to be substantially more sensitive to Cys-targeting therapies than other cells in the body. Moreover, it is important to understand mechanistically how Cys-targeting impacts cell physiology systemically, such that its targeting for cancer therapy can be effectively directed toward having more potent impact on the cancer cells than on other cells in the patient.

Metabolic properties of many cancer cells result in their having chronically elevated levels of reactive oxygen species (ROS). It is logical that this is a liability that might be targetable for therapy (Trachootham, Alexandre, & Huang, 2009). Cellular ROS in the form of peroxides (ROOH) are detoxified in mammalian cells predominantly by the two families of abundant peroxidases, the GSH peroxidases (GPXs) and the peroxiredoxins (PRXs) (Winterbourn, 2013). These, in turn, obtain electrons to drive their reduction reactions from reduced nicotinamide adenine dinucleotide phosphate (NADPH), either via GSH and glutathione disulfide reductase (GSR; $\text{NADPH} \rightarrow \text{GSR} \rightarrow \text{GSH} \rightarrow \text{GPX} \rightarrow \text{ROOH}$) or via thioredoxin-1 (Trx1) and thioredoxin disulfide reductase (TrxR1; $\text{NADPH} \rightarrow \text{TrxR1} \rightarrow \text{TRX1} \rightarrow \text{PRX} \rightarrow \text{ROOH}$) (Winterbourn, 2013). Because GSH links NADPH with GPXs, and Cys is required for GSH synthesis, it has been proposed that limiting Cys will lower cellular GSH levels, thereby indirectly impeding GPX activity and increasing ROS levels to yield oxidative stress and cell death (Jyotsana et al., 2022; Rosell et al., 2023; Stockwell & Jiang, 2020; Xu et al., 2019). Cancer cells generating increased levels of ROS, therefore, should be particularly susceptible to diminished peroxidase activity. Because ferroptosis is associated with accumulation of phospholipid hydroperoxides (discussed further in Section 1.4) and, of all the peroxidases in either family, only GPX4 shows substantial reduction of

phospholipid hydroperoxides (Brigelius-Flohe, 2006; Flohe, Toppo, Cozza, & Ursini, 2011; Schwarz et al., 2023), it seems reasonable that lowering the GSH levels might be particularly effective at promoting ferroptosis even if the $\text{NADPH} \rightarrow \text{TrxR1} \rightarrow \text{Trx1} \rightarrow \text{PRX}$ system remains intact. However, several observations suggest that this notion may be too simplified, as we shall further discuss herein.

The unique attribute of Cys in the proteome is the free thiol-S (-SH) in its side chain. Methionine (Met), the other proteinogenic S-amino acid, has a thioether-S which, although being at the same redox state as a thiol, is much less reactive with oxidants compared with Cys (Kaya, Lee, & Gladyshev, 2015). This allows Met to remain reduced extracellularly also when ingested as a nutrient in food, or when present in proteins or blood plasma. However, Met may nonetheless become oxidized into its sulfoxide derivatives, which in turn can be repaired by TrxR1/Trx1-dependent methionine sulfoxide reductases (Kim & Gladyshev, 2007; Le et al., 2008). Cys being more prone to oxidation than Met, is less utilized in proteins (Miseta & Csutora, 2000) and is typically found only in positions that require the unique chemistry of the thiol: within *i*) active sites, *ii*) regulatory sites that are influenced by derivatization of the thiol, or *iii*) in structural positions where they form intra- or inter-molecular disulfide bonds (Fomenko, Marino, & Gladyshev, 2008).

In the reducing environment of the cytosol, nearly all Cys residues will be in the reduced thiol state. Whereas the Cys-thiol is thermodynamically susceptible to oxidation by biological oxidants like superoxide radical (O_2^-) or H_2O_2 , the reaction rates for these oxidants with “typical cellular Cys-thiols” is between 10^0 and $10^1 \cdot \text{mol}^{-1} \cdot \text{sec}^{-1}$ (Winterbourn & Hampton, 2008; Winterbourn & Metodiewa, 1999; Winterbourn, 2013). Whereas this rate is sufficient to effectively oxidize most thiols to disulfides in the oxidizing environment of blood plasma, these rates are too slow for contributing to intracellular processes (Nagy et al., 2011; Winterbourn & Hampton, 2008; Winterbourn, 2013). “Typical” cellular Cys-thiols include free Cys, GSH, and nearly all protein-Cys-thiols; indeed the total intracellular protein-derived free thiol concentrations has been estimated to be significantly higher than that derived from GSH (Requejo, Hurd, Costa, & Murphy, 2010). Such thiol groups on proteins can clearly participate in redox reactions and thus contribute to the total antioxidant defense capacity of the cells (Requejo et al., 2010). In addition, the PRX and GPX families of “professional peroxidase” proteins exhibit active-site Cys (PRXs) or Sec (most GPXs) residues with $\sim 10^7$ -fold more reactivity towards their

peroxide substrates than that seen with typical non-catalytic thiols (Nagy et al., 2011; Winterbourn, 2013). In the case of PRX, the H_2O_2 -reactive Cys, termed the “peroxidatic Cys”, achieves its high rate through a specialized microenvironment in the active site that both activates the Cys by lowering its pKa, resulting in a more reactive nucleophilic “thiolate anion”, and activates the enzyme-bound H_2O_2 molecule for facilitated catalysis (Nagy et al., 2011). GPXs likely use similar active site specialization, along with the higher intrinsic reactivity of the Se of Sec versus the S atom of Cys, combined with additional enzyme features to achieve very high rates of reactivity with their peroxide substrates (Cozza et al., 2017; Toppo, Flohe, Ursini, Vanin, & Maiorino, 2009). Finally, a small number of other proteins, including glyceraldehyde-3 phosphate dehydrogenase (GAPDH) and protein-tyrosine phosphatase 1B (PTP1B), have specialized microenvironment contexts that similarly increase their reactivity with H_2O_2 by several orders of magnitude, making these also directly reactive with H_2O_2 at physiologically significant rates and concentrations (Dagnell et al., 2017; Doka et al., 2020; Netto & Machado, 2022; Talwar et al., 2023).

1.1 Sources of intracellular cysteine and modes of its entry into cells

1.1.1 *De novo Cys synthesis*

Many plants, fungi, eubacteria, and archaea reduce inorganic sulfur species into sulfide, which is then used for primary synthesis of Cys-thiol from serine (Ser) (Liu et al., 2021b; Russel, Model, & Holmgren, 1990; Wray, Campbell, Roberts, & Gutierrez-Marcos, 1998). Most if not all plants, fungi, eubacteria, and archaea are also able to use the thiol-S from Cys for de novo synthesis of Met (Miller & Schmidt, 2019). Perhaps due to the strictly heterotrophic life-history of metazoans providing an adequate source of protein-Cys, metazoans lack the ability to reduce inorganic sulfur, or to convert Cys to Met. Instead, the Cys-to-Met pathway was effectively reversed at the metazoan transition, exchanging the ability to synthesize Met from Cys for the ability to synthesize Cys de novo using Met as the S-source (Miller & Schmidt, 2019; Miller & Schmidt, 2020). As such, Met is an essential amino acid in metazoans.

De novo synthesis of Cys from Ser + Met in metazoans is a 5-step process in which Met-derived homocysteine (Hcy) is shunted-off of the Met cycle into the transsulfuration pathway (Fig. 1A). The enzyme cystathionine- β -synthase (CBS) covalently ligates Hcy to Ser by elimination of the Ser sidechain-hydroxyl and formation of a C-S bond with the Hcy-thiol, resulting in the thioether

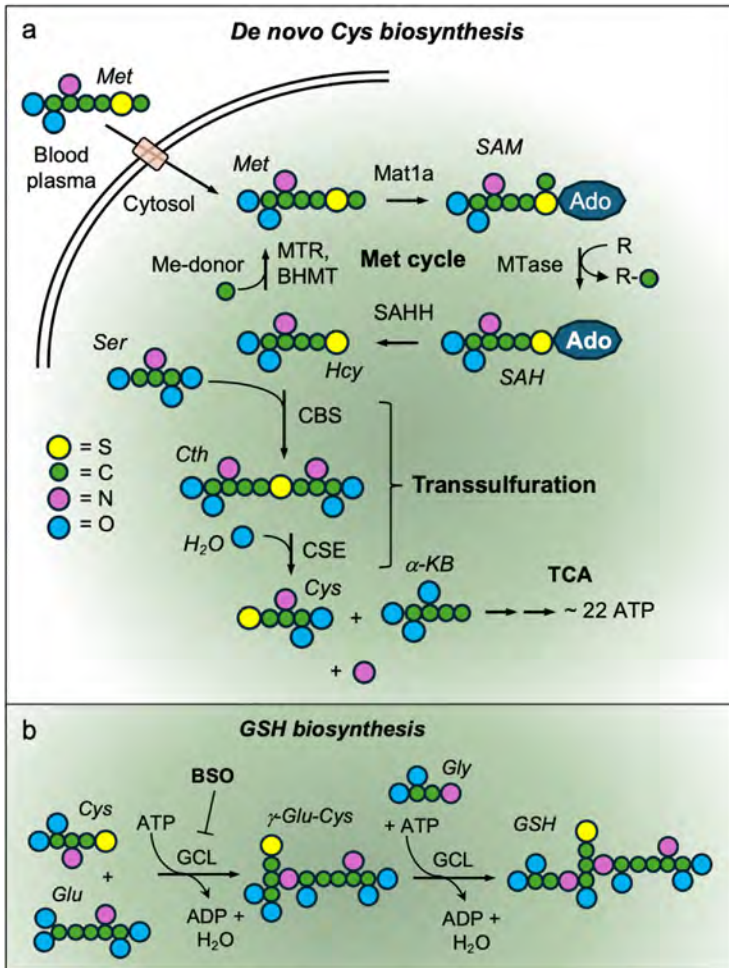


Fig. 1 Cellular synthesis of Cys and GSH. (A) Atomic ball diagram of de novo Cys synthesis. Splanchnic organs, in particular liver, synthesize Cys de novo using the S from Met, an essential amino acid, and the amino acid backbone from Ser. In this 5-step pathway Met transits the Met-cycle to Hcy, whereat rather than being re-methylated to Met, it is shunted to transsulfuration. Only the S from Met appears in Cys; most of the Met amino acid backbone eventually enters the TCA cycle where its oxidation generates an estimated 22 ATP phosphodiester bonds. Abbreviations not in text: Ado, adenosine; α-KB, α-ketobutyrate; BHMT, betaine hydroxymethyltransferase; MAT1a, Met-adenosyltransferase-1a; Me, methyl; MTase, SAM-dependent methyltransferases; MTR, 5-methyltetrahydrofolate-homocysteine methyltransferase; SAH, S-adenosylhomocysteine; SAHH, SAH-hydrolase; SAM, S-adenosylmethionine; (B) Atomic ball diagram of de novo GSH synthesis. Both Cys and GSH biosynthesis are strictly cytosolic.

