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A guideline on the molecular ecosystem regulating ferroptosis

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Competing interests statement

B.R.S. is an inventor on patents and patent applications involving ferroptosis; co-founded and serves as a consultant to ProJenX, Inc. and Exarta Therapeutics; holds equity in Sonata Therapeutics; serves as a consultant to Weatherwax Biotechnologies Corporation and Akin Gump Strauss Hauer & Feld LLP; B.G. is an inventor on patent applications involving targeting ferroptosis in cancer therapy, and reports personal fees from Guidepoint Global, Cambridge Solutions, and NGM Bio; D.I.G. is an employee and shareholder of AstraZeneca; V.G.S. serves as an advisor to and/or has equity in Branch Biosciences, Ensoma, and Cellarity, all unrelated to the present work; L.G. is/has been holding research contracts with Lytix Biopharma, Promontory and Onxeo, has received consulting/advisory honoraria from Boehringer Ingelheim, AstraZeneca, OmniSEQ, Onxeo, The Longevity Labs, Inzen, Imvax, Sotio, Promontory, Noxopharm, EduCom, and the Luke Heller TECPR2 Foundation, and holds Promontory stock options; A.I.B. holds shares in Cogstate Ltd, Alterity Ltd and a profit share with Collaborative Medicinal Development LLC and acts as a paid consultant to Collaborative Medicinal Development LLC. The remaining authors declare no competing interests. X.J. holds inventorship of patents related to autophagy and cell death, and holds equity as well as consults for Exarta Therapeutics and Lime Therapeutics. G.K. has been holding research contracts with Daiichi Sankyo, Eleor, Kaleido, Lytix Pharma, PharmaMar, Osasuna Therapeutics, Samsara Therapeutics, Sanofi, Tollys, and Vascage. G.K. is on the Board of Directors of the Bristol Myers Squibb Foundation France. G.K. is a scientific co-founder of everImmune, Osasuna Therapeutics, Samsara Therapeutics and Therafast Bio. G.K. is in the scientific advisory boards of Hevolution, Institut Servier and Longevity Vision Funds. G.K. is the inventor of patents covering therapeutic targeting of aging, cancer, cystic fibrosis and metabolic disorders. G.K.'s wife, Laurence Zitvogel, has held research contracts with Glaxo Smyth Kline, Incyte, Lytix, Kaleido, Innovate Pharma, Daiichi Sankyo, Pilege, Merus, Transgene, 9 m, Tusk and Roche, was on the on the Board of Directors of Transgene, is a cofounder of everImmune, and holds patents covering the treatment of cancer and the therapeutic manipulation of the microbiota. G.K.'s brother, Romano Kroemer, was an employee of Sanofi and now consults for Boehringer-Ingelheim. All other authors have disclosed no conflicts of interest, whether financial or non-financial. The funders were not involved in the preparation of the manuscript.

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Abstract

Ferroptosis, an intricately regulated form of cell death characterized by uncontrolled lipid peroxidation, has garnered substantial interest since 2012 the term coined. Recent years have witnessed remarkable progress in elucidating the detailed molecular mechanisms governing ferroptosis induction and defense, with particular emphasis on the roles of heterogeneity and plasticity. Within the molecular ecosystem of ferroptosis, present and future advancements promise to unlock safe and effective therapeutic strategies across a broad spectrum of diseases.

Keywords

antioxidant; cell death; disease; ferroptosis; lipid peroxidation

Introduction

Ferroptosis, coined in 2012, is a distinct form of regulated cell death observed in cancer cells, relying on iron but differing from apoptosis and necroptosis[1]. Unlike lytic cell death mechanisms dependent on pore-forming proteins, ferroptosis is driven by toxic, oxidized lipids and their byproducts, notably 4-hydroxynonenal (4HNE)[2], along with lipidated proteins formed through covalent binding to electrophilic lipid peroxidation breakdown products[3].

Ferroptosis has significant implications in preclinical studies across diseases, including cancer, neurodegenerative disorders, and conditions associated with ischemia-reperfusion (I/R) injury. It offers promise as a therapeutic approach against drug-resistant cancer cells deficient in apoptosis[4, 5], while its inhibition holds potential for managing infection-related diseases, sterile inflammation linked to iron overload or lipid toxicity[6–8]. Additionally, ferroptosis plays a vital role in tissue homeostasis and development[8–10].

In this review, our aim is to offer an updated overview of ferroptosis, covering its fundamental mechanisms, heterogeneity, and plasticity. We will also delve into the integrated antioxidant and membrane system's role in regulating ferroptotic sensitivity, along with discussing disease implications, therapeutic prospects, and associated challenges.

The core mechanism of ferroptosis

Erastin and RSL3 are common small molecules used to induce ferroptosis. Originally discovered in screens targeting RAS mutant cancer cells, these compounds trigger a non-apoptotic, iron-dependent form of cell death, leading to the term 'ferroptosis'[1, 11, 12]. At the same time, genetic inactivation of GPX4 was found to induce oxidative, non-apoptotic cell death[13], and overexpression of system x_c^- to protect cells from a similar non-apoptotic cell death[14], highlighting the generality of this process as a potential cancer therapy targeting RAS mutations while sparing normal cells.

Further research has revealed that ferroptosis is highly context-dependent. Metal ions like zinc and copper, in addition to iron, can induce ferroptosis in specific conditions[15, 16]. Both *RAS* wild-type and mutant cells, including cancer and non-cancer cells, can undergo ferroptotic death. Conditional knockout of *Gpx4* in various (e.g., kidney[9]) or cells (e.g., T cells[8] or B cells[10]) can cause ferroptotic damage, highlighting its role in developmental biology.

Ferroptosis is closely linked to autophagy, and heightened autophagy levels often correlate with increased ferroptosis sensitivity[17]. Specific types of selective autophagy, such as ferritinophagy[18, 19], lipophagy[20], and clockophagy[21], can lead to iron accumulation and lipid peroxidation, inducing ferroptosis. Genome-wide CRISPRi/a screens in human neurons revealed that so-called ATG (autophagy related) family members (e.g., BECN1 [beclin 1]) and lysosomal proteins (e.g., PSAP [prosaposin]) are involved in ferroptosis by triggering the formation of lipofuscin or increasing iron accumulation[22]. In certain conditions, the depletion of *ATG* genes has no effect on cell death, including ferroptosis.

These findings underscore the adaptable and context-dependent nature of ferroptosis, but its initiation involves three essential elements, which will be discussed below.

Reactive oxygen species

The first crucial element in ferroptosis induction is the presence of initiation signals that stimulate the production of ROS from various sources (Fig. 1):

1) Mitochondria: Mitochondria serve as a major source of ROS, primarily superoxide anion/$O_2^{\bullet-}$ during oxidative phosphorylation. Mitochondrial SOD converts superoxide

into other ROS, including hydrogen peroxide (H_2O_2). Mitochondrial ROS can trigger ferroptosis, with glutaminolysis promoting ferroptosis induced by cyst(e)ine deprivation cyst(e)ine deprivation[23, 24]. Mitochondrial quality is regulated by mitophagy, which has a dual role in ferroptosis. Whereas mitochondrial fission promotes apoptosis, mitochondrial fusion can increase cellular sensitivity to ferroptosis[25]. Mitochondrial energy stress inhibits ferroptosis through AMPK-mediated phosphorylation of ACACA/ACC (acetyl-CoA carboxylase alpha)[26], but AMPK can also promote ferroptosis by targeting BECN1[27] or by disrupting pyrimidinosome assembly, hindering pyrimidine intermediate synthesis[28].

2) NOX (NADPH oxidase): Overexpression of NOX increases ROS levels, heightening ferroptosis sensitivity. The activity of NOX in ferroptosis is regulated by multiple factors, such as TP53 (tumor protein p53)[29] and ALDH1B1 (aldehyde dehydrogenase 1 family member B1)[2]. *Trp53/TP53* deficiency promotes the accumulation of DPP4 (dipeptidyl peptidase 4) on the cell membrane, forming a complex with NOX1 and causing ferroptotic death[29]. ALDH1B1 inhibits the ferroptosis-inducing effect of NOX1 activity by catalyzing the oxidation of aldehydes, converting them into carboxylic acids[2].

3) Enzymatic reactions: ROS can be byproducts of enzymatic reactions, such as cytochrome P450 and its reductase involved in drug metabolism. POR (cytochrome P450 oxidoreductase), a flavoprotein, induces lipid peroxidation and ferroptosis by generating superoxide radicals[30, 31].

4) The Fenton reaction. This reaction involves the interaction between H_2O_2 and a transition metal, typically iron (Fe^{2+}), leading to the generation of highly reactive hydroxyl radicals/$^{\bullet}OH$. An extensively studied iron metabolism mechanism during ferroptosis is ferritinophagy, where autophagy degrades the iron storage protein ferritin. This liberates free iron, converting one ROS type into another, thereby inducing ferroptosis in both cancer and non-cancer cells[18, 19].

Oxidizable lipids

The second key element in ferroptosis is the presence of easily oxidizable polyunsaturated lipids (Fig. 2). Cell membranes, the primary target of oxidative damage in ferroptosis, can be influenced by metabolic pathways that promote lipid synthesis, particularly the generation of polyunsaturated fatty acids (PUFAs), increasing cell sensitivity to ferroptotic inducers. While the exact threshold for PUFA breakdown required to initiate ferroptosis remains obscure, one well-established positive regulator is ACSL4. ACSL4 activates long-chain fatty acids by converting them into acyl-CoA esters, facilitating their entry into various metabolic pathways[32–35].

ACSL4 mediates two downstream pathways, yielding different PUFA-related acyl-CoA esters. One involves LPCAT3 (lysophosphatidylcholine acyltransferase 3), incorporating PUFA into phosphatidylethanolamines (PEs)[32, 35], while the other activates SOAT1 (sterol O-acyltransferase 1), producing PUFA-cholesteryl esters (CEs) instead of PUFA-PEs[36]. Both pathways contribute to lipid peroxidation, acting as substrates depending on the context. In the lipid flippase *SLC47A1*-deficient human pancreatic cancer cells, ACSL4-driven PUFA-CE production is particularly relevant[36]. ACSL4 activation is a

strategy to enhance chemotherapy or immunotherapy efficacy by inducing ferroptosis in solid cancers[37]. PRKCB/PKCβII enhances ACSL4 activity via Thr328 phosphorylation[38], while HPCAL1 phosphorylation at Thr149 by PRKCQ induces ferroptosis by autophagic degradation of CDH2, altering membrane tension in cancer cells[39].

ACSL3 synthesizes monounsaturated fatty acids (MUFAs), which may competitively inhibit PUFA peroxidation, protecting against ferroptosis initiation[40, 41]. The mitochondrial glutamate transporter SLC25A22 inhibits ferroptosis in pancreatic cancer cells by enhancing GSH and MUFA synthesis[42]. MBOAT1 (membrane bound O-acyltransferase domain containing 1) and MBOAT2, upregulated by sex hormone receptors, inhibit ferroptosis in cancer cells by remodeling the cellular phospholipid profile to produce MUFA-containing phospholipids[43]. ACSL4-independent pathways add complexity to the understanding of lipid metabolism in cell death regulation[44].

Peroxisomes, involved in fatty acid breakdown, hydrogen peroxide production, and PUFA plasmalogen biosynthesis, can increase ferroptosis sensitivity[45]. They also contain antioxidant enzymes like CAT, which inhibit ferroptosis, as well as MUFA plasmalogens, which prevent ferroptosis[46]. Thus, peroxisomes or plasmalogens influence ferroptosis positively or negatively depending on the context.