cystathionine (Cth). In a second step, Cth is cleaved at the other C-S bond by cystathionine- γ -lyase (CSE, cystathionase), resulting in Cys, α -ketobutyrate, and NH_3 (Miller, Holmgren, Amer, & Schmidt, 2018). Transsulfuration consumes no NADPH and requires no disulfide reduction step (Miller et al., 2018). Only the S atom of Met is passed on to the newly synthesized Cys. Whereas the pathway consumes Met, Ser, and 3 phosphodiester bonds from ATP, the Met-derived α -KB is metabolized into succinyl-CoA, whose subsequent oxidation in the tricarboxylic acid (TCA) cycle is predicted to generate 22 ATP phosphodiester bonds (Eriksson, Prigge, Talago, Amer, & Schmidt, 2015), suggesting that if Met and Ser are not limiting, this pathway is likely to be energetically favorable (Fig. 1A). Moreover, this uniquely metazoan pathway provides an NADPH-independent source of Cys, which contributes to long-term survival in mouse livers having genetic disruptions in the disulfide reducing systems (Miller et al., 2018); shown to contribute to the sustained long-term homeostasis in mouse livers lacking both TrxR1 and GSR (Eriksson et al., 2015). It is also thought to be important in supporting hepatocyte survival under severe oxidative stress or upon toxic insults that either deplete NADPH or compromise TrxR1 and GSR in the liver (Miller & Schmidt, 2020). However, whereas transsulfuration in hepatocytes is supported by an active Met-cycle including the specialized liver-specific Met-adenosyltransferase-1a enzyme (MAT1a), and provides an estimated 30–50% of the Cys in liver, few other cell types, unless stressed, express substantial levels of both CBS and CSE (Finkelstein, 1998; Kabil, Vitvitsky, Xie, & Banerjee, 2011). A predicted rationale for transsulfuration in the liver and, to a lesser extent, in kidney, pancreas, and small intestine, is that these splanchnic organs support intermediary metabolism of Cys (Finkelstein, 1998). The ability to use either dietary disulfides or Met + Ser in order to generate Cys might help ensure reliable systemic Cys availability. Interestingly, it has been reported that cancers arising from normally transsulfuration-proficient cells (e.g., liver, pancreas, kidney) lose the expression of one or both transsulfuration enzymes during transformation, making reliance on CSSC reduction a potential acquired liability (Combs & DeNicola, 2019). Irrespective of the source of Cys in the cytosol, it will be readily available for GSH synthesis in a two-step pathway requiring glutamate, glycine and ATP (Fig. 1B).

1.1.2 Cys in cells and in circulation

Food proteins provide the dietary source of sulfur amino acids. Met is essential (must be provided by diet); Cys must either be provided from the diet or synthesized *de novo* using S derived from dietary Met (see above). Following digestion to free amino acids or (sub-antigenic) di- and oligo-

peptides in the gut lumen, with subsequent absorption by the small intestine, these nutrients enter the enterohepatic circulation (Trommelen, Tomé, & van Loon, 2021). First-pass absorption goes from the enterohepatic circulation to splanchnic tissues (small intestine, liver, and pancreas); systemic circulating amino acids and peptides have either escaped first-pass absorption, or were re-introduced to the circulation by the splanchnic tissues, in particular liver, for use in intermediary metabolism (Trommelen et al., 2021).

For Cys to be useful in intermediary metabolism, several criteria must be met: (i) The form of Cys present in circulation needs to be in a thiol- or disulfide-state; higher oxidation states cannot be used as amino acid sources in mammals (Miller & Schmidt, 2020); (ii) cells exporting Cys to circulation need to have a mechanism of producing and exporting it in that form (thiol- or disulfide-state); and (iii) A recipient cell requiring Cys needs to have a mechanism of assimilating Cys from circulation as well as recovering

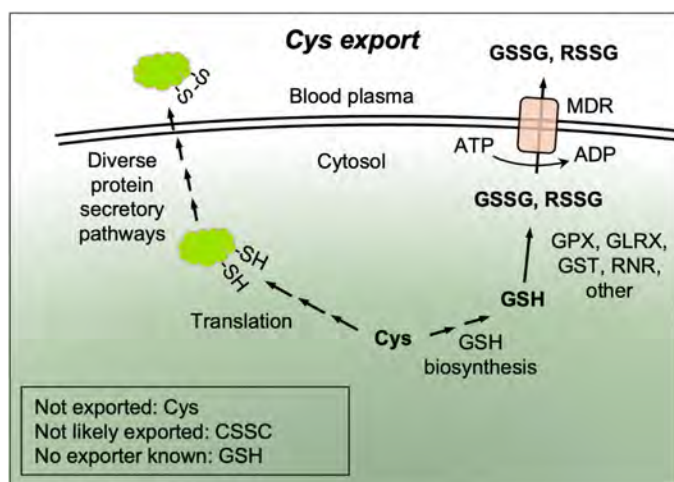


Fig. 2 Cellular Cys export routes. Diagram shows established routes of Cys export from cells. Although CSSC can, in principal, be exported by xCT in exchange for extracellular Glu, there is no known mechanism for producing CSSC in the cytosol and the exceptionally low CSSC concentration in cytosol favors CSSC import by xCT. Also, the cytotoxicity associated with disrupted Cys-catabolism indicates cells cannot likely excrete Cys nor CSSC. GSSG is exported by MDRs in ATP-dependent reactions. Although GSSG and GSSR are very low abundance in cytosol, they are catalytically generated by GPX, GLRX, GST, RNR, and other enzymes, which might drive export. GSH exists extracellularly but this could be derived from thiol-disulfide exchange between GSSG and plasma protein thiols; to date a GSH exporter has not been validated.

the free Cys in its reduced thiol form, in order to make use of it. To date, only few routes are known to meet these criteria for Cys export (Fig. 2).

The most well-established mechanisms of exporting Cys from cells are via secretion of Cys-containing proteins through secretory pathways, which can then be assimilated by pinocytosis, and via secretion of GSSG or other GSH-conjugates by ATP-binding cassette (ABC) exporters, in particular the multidrug resistance protein-1 (MDR1) (Oestreicher & Morgan, 2019).

CSSC is also found in blood plasma but its sources remain unclear. In principle, CSSC could be secreted by hepatocytes via xCT in exchange for extracellular Glu, especially if extracellular Glu and intracellular CSSC are both relatively high. However, this seems unlikely because cytosolic CSSC levels are exceptionally low in normal cells (Kabil et al., 2011), thereby likely favoring xCT to import CSSC and export Glu (Fig. 3A). Recently, a dedicated CSSC exporter, cystinosin, has been reported allowing secretion of CSSC from the lumen of intracellular vesicles into the cytosol, e.g. following pinocytosis or autophagy (Fig. 3B); however cystinosin is not reported to support extracellular excretion (Guo et al., 2022). Moreover, there is no known catalytic mechanism to generate CSSC from cytosolic Cys to allow for its excretion. Theoretically, the more oxidizing environment in the lumen of the endoplasmic reticulum (ER) might support an ER-mediated route of CSSC excretion (Reznik & Fass, 2022); however, we remain unaware of evidence supporting this route, as yet. Based on current knowledge, plasma CSSC appears to predominantly arise directly from intestinal protein digestion.

It has been proposed that free Cys in plasma can be assimilated into cells via neutral-amino acid transporters and perhaps SLC1A1 (Combs & DeNicola, 2019), although evidence supporting either yet remains unclear (Fig. 3A). Regardless, because Cys is neither stable nor abundant in plasma, such direct uptake likely contributes insignificantly to cellular Cys pools. Of interest, the transporter SLC1A5, originally named “Ala, Ser, and Cys Transporter-2” (ASCT2) is now known to be a Na^+ -dependent antiporter that imports Gln in exchange for Ser, Asn, or Thr; Cys is not a substrate but increased intracellular Cys modulates ASCT2, causing it to, instead, export Gln (Scalise, Pochini, Console, Losso, & Indiveri, 2018). The instability and low concentration of Cys in extracellular fluids is due to the oxidizing plasma environment, which favors oxidation of Cys into either CSSC or mixed disulfides; the flavoenzyme quiescin-sulfhydryl oxidase 1 (QSOX1, ~25 nM in human serum) also efficiently catalyzes oxidation of plasma thiols to disulfides with the coincident reduction of molecular oxygen to H_2O_2

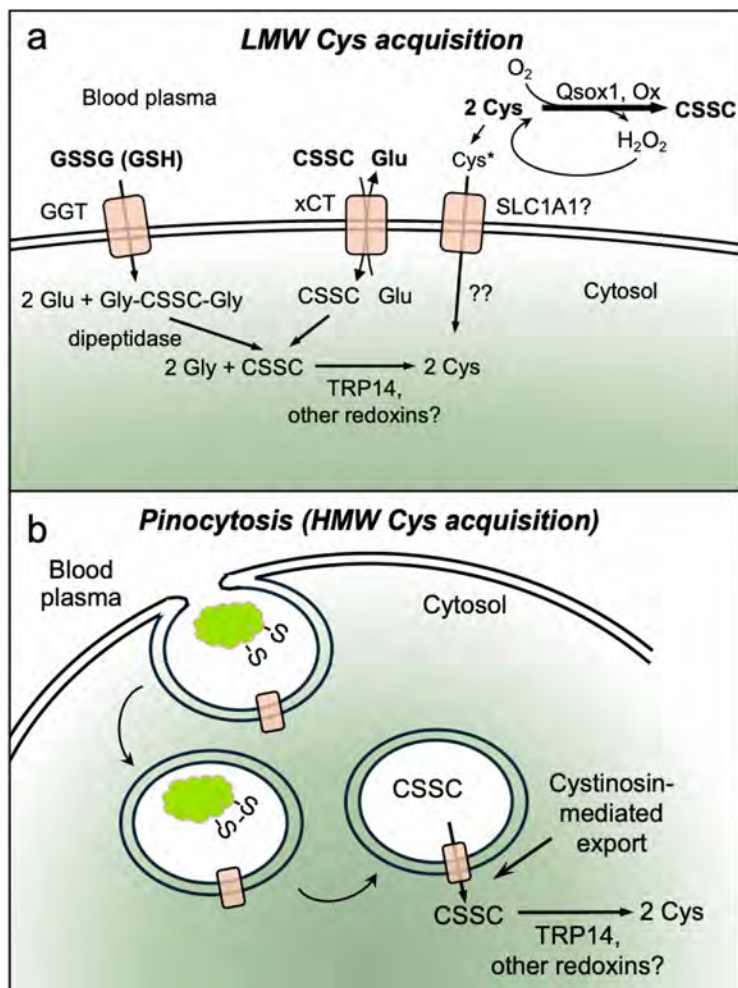


Fig. 3 Cellular Cys acquisition. (A) Cys can be acquired by assimilation of circulating GSSG or CSSC via GGT or xCT, respectively. Extracellular GSH can also be assimilated by GGT and extracellular Cys might be assimilated by neutral amino acid transporters or SLC1A1, when present. However Cys and other thiols are low-abundance in the plasma due to chemical oxidation and enzymatic oxidation via Qsox1 which, in the case of Cys, generate CSSC. Asterisk, Cys oxidation in the extracellular environment casts doubt on the physiological availability of extracellular Cys; question marks, uncertain activities. (B) Plasma proteins can be assimilated by pinocytosis. Following proteolysis to amino acids in the lysosome, the CSSC is exported into the cytosol by cystinosin. Regardless the route of entry, CSSC needs to be converted to Cys intracellularly. TRP14, fueled by NADPH-TrxR1, appears to be the predominant CSSC reductase. (A) Assimilation of LMW sources. (B) Assimilation of HMW sources.