Lipophagy selectively degrades lipid droplets, releasing lipids for peroxidation, making cells, especially hepatocellular carcinoma cells, more susceptible to ferroptosis[20]. Increased lipid storage in lipid droplets by ACSL3 can limit ferroptosis in clear cell renal cell carcinoma cells[47].

Furthermore, TMEM164 acts as a positive regulator of ferroptosis by functioning as an acyltransferase, synthesizing C20:4 ether phospholipids[48], and promoting the formation of membrane-driven phagophores[49]. These phagophores are essential for the subsequent creation of autophagosomes in pancreatic cancer cells in response to ferroptotic stimuli, rather than nutrient starvation[49].

Lipid peroxidation

Several enzymes, including ALOXs, PTGS/cyclooxygenase, and cytochrome P450 enzymes, play a key role in catalyzing lipid peroxidation during ferroptosis (Fig. 3).

ALOXs are enzymes catalyzing PUFA oxygenation, initiating lipid peroxidation by introducing hydroperoxy-groups (-OOH) into fatty acid chains. Humans have six ALOX isoforms (ALOX5, ALOX12, ALOX12B, ALOX15, ALOX15B, and ALOXE3) with distinct substrate preferences and catalytic activities, contributing to ferroptosis in various cells or tissues[41, 44, 50, 51]. PEBP1 (phosphatidylethanolamine binding protein 1) forms catalytic complexes with ALOX15, efficiently peroxidizing PUFA-PE[52]. Inhibitors targeting ALOX15-PEBP1 complexes effectively prevent phospholipid peroxidation and mitigate injuries from total body irradiation *in vivo*[53]. However, the deletion of *Alox15* does not prevent *Gpx4* deletion-driven ferroptosis in kidney or T cells[8, 9]. Therefore, profiling ALOX expression in experimental models is crucial to assess the requirement of different ALOX members in ferroptosis.

PTGS/cyclooxygenase enzymes catalyze lipid peroxidation by oxygenating free PUFAs, generating lipid hydroperoxides. However, their primary function is prostaglandin synthesis, playing a secondary role in lipid peroxidation. PGE2 production can inhibit ferroptosis through PTGER1 and PTGER2 in cerebral I/R[54], but promote it in acute kidney injury[55].

Cytochrome P450 enzymes, involved in drug metabolism, can catalyze lipid peroxidation by introducing oxygen into fatty acid chains, generating lipid hydroperoxides and 4HNE, known ferroptosis mediators. As discussed earlier, POR plays a role by supplying electrons to molecular oxygen, facilitating H_2O_2 production for ferroptosis induction[30, 31].

Regardless of the enzyme catalyzing lipid peroxidation, lipid hydroperoxides initiate a chain reaction. They undergo cleavage reactions, often catalyzed by transition metals like iron, generating highly reactive lipid radicals. These radicals react with nearby lipids, amplifying lipid peroxidation in a self-propagating process[56]. Electrophilic, oxidatively-truncated phospholipid variants then form, reacting with amino acid residues in proteins to induce protein lipoxidation[3]. This series of reactions damages cell membranes, altering membrane tension, compromising membrane repair, and ultimately leading to ferroptotic plasma membrane permeabilization[57–59]. The ER is proposed as the initial site that could potentially result in subsequent oxidative membrane damage in other organelles[60].

Antioxidant systems in ferroptosis

Enzymatic antioxidants

The key enzyme involved in the antioxidant defense against ferroptosis is GPX4, which reduces lipid hydroperoxides to alcohols in biological membranes[61] (Fig. 4). GPX4's active center contains selenocysteine[62, 63]. Low selenium levels lead to ribosome stalling at GPX4's inefficiently decoded selenocysteine UGA codon, causing ribosome collisions, premature translation termination, and proteasomal clearance of the N-terminal GPX4 fragment[64]. The molecular chaperone HSPA5 directly stabilizes GPX4 protein[65], while autophagy[66, 67] or the ubiquitin-proteasome system[68] mediate GPX4 protein degradation, increasing ferroptosis sensitivity. CKB-mediated phosphorylation of GPX4 at serine residue 104 inhibits autophagy-mediated GPX4 degradation and subsequent ferroptosis[67].

The R152H mutation in GPX4 can cause Sedaghatian-type spinal metaphyseal dysplasia/SSMD, a rare and fatal disease in newborns[69]. *In vitro* studies suggest that this R152H mutation does not affect the catalytic activity of the enzyme in a direct fashion but rather interferes with its allosteric activation by cardiolipin[70]. Further examination is necessary to determine if excessive cardiolipin peroxidation by dysfunctional mitochondrial GPX4 contributes to the disease's development.

Constitutive knockout of the *Gpx4* gene in mice leads to embryonic death around 7.5–8.5 days[71]. *In vivo* evidence linking *Gpx4* deficiency to ferroptosis was first observed in mice with a conditional knockout of *Gpx4* in the kidney, combined with a vitamin E-deficient diet, leading to kidney damage[9]. This phenotype was reversed by vitamin E supplementation or the ferroptosis inhibitor liproxstatin-1[9]. Similarly, ferroptosis of activated T cells in the absence of *Gpx4* in mice is prevented by a vitamin E enriched diet[8]. Under normal breeding

conditions and chow feeding, conditional knockout of *Gpx4* in several cell types (e.g., myeloid, pancreatic epithelial cells or hepatocytes) is not lethal[72–74]. However, the inducible conditional knockout of *Gpx4* in neurons or homozygous conditional deletion of *Gpx4* in gut epithelium under the standard chow diet is lethal[75, 76]. Thus, GPX4 and its defense against lipid peroxidation play a context-dependent role in regulating tissue development.