(Fig. 3A) (Israel, Jiang, Gannon, & Thorpe, 2014). Moreover, although dietary sources might release some reduced Cys into the enterohepatic circulation, there is no means for cells to excrete reduced Cys into plasma or interstitial fluids to support its use in intermediary metabolism. No Cys exporter is known, and the cytotoxic phenotype associated with livers or cells having Cys catabolism deficiencies indicate that Cys, itself, cannot be exported from cells. Rather, elimination of excess intracellular Cys proceeds by Cys-dioxygenase (CDO)-catalyzed oxidation to Cys-sulfinate, a non-reversible oxidation state, followed by metabolism to hypotaurine (Htau) and then taurine (Tau) prior to final excretion (Stipanuk & Ueki, 2011; Stipanuk et al., 2006). Thus, it is evident that tissues contributing to Cys intermediary metabolism excrete forms other than free Cys (for more discussions on this topic, see below).

1.2 GSH in cells and in circulation

GSH is found across all phyla; in most cells it is the most abundant low molecular weight (LMW) thiol, present in the cytosol at a steady state concentration of 3–10 mM (Oestreicher & Morgan, 2019; Stenersen, Kobro, Bjerke, & Arend, 1987; Thuillier et al., 2011). By contrast, Cys concentrations are only 20–100 μ M (Stipanuk et al., 2006), i.e. $\sim$ 0.2–3% that of GSH. Neither GSH nor Cys are particularly reactive with cellular oxidants in direct reactions (see above) (Winterbourn & Metodiewa, 1999); however, unlike free Cys, GSH can support GPXs to drive reduction of peroxides (Brigelius-Flohe & Maiorino, 2013; Toppo et al., 2009). Thus, whereas it is unlikely that Cys could replace GSH for protecting cells against oxidative stress, it is plausible that Cys restriction might lead to secondary GSH depletion. Consistent with this, it has been suggested that cytosolic Cys depletion, by leading to GSH depletion, hinders the flow of reducing power via GSH to GPXs (Chiang et al., 2022).

One of the most surprising findings about GSH came with the discovery nearly 50 years ago of the drug buthionine sulfoximine (BSO), which effectively inhibits Glu-Cys ligase (GCL), the first committed and rate-limiting step in GSH biosynthesis (Fig. 1B) (Griffith & Meister, 1979b; Lu, 2013). BSO treatment of cells results in a precipitous drop in GSH levels, often to a few percent of the normal level; importantly, usually without causing cell death (Griffith & Meister, 1979a). Indeed, long-term high-dose BSO treatment in mice results in severe systemic GSH depletion throughout most organs, yet is well tolerated (Griffith & Meister, 1979a). Also, BSO is approved for use in human patients, wherein it has been used

over the past 40 years (Green et al., 1984). More recently, genetically engineered mouse models were developed with either attenuated or disrupted GCL activity in specific cell types. Consistent with the mild consequences of pharmacologic inhibition of GCL by BSO, however, reported phenotypes do not include catastrophic cell death (Botta et al., 2008; Franklin et al., 2009; Harris et al., 2015; Kurniawan et al., 2020; Nakamura et al., 2011). Much clearly remains to be understood about how diverse cells as well as complex multicellular organisms tolerate the loss of this normally abundant thiol—nearly universally, GSH depletion is benign. This should be considered when evaluating models wherein a modest disruption in synthesis rates or slightly lowered steady state levels of GSH might induce oxidative stress or cell death (Lee & Roh, 2022).

In the cytosol, the GSH:GSSG ratio is highly favored towards the reduced state and has been estimated to be between 100:1 and 1000:1 (Morgan et al., 2013), or even as much as up to 40,000:1 (Morgan, Sobotta, & Dick, 2011); by contrast, in blood plasma the ratio is ~20:1 (Jones et al., 2000). We are aware of no reports of blood plasma having measurable levels of NADPH, an NADPH-generating system, nor a primary NADPH-dependent disulfide reductase system. Thus, plasma thiols must arise either from cytosolic disulfide reducing power or from primary protein digestion in the gut (see above). In combination, this suggests that cells can excrete reduced GSH; however, a mechanism for this remains to be found (Fig. 2). Previous reports of organic-anion transporter proteins (OATP), MDR1, or other transporters excreting GSH, have more recently been experimentally unsupported or refuted (Mahagita, Grassl, Piyachaturawat, & Ballatori, 2007). Until a GSH exporter is validated, the only known sources of plasma thiols are thus either dying cells or other mechanisms bluntly releasing all or parts of their cytosolic contents, secreted proteins with reduced Cys, or from primary digestion of protein in the gut. However, we should not exclude that a dedicated GSH exporter might exist and be identified in the future. Indeed, although the machinery for GSH synthesis exists only in the cell cytosol, GSH is found also in organelles, including mitochondria. Only very recently has a putative transporter been identified for moving GSH into the mitochondria (Liu et al., 2023). Inside the mitochondria, mitochondrial GSR recycles GSSG within the mitochondrial matrix independent of the cytosolic GSH/GSSG pool (Kelner & Montoya, 2000).

Extracellular GSSG can undergo disulfide-thiol exchange with plasma thiols—in particular with protein thiols on secreted proteins—thereby liberating one reduced GSH moiety while also glutathionylating the protein thiol

with which it may have reacted. As such, extracellular GSH might arise not from GSH excretion, but from GSSG excretion followed by extracellular disulfide-thiol exchange. By this mechanism, however, the cytosol of the cell exporting a protein with a reduced Cys must have supplied the reducing power for converting $\text{GSSG} + \text{protein-SH}$ into $\text{protein-SSG} + \text{GSH}$. Thus, disulfide-thiol exchange reactions like this cannot generate a net increase in free thiols, but can move the reduced thiol group between cells and onto a different molecular species.

1.3 GSSG in cells and in circulation

GSSG is found in blood plasma and, importantly, there are known routes of export of GSSG from hepatocytes. Like CSSC, GSSG is not abundant in cytosol (at least 1:100–1:1000 ratio for GSH:GSSG, see above); however unlike for CSSC, hepatocytes have numerous efficient enzymes for oxidizing reduced GSH into either GSSG or different mixed disulfide glutathionylated molecular species (GSSR). These enzymes include glutaredoxins (GLRX), GPXs, glutathione-S-transferases (GSTs), ribonucleotide reductase (RNR), and others (Cassier-Chauvat, Marceau, Farci, Ouchane, & Chauvat, 2023). The production of disulfides formed upon the catalytic cycles of these enzymes will be counteracted by the disulfide reductase systems; yet, having catalytic machineries producing GSSG or GSSR will, in contrast to the case for CSSC, make it plausible that these products can also be excreted from cells as a result of their continuous production (Fig. 2). Furthermore, unlike for either GSH or CSSC, there are well-characterized effective exporters for moving either GSSG or GSSR to the blood plasma. Several Multi-Drug Resistance (MDR, also called ATP-Binding Cassette, ABC) exporters secrete glutathionylated substrates out of cells (Van Der Kolk, Vellenga, Muller, & de Vries, 1999). Whereas their substrate specificities are low, one key excreted conjugate is GSSG (Fig. 2) (Ballatori, Krance, Marchan, & Hammond, 2009; Cole & Deeley, 2006). Indeed, effective export of GSSG might contribute to the maintenance of a highly reduced steady-state ratio of GSH:GSSG in cells or in subcellular compartments also under conditions wherein disulfide reduction is compromised (Eriksson et al., 2015; Kojer et al., 2012; Miller & Schmidt, 2020; Morgan et al., 2013).

There is also a well characterized mechanism for cells to acquire Cys from circulating GSSG or GSH (Fig. 3A). The ubiquitous outer membrane protein γ -glutamyl-transpeptidase (GGT) takes GSSG or GSH from the blood plasma, cleaves the γ -glutamyl bond and thereby generates, in the case of GSSG, 2 Glu + diglycylcystine (Gly-CSSC-Gly). The 2 Glu and the

Gly-CSSC-Gly are then imported into the cell, wherein the peptide bonds between Gly and CSSC are cleaved by cytosolic dipeptidases (Kobayashi, Ikeda, Shigeno, Konno, & Fujii, 2020; Tate & Meister, 1981). The net result is import of CSSC, 2 Gly and 2 Glu; the CSSC is then reduced by the cytosolic disulfide reductase systems to give 2 Cys. In the case of GSH, the products acquired by the cell are instead Glu, Gly, and Cys. Of note, unlike the situation for xCT, which must export Glu to obtain CSSC, GGT imports CSSC, 2 Glu and 2 Gly, thus providing all three amino acids that are needed for GSH biosynthesis in the cell (Anderson & Meister, 1980; Deneke & Fanburg, 1989; Griffith & Meister, 1979a; Griffith, Bridges, & Meister, 1981; Meister, Griffith, Novogrodsky, & Tate, 1979). Hence, GSSG appears to be a favorable molecule for mediating the intermediary metabolism of Cys during export, import, and utilization (Fig. 3A).

1.4 Other possible sources of cytosolic cys

A recent study on pancreatic ductal adenocarcinoma suggested that non-cancer cells in the tumor microenvironment (TME) might directly transfer Cys or CSSC to the cancer cell cytosol in a cell-to-cell transfer reaction (Meira et al., 2021). The mechanisms, cell-type compatibility, and proportional contributions of this system remain to be resolved, but it would be consistent with cytosolic exchange between cancer and non-cancer TME cells, e.g., via desmosomes or microvesicle-mediated intercellular transport.

1.5 Roles of NADPH, the NADPH-dependent disulfide reductase systems, and redoxins in CSSC reduction

NADPH is the “universal currency” for anabolic reduction reactions (Miller et al., 2018). For disulfide reduction, including the reduction of CSSC $\rightarrow$ 2 Cys, nearly all reducing power is transited to the process from NADPH by one of mammalian cells only two families of NADPH-dependent disulfide reductases—the GSR and TrxR families (Fig. 4) (Arner & Holmgren, 2000; Holmgren, 2000; Miller et al., 2018). Cells and mouse model systems genetically lacking either GSR or TrxR1 are viable and robust (Bondareva et al., 2007; Jakupoglu et al., 2005; Mandal et al., 2010; Rogers, Tamura, Rogers, Welty, & Hansen, 2004; Suvorova et al., 2009), and TrxR1 inhibitors, including auranofin (AFN) and the more specific TrxR1-inhibitor-1 (TRi-1) are well-tolerated by healthy cells, animals, and for AFN humans (Arnér, 2009b; Sabatier et al., 2021; Stafford et al., 2018), indicating that loss of either TrxR1 or GSR does not cause either a Cys-insufficiency nor catastrophic cell death in any of these situations.