GSH, a tripeptide composed of glutamate, cysteine, and glycine, acts as a GPX4 cofactor. Cysteine, a critical precursor for GSH synthesis, can limit GSH production and is derived from methionine metabolism. In addition, and more importantly, cells import extracellular cystine via the cystine/glutamate antiporter system xc^-, composed of SLC7A11 and SLC3A2 subunits. Imported cystine is subsequently reduced to cysteine. Pharmacological agents like erastin or sulfasalazine can inhibit system xc^- [1, 77, 78]. At high concentrations, sorafenib reportedly inhibits the activity of system x_c^- in an indirect fashion[77], but a recent study indicated that sorafenib fails only to induce ferroptosis in certain cancer cells[79]. GSH is primarily synthesized in the cytosol through enzymatic reactions[80] and system xc^- is crucial for maintaining GSH levels to prevent ferroptosis before it begins, as GSH synthesis during ferroptosis onset is too slow.

Whereas GSH depletion contributes to ferroptosis, GPX4 is not the exclusive target of GSH, suggesting the existence of GPX4-independent protective pathways against ferroptosis (Fig. 4). Among them, AIFM2/FSP1 relocates from mitochondria to the cell membrane in *Gpx4*-deficient cells, reducing COQ10 and inhibiting ferroptosis[81, 82]. STARD7 (StAR related lipid transfer domain containing 7), found in both mitochondrial intermembrane space and cytosol after cleavage by PARL (presenilin associated rhomboid like), participates in COQ10 synthesis and transport to the plasma membrane, also hindering ferroptosis[83]. Additionally, AIFM2 contributes to membrane repair[84] and the canonical vitamin K cycle[85], enhancing its antiferroptotic effects. AIFM2's activity in ferroptosis relies on phase separation and can be initiated by N-terminal myristoylation, facilitated by compound icFSP1[86].

DHODH (dihydroorotate dehydrogenase (quinone)) is a mitochondrial enzyme involved in pyrimidine biosynthesis, crucial for DNA and RNA formation. The activity of DHODH has an influence on the ferroptotic susceptibility of cancer cells expressing low levels of GPX4, likely due the DHODH-catalyzed utilization of COQ10 as an electron acceptor[87]. Inhibiting DHODH reduces COQ10, increasing susceptibility to lipid peroxidation and ferroptosis. However, DHODH inhibitors' potential off-target effects on AIFM2 remain debated[88, 89].

Several antioxidant enzymes beyond GPX4, AIFM2, and DHODH play roles in suppressing ferroptosis. GCH1 (GTP cyclohydrolase 1) is involved in tetrahydrobiopterin/BH4 biosynthesis, contributing to cellular redox balance and ferroptosis inhibition[90]. Mitochondrial SOD2 defends against heat-stress-induced ferroptosis[91]. NOS2/iNOS (nitric oxide synthase 2) represses ferroptosis in macrophages by suppressing ALOX15-mediated lipid peroxidation[92]. NFE2L2/NRF2-mediated upregulation of MGST1 aids cellular detoxification in pancreatic cancer cells in response to ferroptotic activators[93]. GSTZ1/maleylacetoacetate isomerase (glutathione S-transferase zeta 1) inhibits ferroptosis in bladder cancer cells[94], while TXNRD1 (thioredoxin reductase 1), TXNDC12 (thioredoxin

domain containing 12), and peroxiredoxins (PRDX) also have context-dependent roles in ferroptosis inhibition[95, 96]. Additionally, Ca^{2+}-independent PLA2G6/iPLA2β/PNPLA9 (phospholipase A2 group VI) plays a role in eliminating ferroptotic death signals by hydrolyzing peroxidized membrane phospholipids, potentially mediated by TP53 regulation[97, 98]. Understanding the synergistic effects of different antioxidant systems in ferroptosis remains a central theme or challenge in translational medicine.

Non-enzymatic antioxidants

Non-enzymatic antioxidants counteract harmful ROS and protect cells from oxidative damage, maintaining cellular redox balance. Examples in ferroptosis include vitamin E[9], vitamin K[99], GSH[1], COQ10[81, 82, 87], and NADPH[100]. They collaborate with enzymatic antioxidants to prevent or alleviate oxidative stress. Antioxidants scavenge radicals when reduced, but their oxidized form may increase oxidative stress, emphasizing the importance of monitoring redox reactions dynamically.

Metal chelators

Metal ions like iron and copper participate in Fenton or Haber-Weiss reactions, producing highly reactive hydroxyl radicals. Metal-binding proteins, such as TF (transferrin) and ferritin, sequester free iron to prevent these damaging reactions[18, 19]. Intracellular metal homeostasis is tightly regulated by specialized proteins, including metal chaperones that deliver metals to their target proteins[101]. Metallothioneins also help control metal ion availability, reducing their contribution to oxidative damage and ferroptosis[78]. Additionally, metal chelator drugs like deferoxamine, deferiprone, deferasirox, and ciclopirox, used in clinical settings, have shown promise in regulating ferroptosis by countering lipid peroxidation processes.

Transcriptional regulators

NFE2L2: In response to oxidative stress or exposure to electrophilic compounds, NFE2L2 is released from KEAP1 and translocates into the nucleus. SQSTM1 (sequestosome 1)-mediated protein degradation regulates the levels of KEAP1, and impaired autophagy leads to SQSTM1 accumulation, resulting in KEAP1 degradation and increased NFE2L2 protein stability[102]. In the nucleus, NFE2L2 binds to specific DNA sequences known as antioxidant response elements/AREs or electrophile response elements/EpREs in the promoter regions of target genes. This binding activates the transcription of a set of genes involved in both GPX4-dependent and GPX4-independent pathways to inhibit ferroptosis[103, 104]. A key unanswered question is how NFE2L2 selectively activates target genes to inhibit ferroptosis rather than other types of cell death.