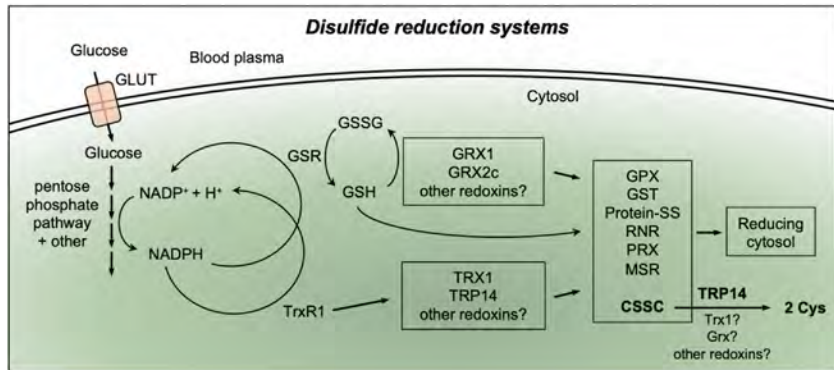


Fig. 4 Cytosolic disulfide reductase systems. Generation of cytosolic disulfide reducing power uses NADPH, generated largely from oxidation of glucose in the Pentose Phosphate Pathway. TrxR1 and GSR are the only two enzymes that can use NADPH to drive reduction of a disulfide bond. TrxR1 reduces Trx1-, TRP14- and other cytosolic TRX-disulfides; GSR reduces GSSG. GSH and reduced redoxins sustain the chemically reducing status of the cytosol and drive specific other reduction reactions. Redoxins reduce CSSC → 2 Cys, with TRP14 being the predominant CSSC reductase.

Neither GSR nor TrxR1 can directly reduce CSSC (Arner & Holmgren, 2000; Arner & Holmgren, 2006; Arnér, 2009b). Instead, reducing power must be transferred from these “primary reductases” via their downstream redoxin-family members of Trx-fold proteins (GLRXs, TRXs, or more distantly related family members) that finally would be able to reduce the CSSC disulfide bond. The details of how CSSC is reduced in cells remains unclear; however not all redoxins show equal reactivity with CSSC (Fig. 4). The TRX-related protein of 14 kDa (TRP14, encoded by *TXNDC17*), although unable to reduce disulfides in many protein-disulfides that are substrates for TRX1, shows at least a 5-fold greater reactivity with CSSC than does TRX1 (Eriksson et al., 2015; Pader et al., 2014). TRP14 is ubiquitously expressed and conserved across phyla, suggesting that TRP14 might be the predominant CSSC reductase in vivo, which we have recently confirmed (Marti-Andres, 2024). Notwithstanding, it is noteworthy that TRP14-null cells and mice are viable (Doka et al., 2016; Doka et al., 2020), showing that loss of TRP14 causes neither Cys-insufficiency nor catastrophic cell death. Clearly there must be other ubiquitous processes that cells can use to obtain sufficient Cys in the absence of TRP14. Even more dramatically, we have found that livers lacking both TrxR1 and GSR “TR/GR-null” (Eriksson et al., 2015; McLoughlin et al., 2019), TRX1/TR/GR-null

livers (McLoughlin et al., 2019; Prigge et al., 2017), or even TRP14/TRX1/TR/GR-null livers (EES, unpublished data) remain viable, indicating that liver hepatocytes are able to obtain Cys in the complete absence of cytosolic NADPH-dependent disulfide reductase systems (Miller & Schmidt, 2019). Sustained hepatic Cys levels in some of these disulfide reductase-deficient liver models was also verified by metabolomic measurements (McLoughlin et al., 2019). These observations suggest that *de novo* synthesis of Cys can fully support liver homeostasis in the absence of CSSC reduction. Further supporting the importance of *de novo* Cys synthesis in these systems, inhibition of the transsulfuration enzyme CSE becomes highly cytotoxic to the reductase-deficient, but not to healthy wildtype livers (Eriksson et al., 2015). These diverse reductase-compromised mouse and cell models show that some, or most, normal and healthy cell types can thrive under conditions of limited or even fully inhibited CSSC reduction capacity (Eriksson et al., 2015; Miller & Schmidt, 2020; Pader et al., 2014).



2. Ferroptosis

In 2003, as a part of a large screen for small molecules that would kill cancer cells but not normal control cells, a novel compound named “Erastin” (for Eradicator of RAS and small-T antigen-expressing cells) was identified that induced a non-apoptotic type of cell death, particularly in cells that express mutant HRAS and small-T antigen (Dolma, Lessnick, Hahn, & Stockwell, 2003). In 2012, it was found that Erastin-induced cell death in HRAS mutant cancer cells could be antagonized by iron-chelators (Dixon et al., 2012). Based on the iron-association, this form of cell death, which was also found to be related to lipid peroxidation, was entitled “ferroptosis” (Dixon et al., 2012). Experimentally, ferroptosis can thus be defined as the type of cell death that is prevented by iron chelators (e.g., deferiprone, deferoxamine) or small lipophilic antioxidants (e.g., ferrostatin, liproxstatin) (Jiang, Stockwell, & Conrad, 2021; Yan et al., 2021).

In the following sections we will discuss the particular features and observations relating to experimental ferroptosis studies, relate these findings to the metabolism of Cys and GSH, also considering cytosolic reducing enzymes, and we will suggest where further scrutiny might be warranted due to a number of yet outstanding questions with regards to the mechanisms involved (Fig. 5).

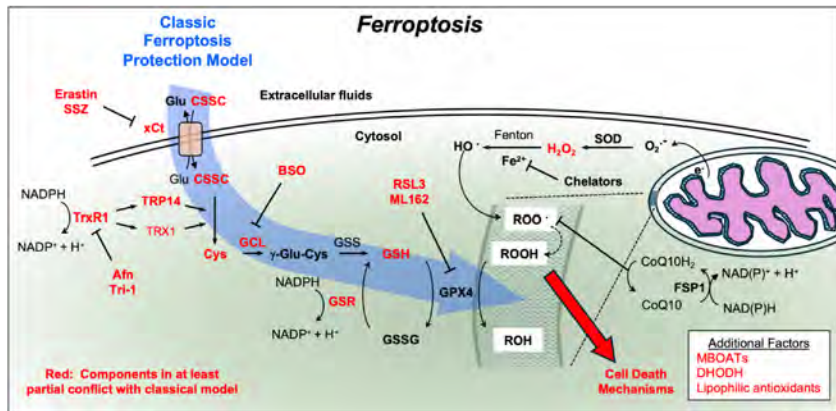


Fig. 5 Ferroptosis mechanisms and outstanding questions remaining to be answered. With ferroptosis defined as a cell death triggered by iron-dependent lipid peroxidation, the roles of Fe^{2+} , iron-chelators, $\text{HO}^\cdot$, and lipid-radicals/lipid peroxidation are clear, as, very likely, is the role of GPX4, as a lipid-peroxidase (center of diagram) and FSP1 + CoQ10 shown to similarly repair lipid radical species. However, because ferroptosis was identified based on data from treating cultured cells with small molecules, including erastin or SSZ, and described as being a non-apoptotic cell death, the classical model of cellular protection against ferroptosis (blue arrow) has many components for which conflicting experimental findings are now known (components in red font, and likely others). [Table 1](#) outlines some of the outstanding questions related to these components.

2.1 Erastin

Erastin (PubChemID: 11214940) is a quinazoline compound that inhibits mitochondrial voltage gated anion channels (VDACs) and was subsequently proposed as an irreversible inhibitor of xCT. In the latter case, brief exposure of cells to erastin was sufficient to see a strong inhibitory effect on CSSC uptake, although it could not be demonstrated if or how erastin directly might have modified any component of xCT ([Sato et al., 2018](#)). Interestingly, a more recent study suggested that erastin's inhibition of VDACs directly leads to oxidative stress and cell death, without involvement of inhibitory effects on CSSC uptake ([DeHart et al., 2018](#)). Knockdown of either TRX-domain protein 12 (TXNDC12) ([Yu, Zhu, Wang, & Jiang, 2023](#)) or GLRX5 ([Lee, You, Shin, & Roh, 2020](#)) have furthermore been shown to sensitize cells to ferroptosis as triggered by erastin, showing how the different cellular redox systems are functionally linked and that the exact molecular mechanisms of erastin leading to ferroptosis are yet not fully understood. In this section, we aim to specifically scrutinize a few aspects of erastin mechanisms of action that warrant further studies.

2.1.1 On- and off-target activities of erastin

With VDACs and xCT having been implicated as targets of erastin, as discussed above, one should consider that the compound, as for all small molecules, might also have other protein targets in cells. In recent non-biased proteome analyses performed with crude cell lysates, erastin was found to clearly affect 3061 proteins, while another ferroptosis-inducing compound ML210 impacted 2828 proteins, and 2550 proteins were altered upon treatment with BSO; among these proteins as many as 2278 were altered in all three experiments (Kudryashova et al., 2023). Such data suggest that proteome changes during ferroptosis are far-reaching, and perhaps surprisingly reproducible, but the actual protein targets of the compounds used in these cases to trigger the cell death are not identified through such analyses. A more direct method evaluates shifts in thermostability of specific proteins upon treatment of cells with a specific compound, assuming that when a compound binds a specific protein the stability of that protein becomes altered. Another method to study target engagement employs alkyne-derivatives and click chemistry to directly link a compound in question to its plausible protein targets. Using these approaches to detect potential protein targets of erastin, a study recently found more than 800 potential erastin targets in cells, of which five were identified with higher confidence and further validated in subsequent experiments. These five additional erastin targets were identified as monoacylglycerol lipase ABHD6, the epoxide hydrolase 1 EPHX1, the mitochondrial-processing peptidase subunit α -PMPCA, the puromycin-sensitive aminopeptidase NPEPPS, and the saccharopine dehydrogenase-like oxidoreductase SCCPDH (Li, Liu, Wang, & Wang, 2024). It remains unknown how targeting any of these proteins, in addition to VDACs and xCT, might contribute to the cellular or pharmacological effects of erastin.