TP53: TP53 has a dual role in regulating ferroptosis susceptibility. For instance, the acetylation-deficient TP53 variant, TP53[3KR], lacks the ability to induce apoptosis and cell cycle arrest. However, it retains its capacity for tumor suppression similar to wild-type TP53 by suppressing SLC7A11 expression, thereby increasing ferroptosis sensitivity in certain cancer cells[105]. TP53-mediated downregulation of VKORC1L1 also increases ferroptosis sensitivity in cancer cells through vitamin K metabolism[106]. Additionally, TP53 positively regulates ferroptosis by inducing the expression of SAT1, a rate-limiting enzyme

in polyamine catabolism that can produce ROS[107]. Conversely, under certain conditions, TP53 inhibits ferroptosis. For instance, in human colorectal cancer cells, *TP53* deletion increases sensitivity to erastin-triggered ferroptosis through the activation of the DPP4-NOX1 pathway on the cell membrane[29]. The classical TP53-inducible gene, CDKN1A/p21, also inhibits ferroptosis in cancer cells[108]. Furthermore, *TP53* mutation (R175H) yields a modified TP53 protein that functions as a suppressor of ferroptosis by preventing BACH1-mediated downregulation of SLC7A11, thus promoting tumor growth[109]. These findings underscore the wide implications of TP53 in the modulation of ferroptosis.

ATF4: ATF4 (activating transcription factor 4) plays a crucial role in ER stress and amino acid metabolism. ATF4 activation by ER stress upregulates anti-ferroptotic genes, such as HSPA5[65], SLC7A11[110], or TXNDC12[96]. This pathway protects against ferroptosis in cancer cells and mitochondrial cardiomyopathy[111, 112]. Sublethal cytochrome c release induced by pro-apoptotic BH3 mimetics (ABT-737 and S63845) can lead to ATF4-dependent chemotherapy resistance in cancer cells[113]. Considering the importance of the ER as a critical organelle for ferroptosis[60], ATF4 likely plays a specific role in transcriptional regulation, preserving cellular viability and conferring ferroptosis resistance.

Other important transcription factors, including HIF1A[114], NFKB/NF-κB[115], YAP1[116, 117], WWTR1[116, 117], and SREBF1[118], also play a context-dependent role in shaping the ferroptotic response through multiple targeted genes.

Membrane repair system

Ca^{2+} is the key initiator of the membrane repair response. When the plasma membrane is damaged, Ca^{2+} enters the cytoplasm from outside sources, signaling downstream repair processes, such as endosomal sorting complexes required for transport (ESCRT)-III[58, 59] and exocytosis[119], thereby enhancing ferroptosis resistance. Efficient membrane repair is vital for cell function, and its disruption may be irreversible. However, Ca^{2+} signaling from different organelles has a dual role in the control of ferroptosis sensitivity, underscoring the importance of timely monitoring.

Therapeutic opportunities and challenges

Therapeutic opportunities

Preclinical studies suggest that targeting ferroptosis has broad implications for various diseases, notably in oncology, neurodegenerative disorders, and I/R injury, as elaborated below.

Cancer cells often undergo metabolic changes that disrupt redox balance and increase their reliance on antioxidants, making them vulnerable to ferroptosis induction. Targeting ferroptosis offers a novel approach to overcome treatment limitations[105, 120–124], despite occasional resistance mechanisms (e.g., due to enhanced biosynthesis of pyrimidines[28] or hydropersulfides[125]). Furthermore, specific mutations in genes like *KRAS* and *TP53* in certain solid cancers are associated with ferroptosis sensitivity, offering potential for precision medicine strategies[1, 105, 109].

Neurodegenerative disorders, such as Alzheimer, Parkinson, and Huntington diseases, involve neuronal destruction and protein aggregation in the brain. Oxidative stress plays a key role in this degeneration, leading to lipid peroxidation and ferroptotic cell death. Therapies targeting ferroptosis inhibition aim to reduce oxidative damage and enhance neuron survival[62, 126]. Modulating ferroptosis pathways may help mitigate the accumulation of harmful byproducts like lipid peroxides and reactive aldehydes, potentially slowing neurodegeneration, including in conditions like multiple sclerosis[127].

I/R events trigger oxidative stress and cell death, making ferroptosis-targeting therapies promising for mitigating oxidative damage and preserving tissue function in conditions like stroke, myocardial infarction, and kidney and liver injuries. Combining ferroptosis and necroptosis inhibition has shown particular effectiveness[128, 129]. For kidney tubules, ferroptotic cell death propagation follows a unique pattern that has been referred to as a “wave-of-death” and has since also been described in other systems[56]. These studies highlight the therapeutic potential of ferroptosis inhibitors in I/R-related diseases.

Therapeutic challenges

Specificity and selectivity: High specificity and selectivity are needed to minimize off-target effects and potential toxicity. For instance, there are concerns about off-target effects of RSL3 and ML162 on the TXNRD1 protein[130]. Imidazole ketone erastin (IKE) is a widely used *in vivo* ferroptosis inducer[131], but its activity relative to other *in vitro* activators needs further study. Additionally, inhibiting ferroptosis through antioxidant mechanisms may impact non-ferroptotic pathways, including apoptosis[132] and necroptosis[128, 133].

Drug delivery: Developing targeted drug delivery systems is essential to enhance therapeutic effectiveness and reduce systemic side effects. Recent research has shown promise in using nanoparticles, including liposomes, micelles, and polymer-based carriers, to address these challenges. Nanoparticles provide advantages like enhanced drug stability, solubility, and targeted delivery.

Biomarker identification: Several biomarkers, such as TFRC[134], ACSL4[34], and PTGS2[61], hyperoxidized PRDX3[135], have been measured at the mRNA or protein levels to monitor ferroptosis responses. Theoretically, blood-based biomarkers have strong translational potential for clinical use, particularly danger signals like HMGB1[136], ATP[137], SQSTM1[138], and DCN (decorin)[139], which can indicate plasma membrane rupture during ferroptosis. DCN is notable for its ability to distinguish ferroptosis from other cell death types, especially in early stages[139]. LC-MS-based redox lipidomics is a valuable tool for characterizing ferroptotic biomarkers *in vivo*, especially in various disease conditions[3].

Side effects: Current widely used ferroptosis activators lack cell or tissue selectivity, potentially causing unintended cell death in various immune cell types, such as neutrophils[140], $CD8^+$ T cells[141, 142], natural killer cells[143] and dendritic cells[144]. Strategies are needed to selectively target tumor cells while preserving immune cell integrity and anticancer immune responses. A compound called N6F11 shows promise in selectively inducing ferroptosis in cancer cells, not immune cells, by triggering TRIM25-dependent

GPX4 degradation[68]. Ferroptosis therapy can also lead to adverse effects like early-onset cachexia[145], stem cell death[146], bone marrow injury[147], hematopoiesis disruption[146], and inflammation-driven tumorigenesis[73, 74, 112].

Clinical translation: While some FDA-approved drugs like sorafenib[77], sulfasalazine[77], artesunate[148], and zalcitabine[50] have shown potential in preclinical ferroptosis induction, their effects may be linked to adverse off-target effects. Identifying safe drugs for patients is crucial, as is considering co-administration of medications to mitigate systemic toxicity and exploring intermittent treatment regimens for better tolerability. Future research should address these aspects to understand ferroptosis in human diseases. Well-designed clinical trials are essential to evaluate the effectiveness, safety, and long-term outcomes of ferroptosis-targeting agents. These trials should enroll specific patient populations, identify sensitive ferroptosis biomarkers, and measure them alongside clinical outcomes.

Conclusion and outlook

In recent years, the field of ferroptosis research has witnessed a remarkable surge. This surge reflects the establishment of a genuine ferroptosis-focused research era[149, 150]. However, the initial definition of ferroptosis as Fe(II)-dependent regulated necrosis accompanied by lipid peroxidation is now recognized as incomplete. Although iron-induced oxidative stress remains a prominent trigger, other iron-independent stimuli or stresses are undoubtedly involved in ferroptosis. Considering that the core downstream feature of ferroptosis is structural damage to cellular membranes resulting from uncontrolled lipid peroxidation, the term “lipotoxicity” may also reflect its core mechanism.

Molecular mechanisms of ferroptosis have expanded beyond the original GPX4 regulatory pathway. This review explores the interplay between pro-ferroptotic and anti-ferroptotic mechanisms, categorized as GPX4-dependent and GPX4-independent, encompassing historical insights and recent findings. However, questions about when, where, and how these pathways activate persist.

Numerous regulatory molecules linked to ferroptosis also play roles in other types of cell death, emphasizing the complexity of intercellular crosstalk. Untangling these mechanisms requires well-designed experiments, stringent controls, and the validation of specific biomarkers. Understanding how physiological and pathological stressors influence ferroptosis in real-world situations remains a challenge. Additionally, the intricate connections between stress pathways leading to ferroptotic and non-ferroptotic cell death require further elucidation.

Despite occasional research limitations and conflicting hypotheses, we maintain optimism about the future prospects of ferroptosis. We believe that the principles of ferroptosis will eventually find clinical applications beyond their heuristic value.

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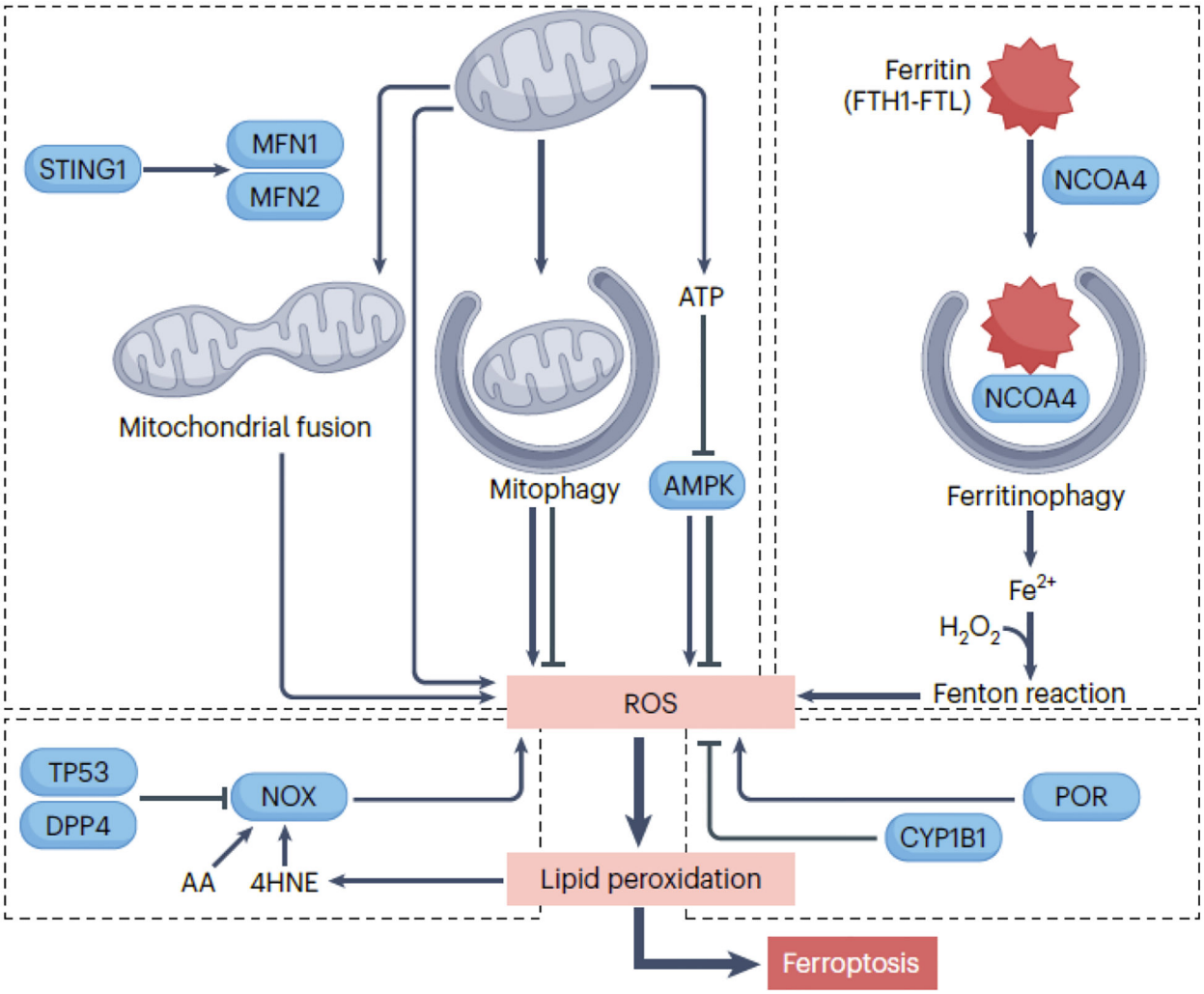