2.1.2 Sulfasalazine—similar results as erastin?

Sulfasalazine (SSZ) is, citing its PubChem entry “an azobenzene consisting of diphenyldiazene having a carboxy substituent at the 4-position, a hydroxy substituent at the 3-position and a 2-pyridylaminosulphonyl substituent at the 4'-position, known to act as a non-steroidal anti-inflammatory drug, an antiinfective agent, a gastrointestinal drug, a glutathione transferase inhibitor, a drug allergen, and a ferroptosis inducer” (PubChem ID: 5339). SSZ has been used since the 1950s as an anti-inflammatory drug and is still proscribed for use in treatment of ulcerative colitis and rheumatoid arthritis (<https://www.drugs.com/sulfasalazine.html>). Although well tolerated in animals and

patients, in cell cultures, the death-triggering effects of SSZ were suggested, similarly to erastin, to be due to direct inhibition of xCT and thus inhibition of the cellular uptake of cystine (Ogihara et al., 2019). Also like erastin, it was shown that iron plays a role in SSZ-triggered cell death, hence pointing to ferroptosis (Liu et al., 2022). One study suggested that these effects could, in turn, be modulated by the expression level of the estrogen receptor (Yu et al., 2019). Early proteomics analyses using 2-D gel electrophoresis combined with mass spectrometry, showed that SSZ affected the levels of hundreds of proteins in treated cells, with a profile compatible with its anti-inflammatory properties (Endo et al., 2014). In more recent proteome analyses, SSZ was again shown to have wide-ranging effects and also triggered significant redox-related changes in many proteins; interestingly in the opposite direction as occurred with treatment of the cells with H₂O₂ in an attempt to directly trigger oxidative stress (Sun et al., 2019). In the context of the present review, we here wish to conclude that both erastin and SSZ can trigger ferroptosis coincident with their inhibition of xCT, but the cellular effects of both compounds are wide-ranging. We suggest that a mere inhibition of Cys uptake is unlikely to be the sole mechanism of action of these compounds.

2.1.3 Impacts of erastin or SSZ on cellular cystine uptake and Cys or GSH levels

Both erastin and SSZ are usually suggested to induce ferroptosis through inhibition of cystine uptake and, thus, by compromising cellular Cys availability. What is the evidence for this? One important original study showing these effects reported that erastin, sulfasalazine and the kinase inhibitor sorafenib, all triggered typical ferroptotic cell death; this was also coupled with blockage of cystine uptake and Glu release. Furthermore, similar effects on Glu release were induced by silencing the SLC7A11 component of xCT with siRNAs, but not by silencing the large-neutral amino acid transporter SLC7A5 (LAT1) (Dixon et al., 2014). What could the arguments be that other mechanisms of action would be involved? For one, whole-body SLC7A11 knockout mice are viable (Chen et al., 2023; Hamashima et al., 2017; Zhang et al., 2022b), suggesting that merely inhibition of xCT cannot likely be sufficient to trigger ferroptosis in any critical cell types in the body, at least in normal conditions. Second, cytotoxicity of sulfasalazine was found to depend upon ASCT2-dependent glutamine (Gln) uptake and Glu dehydrogenase (GLUD)-mediated α -ketoglutarate (α -KG) production, suggesting that effects on glutaminolysis and not on Cys uptake are involved (Okazaki et al., 2019). Third,

could perhaps an additional reason for erastin- and sulfasalazine-mediated inhibition of Cys uptake be that these compounds trigger NRF2 activation, driving *de novo* Cys biosynthesis by transsulfuration (see above), thus blocking uptake of cystine from the extracellular space by competitive inhibition from intercellularly synthesized cysteine? We propose that this is theoretically possible, although having no definite evidence for it to be the case. We merely conclude that it might be wise not to consider erastin or sulfasalazine as “pure” or “single-acting” inhibitors of xCT, as is unfortunately often done in current ferroptosis literature.

2.2 Protection/prevention against ferroptosis by iron-chelators

With the definition of ferroptosis being a type of cell death blocked by iron chelators (see above), it becomes a truism that iron chelators prevent ferroptosis. But what are the mechanisms of action for the protective effects of iron chelators? A likely explanation should be that chelation of free iron cations prevents their reactivity with H_2O_2 through the well-known Fenton reaction producing the very reactive hydroxyl radical (Merkofer, Kissner, Hider, Brunk, & Koppenol, 2006). If occurring close to a cell membrane, this could produce the lipid peroxides seen in ferroptosis (Gutteridge, 1986), although the exact molecular species involved in the iron-promoted lipid peroxidation may be more complex (Minotti & Aust, 1989). It should be noted that, chemically, copper ions can partake in the same type of peroxidation reactions as iron and, indeed, the term “cuproptosis” has also been coined for the copper-triggered cell death that would otherwise likely be highly analogous to ferroptosis (Chen, Min, & Wang, 2022). In this context, it should be emphasized that cell death triggered by excessive oxidative stress has long been known, described, and discussed (Orrenius, Gogvadze, & Zhivotovsky, 2007; Sies, Berndt, & Jones, 2017; Sies, 2020; Zhivotovsky & Nicotera, 2020). Thus, both ferroptosis and cuproptosis are likely to be forms of cell death triggered by oxidative stress that specifically involve Fenton-chemistry and lipid peroxidation.

2.3 Association with lipid peroxidation

Lipids, especially polyunsaturated fatty acids including phospholipids and other species, can easily be further oxidized with several enzymatic pathways leading to their oxidation through lipoxygenases, such as during metabolism of arachidonic acid as part of the synthesis of leukotrienes, prostaglandins or thromboxane species (Kuhn, Banthiya, & van Leyen, 2015; Radmark, Werz, Steinhilber, & Samuelsson, 2015; Ricciotti & FitzGerald, 2011). Lipids may

also be oxidized in a non-regulated manner through the Fenton reaction-like free-radical mechanisms mentioned above. It has furthermore been proposed that 15-lipoxygenases might specifically produce lipid peroxides as a mechanism to trigger enzymatically regulated initiation of ferroptosis (Bayir et al., 2020; Stoyanovsky et al., 2019). Irrespective of the possible distinctions between “regulated” or “non-regulated” iron-triggered lipid peroxidation, it seems clear that the adverse accumulation of lipid peroxides, presumably mainly in cellular membranes, is the actual event leading to the type of cell death that defines ferroptosis. Using mass spectrometry approaches this has been clearly shown, mainly demonstrating accumulation of phospholipid hydroperoxide species during ferroptosis (Sparvero et al., 2021; Wiernicki et al., 2020), perhaps with specific sites, such as the mitochondrial membrane, being more important than other (Lyamzaev, Panteleeva, Simonyan, Avetisyan, & Chernyak, 2023).



3. Role of GPX4 in ferroptosis

The notion that GPX4 can protect cells from cell death through ferroptosis due to its capacity to reduce lipid peroxides and thereby prevent their accumulation, is widely acknowledged. Still, there are several unanswered questions regarding the exact mechanisms of the links between GPX4 and ferroptosis, as was also previously discussed at detail by Matilde Maiorino, Marcus Conrad and Fulvio Ursini (Maiorino, Conrad, & Ursini, 2018; Ursini & Maiorino, 2020). It is important to note that GPX4 is not the only suppressor of ferroptosis in cells that may act through reduction, directly or indirectly, of lipid peroxides, or prevent their accumulation (see Section 1.6, below). This may help to explain why or how the impact of GPX4 seems to be important in relation to ferroptosis in only certain cell types or growth conditions, and not in others. Here we shall specifically point out a few questions regarding the roles of GPX4 in relation to ferroptosis where we see a clear need for further studies.

3.1 Kinetics and substrate specificities of GPX4

GPX4 was originally discovered in 1982 by Ursini and coworkers as an enzyme reducing phosphatidylcholine hydroperoxide using GSH and in 1982 they showed that the enzyme was a selenoprotein (Ursini, Maiorino, & Gregolin, 1985). Later it was shown that GPX4 specifically associates with phospholipids in membranes to facilitate the reduction of phospholipid hydroperoxides, and that cardiolipin is a preferred binding partner

(Cozza et al., 2017). Interestingly, an Arginine-to-Histidine mutation at residue 152 (R152H), found in cases of Sedaghatian-type Spondylometaphyseal Dysplasia (SSMD) that encompasses severe neurodevelopmental dysfunction in human, mainly leads to dissociation of GPX4 from cardiolipin rather than affecting its catalytic turnover, as such (Roveri et al., 2023). Comparisons of the substrate specificities and enzyme activities of recombinant forms of GPX1, GPX2 and GPX4 side-by-side under identical conditions recently confirmed that GPX4 is the only of these three enzymes that can reduce phosphatidylcholine hydroperoxides; GPX1 is far more efficient than the other GPX isoenzymes in reduction of other peroxides, including H_2O_2 , cumene hydroperoxide, tertbutyl hydroperoxide or other fatty acid hydroperoxides including those derived from linoleic acid, arachidonic acid or eicosapentaenoic acid (Schwarz et al., 2023). Thus, GPX4 seems to be particularly and uniquely specialized for reduction of membrane-associated phospholipid hydroperoxides, which underlies its important role in preventing the accumulation of toxic levels of these molecular species in cells.

3.2 Results that question the exact mechanisms of GPX4 targeting in ferroptosis

RSL3 (PubChem ID: 1750826) was discovered by the Stockwell group in 2008 in a screen for compounds being specifically toxic towards tumor cells harboring mutated small GTPases, and was thus named RSL3 for “RAS-selective lethal” (Yang & Stockwell, 2008). It was later used together with erastin in the study published in 2012 naming ferroptosis (Dixon et al., 2012). In 2014, the group identified GPX4 as the target of RSL3 using chemoproteomics with an RSL3-derivative for identification of cellular protein targets; that study also showed that erastin treatment caused cellular GSH depletion (Yang et al., 2014). These findings, together with the cellular effects of additional ferroptosis inducers, suggested that GPX4 was the target of RSL3 and thus a key suppressor of ferroptosis. The study also showed that RAS-transformed cells were more sensitive to GSH depletion using BSO than control cells, that overexpression or knockdown of GPX4 prevented or accentuated ferroptosis, respectively, and that this cell death was associated with increased lipid peroxidation (Yang et al., 2014). It was thus a surprise when the group of Ursini and coworkers could not find any inhibition of purified GPX4 using RSL3, while if they included cell lysate and/or the 14-3-3 protein under reducing conditions, inhibition could be detected (Vuckovic et al., 2020).

Our own group (ESJA) confirmed Ursini's results using recombinant GPX4 selenoprotein by showing that RSL3 is not a direct inhibitor of the enzyme, but we were even more surprised when we discovered that RSL3 is, instead, a direct inhibitor of TrxR1 both in recombinant form and in a cellular context (Cheff et al., 2023). A similar result was found for another ferroptosis inducing compound, ML162 (Cheff et al., 2023). Noteworthy, while both RSL3 and ML162 triggered typical ferroptosis in cells when used at very low nM-range concentrations; another more selective inhibitor of TrxR1, TRi-1, did not (Sabatier et al., 2021; Stafford et al., 2018). RSL3, ML162 and ML210 (the latter of which could not directly inhibit either GPX4 or TrxR1) all triggered a cell death blocked by ferrostatin or iron chelators, while the cell death triggered by TRi-1 at low μM range was not affected by ferrostatin or iron chelators. When ferrostatin was used to protect cells treated with RSL3, the cytotoxicity of RSL3 was blunted and instead induced cell death at higher concentrations, very closely matching that obtained with TRi-1 (Cheff et al., 2023). Interestingly, we also observed that RSL3, ML162 and ML210 all affected the migration of GPX4 in SDS-PAGE, suggestive of downstream indirect effects on GPX4 when the ferroptosis inducing compounds were used in cells (Cheff et al., 2023).

Very recently the group of Marcus Conrad addressed the question regarding the possible RSL3-GPX4 interaction using HEK293 cells transfected with Streptavidin-tagged GPX4, to enable affinity purification of the enzyme with or without treatment with ferroptosis inducing compounds (Nakamura et al., 2024). They confirmed the altered GPX4 migration in SDS-PAGE after treatment with RSL3, and they show inhibition of the enzyme activity of the purified enzyme from RSL3-treated cells or upon treatment in vitro. However, they used incubation of cells with 10 μM RSL3 (1000-fold higher concentrations than those triggering ferroptosis) or incubation of purified enzyme using 0–1 μM RSL3 in vitro at 37 °C for 30 min, resulting in approximately 60% inhibition of GPX4 activity during an additional 30 min incubation, as judged from the quantitation of remaining phosphatidylcholine peroxide (Nakamura et al., 2024). We judge those results to display a rather weak inhibition of GPX4 by RSL3. At this stage, we wish to conclude that it seems clear that ferroptosis inducing compounds, including RSL3, ML162 and ML210, somehow affect cellular GPX4 and its migration in subsequent SDS-PAGE analyses, but that could be due to indirect mechanisms. The Conrad group posits that mammalian cell-specific posttranslational modifications of GPX4 might be required for RSL3-mediated inhibition of

GPX4, which would explain why the protein affinity purified from mammalian cells is inhibited by RSL3 whereas the *E. coli*-expressed recombinant selenoprotein is not (Cheff et al., 2023). Clearly additional studies will be needed to resolve whether RSL3 interacts directly with GPX4 in vivo, whether that is dependent on mammalian posttranslational modifications, and indeed the exact molecular mechanisms of action by which RSL3 induces ferroptosis.

Only a few selenoprotein-encoding complete genetic knockout models cause embryonic lethality in mice, including *Txnrd1* encoding cytosolic TrxR1 (Bondareva et al., 2007; Jakupoglu et al., 2005), *Txnrd2* encoding mitochondrial TrxR2 (Conrad et al., 2004), and *Gpx4* encoding both the cytosolic, nuclear and mitochondrial forms of GPX4 (Seiler et al., 2008; Yant et al., 2003). A similar phenotype is seen with expression of mutant GPX4 variants with the catalytic Sec residue mutated to either inactive Ala or Ser (Ingold et al., 2015), clearly showing that it is a lack of GPX4 catalytic activity that leads to embryonic lethality. Interestingly, targeted deletion of only the nuclear form of GPX4 yields no overt phenotype and the only effect that could be discovered was that of increased sperm abnormalities (Conrad et al., 2005), which likely relates to the fact that GPX4 is also a moonlighting protein converted during spermatogenesis into a structural component of sperm (Ursini et al., 1999). When mitochondrial GPX4 was deleted, this gave a similar effect with a lack of overt phenotype but abnormal sperm maturation, thus suggesting that the cytosolic form of GPX4 is the essential form (Schneider et al., 2009). Nonetheless it remained unclear whether lack of cytosolic GPX4 was embryonically lethal due to increased ferroptosis, or whether other mechanisms lay behind the lethality upon its deletion. It could be reminiscent of the genetic deletion of *Txnrd1*, which yielded early embryonic lethality without signs of tissue damage due to increased oxidative stress and, instead, exhibited a lack of formation of mesoderm indicating distorted intracellular differentiation or signaling programs (Bondareva et al., 2007; Dagnell, Schmidt, & Arner, 2018).

Further addressing the question of the reasons for lethality upon GPX4 deletions, Marcus Conrad and coworkers replaced GPX4 in a mouse model with a Sec-to-Cys “knock-in” variant of the enzyme (*Gpx4^{cys}* allele), which displays a much lower turnover with hydroperoxide substrates yet is not totally inactive. With an inbred C57Bl/6J mouse strain background, crossing parents heterozygous for this knock-in (*Gpx4^{sec/cys}*), homozygous Sec-Cys mice (*Gpx4^{cys/cys}*), unlike mice

homozygous for either the null-mutation ($Gpx4^{-/-}$) or a totally catalytically inactive Sec-Ser knock-in ($Gpx4^{ser/ser}$), which each exhibit 100% embryonic lethality by embryonic-day 7.5 (E7.5) (Ingold et al., 2015), died not until after organogenesis (E11.5–12.5) (Ingold et al., 2018). This finding was further studied with a Tamoxifen-induced conditional allele-switch strategy in adult mice, wherein most cells in the mice switched from being heterozygous across a functionally wildtype floxed allele, ($Gpx4^{fl}$) (Friedmann Angeli et al., 2014) for either the Sec-to-Cys or the Sec-to-Ser allele ($Gpx4^{fl/cys}$ or $Gpx4^{fl/ser}$, respectively), to being hemizygous for only the Sec-to-Cys or the Sec-to-Ser allele ($Gpx4^{-/cys}$ or $Gpx4^{-/ser}$, respectively). That strategy showed that the induced adult-onset $Gpx4^{-/ser}$ mice, like induced adult-onset $Gpx4^{-/-}$ mice, died within 11 days of Tamoxifen-induced allelic conversion; both showing similar renal pathology. By contrast, the induced adult-onset $Gpx4^{-/cys}$ mice thrived for at least 40 d after Tamoxifen-induced allelic conversion (Friedmann Angeli et al., 2014; Ingold et al., 2018). Moreover, cultured $Gpx4^{cys/cys}$ cells displayed typical ferroptotic cell death when challenged, suggesting that Sec-containing GPX4 indeed protects cells from ferroptosis (Ingold et al., 2018). Notably, the cells overexpressing the Sec-to-Cys variant of GPX4 could also survive genetic co-deletion of the tRNA for selenocysteine, and thus survive deletion of all other selenoproteins (Ingold et al., 2018).

Of note, when the $Gpx4^{cys}$ allele was studied on a hybrid strain-background 129S6SvEV x C57Bl/6J) all $Gpx4^{cys/cys}$ pups survived to birth; however they failed to thrive postnatally and, by postnatal day 18, all had developed severe seizures and needed to be sacrificed (Ingold et al., 2018). Necropsies revealed these pups lacked PV+ interneurons (Ingold et al., 2018). Considering the available data, it can thus be concluded that cytosolic GPX4 is clearly an essential enzyme, unique in being able to reduce phospholipid peroxide species, and that the enzyme typically protects cells in culture from ferroptosis. It has not yet been unequivocally shown that the embryonic lethality of inbred $Gpx4^{cys/cys}$ fetuses, nor the lack of PV+ interneurons in the hybrid-strain $Gpx4^{cys/cys}$ pups, was due to ferroptosis, as assessing either would be extremely technically difficult. However, in the latter case, an increased number of TUNEL-positive cells were seen in the cortex, associated with increased astrogliosis and neuroinflammation (Ingold et al., 2018). This phenotype is consistent with cell death by diverse mechanisms, including apoptosis or ferroptosis, thereafter leading to inflammatory responses.

Interestingly, recent reports suggest that GPX4 knockdown does not induce ferroptosis via a mechanism that might involve failure to reduce lipid peroxides, but rather induces overaccumulation of ferrous iron, leading to ferroptosis (Wei et al., 2022).



4. FSP1 and other enzymes affecting ferroptosis in parallel with GPX4

With ferroptosis being defined as a cell death triggered by iron-dependent lipid peroxidation, this suggests that all proteins or enzymes that may limit either iron reactivity or accumulation of lipid peroxides may prevent ferroptosis. Indeed, several such systems have been found, but shall here only briefly be introduced with this review article mainly being focused on the links between cysteine homeostasis and ferroptosis. These additional enzyme systems acting in parallel with GPX4 are, in contrast to GPX4, typically not considered to be dependent upon GSH.

4.1 FSP1

In 2019, both the groups of Marcus Conrad and of James Olzmann reported that the flavoprotein apoptosis-inducing factor mitochondrial 2 (AIFM2) efficiently protected cells from ferroptosis upon deletion of GPX4, thereby renaming AIFM2 to Ferroptosis suppressor protein 1 (FSP1). They also showed that FSP1 expression levels closely correlate with resistance of cells to ferroptosis, that inhibition of FSP1 sensitizes cells to ferroptosis, and that myristoylated membrane-associated FSP1 acts by reducing ubiquinone coenzyme Q10 (CoQ10) in a reaction using NADPH, with reduced ubiquinol being able to trap lipid peroxyl radicals and thus suppress propagation of lipid peroxidation (Bersuker et al., 2019; Doll et al., 2019). The Conrad group furthermore showed that FSP1 can alternatively use Vitamin K in catalytic lipid peroxide trapping (Mishima et al., 2022). Recently the same group also reported that a number of 3-phenylquinazolinone compounds can target FSP1, trigger its release from the membrane, and lead to aggregation of the enzyme and intracellular phase separation, also correlating with increased sensitivity to ferroptosis (Nakamura et al., 2023). The structural features of FSP1 were solved and the catalytic cycle described (Lv et al., 2023). FSP1, like many other antioxidant enzymes, is also an NRF2 target, and is upregulated in many cancer cells (Bersuker et al., 2019; Koppula et al., 2022; Muller et al., 2023). Thus, pharmacological inhibition of FSP1 makes cancer cells and

tumors more prone to cell death, thereby showing that FSP1 holds promise as an anticancer therapy target (Bersuker et al., 2019; Doll et al., 2019; Hendricks et al., 2023; Muller et al., 2023; Nakamura et al., 2023).

4.2 DHODH

In 2021 it was reported by the group of Boyi Gan that dihydroorotate dehydrogenase (DHODH) could protect cells from ferroptosis by regenerating ubiquinol, independently from FSP1 (Mao et al., 2021). This notion was subsequently challenged by the group of Marcus Conrad, showing that the study had used inhibitors of DHODH at high concentrations, which also inhibited FSP1 (Mishima et al., 2023). This word of caution was replied to by Boyi Gan et al., arguing that not only the use of inhibitors showed that DHODH plays a role in protection of cells against ferroptosis, but also genetic evidence (Mao, Liu, Yan, Olszewski, & Gan, 2023). This topic remains unresolved, but perhaps it can be wise at this stage to remain open to the possibility that DHODH can indeed protect certain cells against ferroptosis, depending upon overall cellular context, metabolic state and expression of other proteins and enzymes affecting iron-dependent peroxidation events and thus steady-state levels of phospholipid hydroperoxides.

4.3 MBOAT1 and MBOAT2

Very recently it was shown that membrane-bound O-acyltransferases MBOAT1 and MBOAT2, being transcriptional targets upregulated by the estrogen and androgen receptors, respectively, can “remodel” cellular phospholipids and thus make cells more resistant to ferroptosis in a GPX4- as well as FSP1-independent manner (Liang et al., 2023). The remodeling reaction catalyzed by the MBOAT enzymes involves acyl transfer reactions, and the enzymes accept several different phospholipids as their substrates, including phosphatidylcholine (Gijon, Riekhof, Zarini, Murphy, & Voelker, 2008), the peroxide of which is a unique GPX4 substrate (see above). There are more than 10 family members of MBOATs in human (Masumoto et al., 2015) and thus the evaluation of their individual possible importance in relation to ferroptosis will require significant additional studies in the forthcoming years.