Figure 1. The production of ROS in ferroptosis.

The initiation of ferroptosis requires an oxidative environment, facilitated by diverse sources of ROS. Mitochondrial ROS, primarily generated through the electron transport chain, can trigger ferroptosis in specific conditions. Mitophagy, involved in removing damaged mitochondria, has a dual role in promoting or inhibiting ferroptosis, while mitochondrial fusion increases cellular sensitivity to ferroptosis. Activation of the mitochondrial STING1 (stimulator of interferon response cGAMP interactor 1) may promote mitochondrial fusion, leading to ROS production implicated in ferroptosis. Mitochondrial energy stress activates AMPK, which can promote or inhibit ferroptosis by phosphorylating different substrates. NOX (NADPH oxidase) enzymes in cell membranes play a crucial role in generating ROS in ferroptosis. TP53 inhibits NOX-mediated ferroptosis by binding to DPP4 (dipeptidyl peptidase 4), while arachidonic acid (AA) and 4HNE enhance NOX1 activity to promote ROS production. POR (cytochrome p450 oxidoreductase) promotes ROS production and ferroptosis, whereas CYP1B1 (cytochrome P450 family 1 subfamily B member 1) inhibits ferroptosis. Ferritinophagy involves the degradation of the iron storage protein ferritin, releasing Fe^{2+} that triggers ROS production through the Fenton reaction.

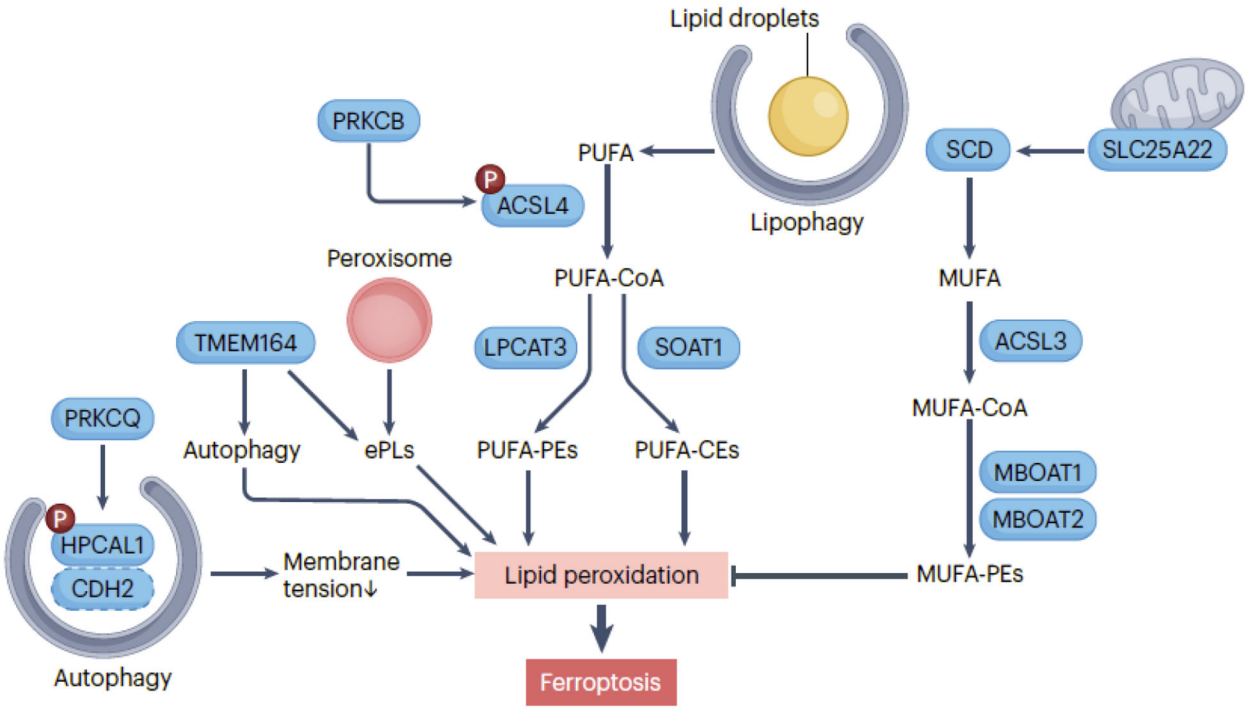

Figure 2. Lipid resources for ferroptosis.