4.4 Proteins affecting iron status

If ferroptosis is defined as being iron-dependent, then iron homeostasis should likely affect the extent of ferroptotic cell death. Indeed, many studies have reported such observations. This includes NRF2-driven HERC2 and VAMP8 indirectly increasing ferritin levels, thus shown to

block ferroptosis (Anandhan et al., 2023); transferrin-knockout mice being more prone to ferroptosis in the liver (Yu et al., 2020); alpha-Enolase 1 (ENO1) moonlighting as an mRNA-binding protein suppressing iron regulatory protein 1 (IRP1) expression leading to inhibition of mitoferrin-1 (Mfrn1) expression and subsequent repression of mitochondrial iron-induced ferroptosis (Zhang et al., 2022a); or prominin-2 leading to export from cells of iron and thereby leading to suppression of ferroptosis (Brown et al., 2019). Additional examples include NUPR1-mediated LCN2 expression that blocks ferroptosis by preventing iron accumulation and subsequent oxidative damage (Liu et al., 2021a), or loss of ferritin through autophagy leading to increased ferroptosis (Hou et al., 2016). There are many examples in the literature showing that distorted iron status can be linked to modulation of ferroptosis.



5. Genetic and pharmacologic models that question the exact roles of cystine, Cys, xCT, GSH, or GSH synthesis in ferroptosis

The discovery of ferroptosis using compounds that disrupt xCT activity and the long-association of ferroptosis with xCT inhibition, has led to the prevalent working hypothesis wherein diminished CSSC is the first step on a chain-reaction that diminishes cellular Cys, cellular GSH, and GSH-mediated transit of reducing power to GPX4 (Dixon et al., 2012; Jiang et al., 2021; Yan et al., 2021). However, as detailed above, aspects of this inhibitor- and cell culture-based relationship have proven recalcitrant to subsequent validations, in particular using other inhibitors, genetic approaches, or whole-animal models. Moreover, the inhibitors that formed the basis of this hypothesis are now known to have often poorly characterized mechanisms of action on xCT, and to have wide-ranging and incompletely defined off-target effects. Similar concerns are now being raised about next-generation ferroptosis-inducing compounds targeting GPX4. In the best-case scenario, the working hypothesis is conditionally correct, but requires other predisposing conditions to result in ferroptosis. As an example of this, one can look to the original discovery of erastin, which reported that it only reliably caused non-apoptotic cell death in cell lines that carried mutant HRAS and the SV40 small-T antigen (Dolma et al., 2003). Clearly there are many missing pieces to the puzzle regarding what exact conditions that will trigger ferroptosis in a living cell. In this

section we will critically discuss the components of the hypothesized chain-reaction from extracellular CSSC to lipid peroxidation and cell death, to better clarify which are critical components; which are, perhaps, only “passenger components” arising from the ferroptosis-inducing treatments, yet not playing a causal role; and which are perhaps “conditionally causal”, for example reliably inducing ferroptosis in cell culture conditions but not necessarily relevant in vivo.

5.1 CSSC and xCT

The hypothesis that restricting access of cells to CSSC can induce ferroptosis, whether these are HRAS-mutant/small-T antigen expressing cancer cells or not, is problematic. As overviewed in [Section 1.1.2](#), above, although CSSC is typically the sole source of Cys in cell culture media and, in these conditions, knockdown of xCT can promote ferroptosis ([Xu, Huang, Zhu, & Guo, 2022](#)), it is unclear whether CSSC is reliably available to cells in vivo. Moreover, it is very clear that xCT is not essential in vivo, as discussed above. Since there is not a mechanism for splanchnic tissues to export CSSC into the blood plasma ([Fig. 2](#)), CSSC in vivo appears to be predominantly supplied by the gut upon digestion of food-protein. Thus, if CSSC restriction either extracellularly or by blockage of xCT induced ferroptosis, then intermittent fasting should induce ferroptosis in vivo, at least in mutant HRAS/small-T antigen expressing cancers, which has not been reported. Moreover, the MGI database of The Jackson Laboratories (<https://www.informatics.jax.org/marker/MGI:1347355>) reports that full-body homozygous xCT (SLC7A11)-null mice have mild pigmentation defects but are overtly healthy, consistent with CSSC being a convenient source of Cys for cells to exploit when available, e.g., after a protein-rich meal, but not a necessary source in any known cell type in vivo. Clearly other sources of Cys can supplant CSSC uptake in vivo. This obvious conclusion, nonetheless, largely annuls approaches targeting xCT to induce ferroptosis in vivo. Additionally, for the model of CSSC/xCT/Cys targeting to induce ferroptosis via lowering GSH levels and, thereby, lowering GSH-mediated trafficking of reducing power to GPX4, these treatments must have impacts on GSH greater than other treatments that do not induce ferroptosis. However, as also discussed above, cells and organisms, including cancer cells in organisms or in patients, are highly tolerant of treatments, notably BSO administration, that precipitously lower GSH levels. We shall in the next section further discuss this aspect in specific relation to ferroptosis.

5.2 GSSG/GSH and GGT

As overviewed in [Sections 1.1](#) and [1.2](#) above, GSH and GSSG are likely to be a more important source of systemic Cys than CSSC, arising from intermediary metabolism in splanchnic tissues. Of these, GSH is more abundant in plasma but, until a GSH exporter can be identified, this appears to arise from secreted GSSG at the expense of reducing power captured from secreted protein-thiols (see above). However, mice homozygous-null for a knockout of GGT1, the predominant GGT enzyme (with a second enzyme, GGT5, and phenotypes associated with its disruption, appearing to be restricted to immune cells ([Shi, Han, Habib, Matzuk, & Lieberman, 2001](#)), show a mild growth retardation phenotype ([Lieberman et al., 1996](#)). It is noteworthy that full-body pharmacologic disruption of GSH biosynthesis in mice with BSO dramatically lowers plasma GSSG+GSH levels, with this being well tolerated and, although BSO treatment compromises growth of some cancer cell lines in culture ([Wei et al., 2022](#)), systemic BSO treatment has not been useful in either killing or diminishing growth in tumor-bearing mice ([Coshan-Gauthier & Kirkpatrick, 1989](#); [Zhu, Hu, Wu, & Hu, 2014](#)). The ability of BSO to sensitize tumors to other therapies has varied ([Geroni et al., 1993](#); [Soble & Dorr, 1988](#); [Tsutsui et al., 1986](#)), and it is unclear which of these effects arise from restricting GSH as a source of Cys versus via restricting its other activities, e.g., in drug metabolism pathways or its other activities ([Tanaka, Kurokawa, Matsuno, Matsumoto, & Hayashida, 2008](#)).

5.3 GCL disruptions

The target enzyme of BSO is the Glu-Cys ligase enzyme (GCL), which is a heterodimer composed of a catalytic subunit, GCLC, that is responsible for the chemical reaction, and a modulatory subunit, GCLM, that confers product (GSH)-inhibition on the holoenzyme ([Chen, Shertzer, Schneider, Nebert, & Dalton, 2005](#)). Genetic models targeting GCL have not supported the working hypothesis of ferroptosis induction (see [Section 2](#), above). Thus, whereas GCLM-null mouse models have exhibited diverse and usually modest alterations in GSH levels, they have not caused ferroptosis ([Botta et al., 2008](#); [Franklin et al., 2009](#); [Nakamura et al., 2011](#)). More recently, using conditional disruption of GCLC in whole mice, the team of Isaak Harris has shown that many cells in whole mouse models tolerate disruption of GCLC. Whereas that result is consistent with decades of research using BSO to inhibit GCL activity, as an inhibitor, the specificities and potential off-target activities of BSO could

never be fully assessed, whereas by disrupting the gene encoding GCLC, the new studies more precisely hone-in on the specific activities of GCL; in this way, they have shown that T cell-specific genetic disruption of GCLC and therefore, of GSH biosynthesis, does not cause cell death but, instead, compromises the functions of regulatory T cells (Kurniawan et al., 2020). Additional insights to the in vivo requirements for GSH biosynthesis are anticipated from the continuation of this work.

5.4 GSR KO

Another tenet in the prevalent working hypothesis that targeting xCT induces ferroptosis by indirectly interfering with GSH-mediated transit of reducing power to GPX4, is that other treatments that interfere with GSH-mediated transit of reducing power to GPX4 should also induce ferroptosis. TrxR1 cannot reduce GSSG (Amér, 2009b), meaning that only one primary disulfide reductase in the cytosol, GSR, can directly reduce $\text{GSSG} \rightarrow 2 \text{GSH}$ (Miller & Schmidt, 2019). Nonetheless, TRX1 can also reduce GSSG (Gromer, Merkle, Schirmer, & Becker, 2002). Importantly, similar to the situation wherein we long recognized that there must be a mechanism to transport GSH into mitochondria (Booty et al., 2015) even though the transporter was only recently identified (Liu et al., 2023) (see Section 1.2.3, above), we know that cells can sustain the highly reduced intracellular status of the GSH+GSSG pool in the absence of GSR (Rogers et al., 2004). Although this has been attributed to “cross-trafficking” of reducing power from the TRX system to the GSH system (Zhang, Du, Zhang, Lu, & Holmgren, 2014), the ability of TrxR1/GSR- and TRX1/TrxR1/GSR-null mouse livers to also sustain the reduced status of the GSH+GSSG pool indicates that completely TrxR1-independent mechanisms should also be at play here (Miller & Schmidt, 2020). To date, the only such TrxR1- and GSR-independent mechanisms that have been implicated involve *de novo* synthesis of Cys and cellular export of GSSG (Sections 1.1 and 1.2, above) which, although still untested, we predict should be less efficient at trafficking disulfide reducing power to target enzymes including GPX4.



6. Potential roles of the thioredoxin system in ferroptosis

With TrxR1 being a highly important selenoprotein for support of reductive power in cells (Arner & Holmgren, 2000; Amér, 2009a; Miller et al., 2018; Nordberg & Arner, 2001) that was suggested together with

GPX4 to be one of only two key essential selenoproteins in human cells (Santesmasses & Gladyshev, 2022), and considering that the ferroptosis inducing compounds RSL3 and ML162 efficiently inhibit TrxR1 (Cheff et al., 2023), it could have been hypothesized that TrxR1 would be another key enzyme for protection of cells against ferroptosis. However, there's no clear data at this moment suggesting this would be the case. When TrxR1 is targeted with inhibitors, cancer cells typically die, but not with ferroptosis features (Cheff et al., 2023; Sabatier et al., 2021; Stafford et al., 2018), and although the enzyme is essential for embryonic development (Bondareva et al., 2007; Jakupoglu et al., 2005) it can be conditionally genetically deleted from adult tissues without typical signs of ferroptosis induction (Dagnell et al., 2018; Eriksson et al., 2015; Iverson et al., 2013; Miller et al., 2018; Prigge et al., 2012; Prigge et al., 2017; Rollins et al., 2010). Importantly, the cell death triggered by TrxR1 inhibitors is not prevented by ferrostatin or iron chelators, in contrast to that seen with low (but not higher) concentrations of RSL3 and ML162 (Cheff et al., 2023). Of special note here, for the *in vivo* functions of TrxR1, is that mouse livers living under TrxR1-deficient conditions have not been found to undergo precipitous ferroptosis nor, remarkably, are disulfide reductase-deficient malignant hepatocellular carcinomas induced in such mouse livers via chemical carcinogenesis (McLoughlin et al., 2019). Based on those robust, living, non-ferroptotic mouse systems, we consider it formally possible, yet nonetheless unlikely, that diminishing import of CSSC via xCT could have a large enough impact on the trafficking of electrons to GPX4 to induce ferroptosis. More likely perhaps, we suggest that alternative mechanisms, in particular ones that do not require CSSC, Cys, or GSH restriction, need to be considered for how cell death can be triggered in normal cells upon TrxR1 targeting, such as during embryogenesis, if at all, such as with the lack of overt phenotypes upon conditional knockout in adult tissues.

We consider that TrxR1 does not seem to be a *bona fide* suppressor of ferroptosis but should rather be viewed of more as an important pillar of antioxidant defense systems and reductive pathways of cells, in general. In cancer cells, however, it may be another case, with several groups having shown that cancer cells are sensitive to TrxR1 targeting, both in cell culture and *in vivo* (Chew, Lu, Bradshaw, & Holmgren, 2008; Gencheva & Arner, 2022; Lu, Chew, & Holmgren, 2007; Mandal et al., 2010; Scalcon, Bindoli, & Rigobello, 2018; Stafford et al., 2018; Urig & Becker, 2006; Wang et al., 2012; Zhang et al., 2016). The mechanisms of cell death also in these cases do not, however, seem to be typical for ferroptosis.

Whereas NADPH-GSR is a likely primary source of reducing power for sustaining the highly reduced status of the GSH:GSSG pool in cells, NADPH-TrxR1 appears to play the predominant role in supplying the reducing power to convert nutritional CSSC entering the cytosol, regardless of from xCT, GGT, or cystinosin, into 2 Cys (Fig. 3). Thus, biochemical studies showed that NADPH-TrxR1/TRP14 is at least 5-fold more efficient at catalyzing reduction of CSSC into 2 Cys than is NADPH-TrxR1/TRX1 and, unlike other redoxins, TRP14 activity with CSSC is not competed by substrates such as protein-disulfides (Pader et al., 2014). If Cys availability becomes limiting for cytosolic Cys production, whether from pathways involving $\text{CSSC} \rightarrow \text{xCT} \rightarrow \text{CSSC}$, $\text{GSSG} \rightarrow \text{GGT} \rightarrow \text{CSSC}$, or $\text{proteolysis} \rightarrow \text{cystinosin} \rightarrow \text{CSSC}$, as often posited for ferroptosis induction, then the redoxin systems, in particular NADPH-TrxR1/TRP14, should be particularly critical for preventing ferroptosis. This prediction, however, has at least not yet been borne out experimentally.

NADPH-TrxR1 is the primary reductase reducing cytosolic TRX1 or TRP14, although a cytosolic isoform of GLRX2, entitled GLRX2c, can also reduce the active site disulfide of TRX1 and, thereby, might serve as a conduit for cross-trafficking reducing power between the TrxR1 and GSH pathways (Zhang et al., 2014). However, decades of treatment of cells, animals, and patients with the potent TrxR1-inhibiting drug AFN have shown that this inhibition does not cause detectable ferroptosis in normal cells (Arnér, 2009b), nor has it yet been shown effective at treating cancers (Arner & Holmgren, 2006), probably suggesting that ferroptosis is not induced by AFN. Another more specific TrxR1 inhibitor, TRi-1, similarly has not been reported to induce ferroptosis (Sabatier et al., 2021; Stafford et al., 2018), nor are other known TrxR1 inhibitors considered to be ferroptosis inducing compounds (Gencheva & Arner, 2022). Also, mouse and cell models with genetic disruptions of TrxR1 have been developed, yet none to our knowledge, including those in cancers, have been reported to cause ferroptosis (Bondareva et al., 2007; Jakupoglu et al., 2005; Mandal et al., 2010; McLoughlin et al., 2019; Peng et al., 2016). Possibly the lack of ferroptosis in all these observations could be explained if the NADPH-GSR system, either via GLRX-mediated reduction of CSSC or via trafficking of reducing power via $\text{GLRX2c} \rightarrow \text{TRP14}$, complemented the effects of loss of TrxR1 on CSSC reduction. However, subsequent development of TR/GR-null mouse liver, cell, or cancer models, clearly shows that, even in the absence of any primary NADPH-driven cytosolic disulfide

Table 1 Presumed players in ferroptosis and outstanding questions regarding their exact roles.

Player	Expected role	Outstanding Questions with regards to ferroptosis	Sections
CSSC	Source of Cys	Cells survive without CSSC. CSSC not likely limiting source of Cys in vivo	1.1, 5.1
Cys	Limit GSH synthesis	Required by all cells. Unclear that cell death by Cys-restriction is normally ferroptosis	1.1
GSH	Limit GPX4 activity	Most cells tolerate near-total GSH depletion	1.2
GSH:GSSG	Limit GPX4 activity	Regulated by GSR and GSSG-export. Not clear this ratio can increase to incapacitate GPX4	1.2, 5.2
H₂O₂	Precursor for HO.-	Few sources of H ₂ O ₂ associated with ferroptosis	2.1.2, 2.2
xCT	Import of CSSC	xCT-null mice survive. CSSC not only source of Cys in vivo	5.1
TRP14	Reduce CSSC→2Cys	TRP14-null mice overtly normal. TRP14-disruption does not cause ferroptosis	1.5
TRX1	Reduce CSSC→2Cys	TRX1-null livers survive. TRX1-disruption does not cause ferroptosis	1.5
TrxR1	Fuels TRP14, TRX1	TrxR1-livers and cells survive. TrxR1-disruption does not cause ferroptosis	1.5
GCL	Synthesis of GSH	GCLM-null cells and mice survive. At least some GCLC-null T cell types survive	1.2, 5.3
GSR	Reduce GSSG→2GSH	GSR-null mice overtly normal. GSR-disruption does not cause ferroptosis	1.5, 5.4
Erastin	Inhibit xCT	Causes ferroptosis, off-target activities. Mechanisms unclear, possibly indirect	1.4.1

SSZ	Inhibit xCT	Off-target activities. Mechanisms of xCT disruption unclear and possibly indirect	2.1
AFN	Inhibit TrxR1	Whole-body AFN tolerated. Inhibits TrxR1 but does not cause ferroptosis	1.5, 6
Tri-1	Inhibit TrxR1	Diverse cells tolerate Tri-1. Inhibits TrxR1 but does not cause ferroptosis	1.5, 3.2, 6
BSO	Inhibit GCL	Depletes GSH in cells and mice, but tolerated and rarely associated with ferroptosis	1.2, 2.1.1
RSL3	Inhibit GPX4	Causes ferroptosis, mechanisms unclear. Inhibits TrxR1; unclear if it inhibits GPX4 directly	3.2, 6
ML162	Inhibit GPX4	Causes ferroptosis, mechanisms unclear. Inhibits TrxR1; unclear if it inhibits GPX4 directly	3.2, 6
Death	non-apoptotic	Death mechanisms? Unclear whether mitochondrial leakage and apoptosis are involved	2, 5

This table summarizes some of the outstanding questions regarding Cys metabolism and its relation to ferroptosis discussed in this review. References are given to the sections of this review for further details. See also [Fig. 5](#).

reductase system, this does not result in an inability to reduce CSSC thus causing detectable ferroptosis ([Eriksson et al., 2015](#); [McLoughlin et al., 2019](#); [Prigge et al., 2017](#)).

The TrxR1 inhibitor, AFN, and the TrxR1- and TR/GR-null models discussed in the previous paragraph all have the characteristic of inducing strong chronic activation of the Nrf2 system, which plausibly could protect cells against ferroptosis ([Cebula, Schmidt, & Arner, 2015](#); [Iverson et al., 2013](#); [Locy et al., 2012](#); [Prigge et al., 2017](#); [Suvorova et al., 2009](#)). However, more recently we have developed fibroblast- and liver-specific mouse models lacking TRX1 ([Prigge et al., 2017](#)), and cell culture- or full body mouse-models lacking TRP14 ([Doka et al., 2016](#); [Doka et al., 2020](#)), none of which show measurable activation of Nrf2 ([McLoughlin et al., 2019](#);

Prigge et al., 2017), yet these also have not resulted in ferroptosis. Moreover, even TRX1/TR/GR-null mouse liver or liver cancer models (McLoughlin et al., 2019; Prigge et al., 2017), or TRP14/TRX1/TR/GR-null mouse livers (EES, unpublished) display overt evidence of ferroptosis. As such, since the thioredoxin system is crucial for CSSC reduction, we must seriously question what role, if any, CSSC reduction plays in triggering ferroptosis.

Based on these robust, living, non-ferroptotic mouse systems, both cancerous and non-cancerous, we consider it formally possible, yet nonetheless unlikely, that diminishing import of CSSC via xCT could have a large enough impact on the trafficking of electrons to GPX4 to induce ferroptosis. Instead, we suggest that alternative mechanisms should be considered for how ferroptosis is induced, in particular ones that do not require CSSC, Cys, or GSH restriction. Several outstanding questions regarding individual chemicals, enzymes, and pharmaceutical compounds, as discussed above, concerning their roles in ferroptosis mechanisms, are summarized in Table 1.



7. Concluding remarks

Ferroptosis is an interesting and provocative topic. In the context of cancer, it is also a potentially targetable physiological response that has been intensely studied for over a decade, but remains mechanistically mysterious. This remarkable mode of cell death was identified as early as 2012, yet targeting ferroptosis-susceptibility to treat cancer remains an elusive goal. Much in the literature, unfortunately, presents ferroptosis as a rather straightforward mode of cell death, with xCT-mediated CSSC import and subsequent potency of the GSH system as a critical limiting factor in supporting GPX4 activity, and with iron-propelled lipid peroxidation leading to cell death prevented by either GPX4 or FSP1. In this review we have tried to emphasize that ferroptosis indeed likely derives from oxidative stress-related damage to cell membranes, yet that that the current rather strict definition or view of ferroptosis conceptually limits the interpretations and understanding of underlying mechanisms of action. Several of the small molecule compounds currently used in studies of ferroptosis are likely not as specific in their target engagements as is often presented. Several suspected mechanistic components, including CSSC, xCT, Cys, and GSH, are potentially “false-flags”, arising from models and conditions that do not reflect cancers in patients. Critically, an oversimplified view of ferroptosis can perhaps distract focus away from its true mechanisms of action. We

have here attempted to bring attention to experimental systems usually not used in ferroptosis research, and the physiology of Cys metabolism, surprisingly often not considered in mechanistic models of ferroptosis, aiming to integrate these topics. We hope that our review will stimulate discussions, kindly asking the many colleagues in the field of ferroptosis research to be conservative in interpretations of the observed effects, and being open minded with regards to the complexity of biological systems.

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