Cell membranes are the primary target of oxidative damage in ferroptosis, influenced by processes and metabolic pathways that promote lipid synthesis. ACSL4 (acyl-CoA synthetase long chain family member 4) plays a critical role in activating polyunsaturated fatty acid (PUFA) by converting them into acyl-CoA esters (PUFA-CoA), which serve as substrates for lipid peroxidation, contributing to the initiation of ferroptosis. Two downstream pathways involve LPCAT3 (lysophosphatidylcholine acyltransferase 3)-mediated PUFA-PEs and SOAT1 (sterol O-acyltransferase 1)-mediated PUFA-CEs. The activity of ACSL4 in ferroptosis is further enhanced by PRKCB (protein kinase C beta)-mediated ACSL4 phosphorylation. HPCAL1 (hippocalcin like 1) phosphorylation by PRKCQ (protein kinase C theta) promotes ferroptosis by inducing autophagic degradation of CDH2 (cadherin 2), leading to alterations in membrane tension in cancer cells. Monounsaturated fatty acid (MUFA) synthesis mediated by SCD (stearoyl-CoA desaturase) and ACSL3 (acyl-CoA synthetase long chain family member 3) counteracts the initiation of ferroptosis by protecting against PUFA peroxidation. The mitochondrial transporter SLC25A22 (solute carrier family 25 member 22) plays a role in inhibiting ferroptosis by facilitating the production of SCD-mediated MUFA. MBOAT1 (membrane bound O-acyltransferase domain containing 1) and MBOAT2 inhibit ferroptosis by remodeling the cellular phospholipid profile to produce MUFA-PEs. Peroxisomes contribute to the biosynthesis of ether phospholipids (ePLs), which are vulnerable to lipid peroxidation. TMEM164 (transmembrane protein 164) functions as an acyltransferase involved in ePLs synthesis or promotes the formation of autophagosomes. Lipophagy, the degradation of lipid droplets, releases lipids that can undergo peroxidation, increasing the susceptibility of cells to ferroptosis.

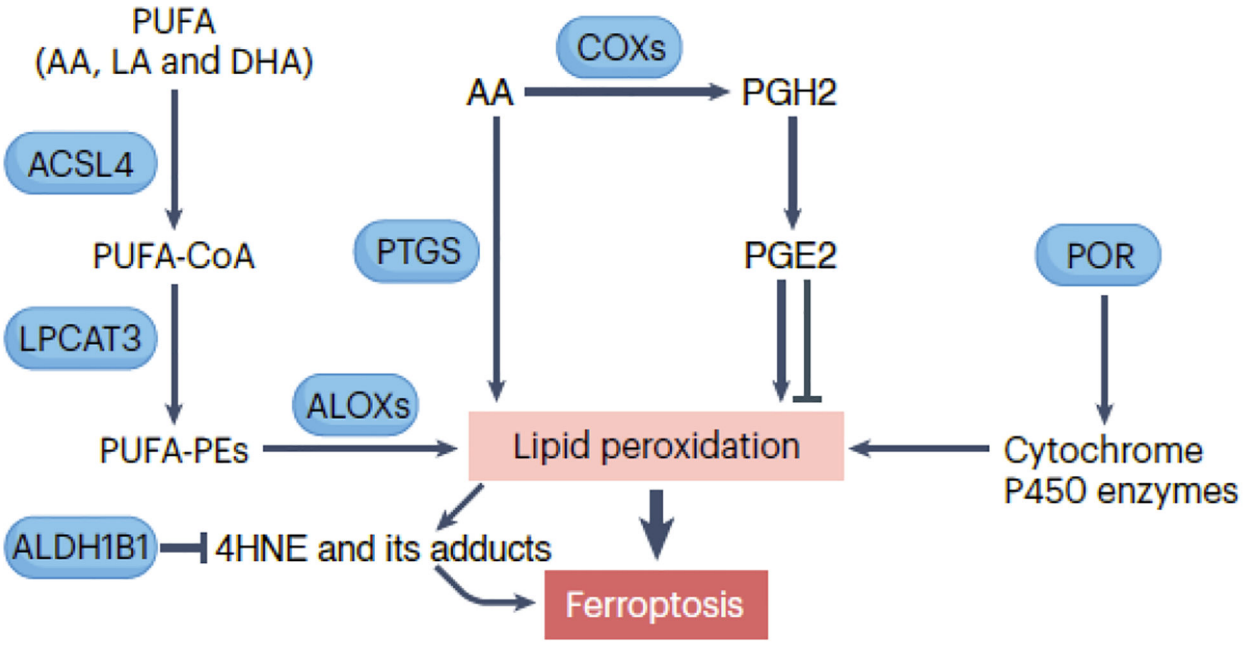

Figure 3. Lipid peroxidation in ferroptosis.

Several key enzymes participate in lipid peroxidation, including ALOX/lipoxygenase, PTGS/cyclooxygenase, and cytochrome P450 enzymes. ALOXs are a family of enzymes that catalyze the oxygenation of polyunsaturated fatty acids (PUFAs), such as arachidonic acid (AA), linoleic acid (LA), and docosahexaenoic acid (DHA), leading to the formation of lipid hydroperoxides. PTGS/cyclooxygenase enzymes are involved in prostaglandin synthesis but can also catalyze lipid peroxidation. The production of prostaglandin H2 (PGH2) and subsequently prostaglandin E2 (PGE2) promotes or inhibits ferroptosis in a context-dependent manner. Additionally, POR plays a role by supplying electrons to cytochrome P450 enzymes involved in the production of lipid hydroperoxides. These hydroperoxides can undergo further reactions, such as decomposition and rearrangement, generating highly reactive lipid radicals. Ultimately, this cascade of reactions can disrupt membrane integrity and contribute to ferroptotic cell death.

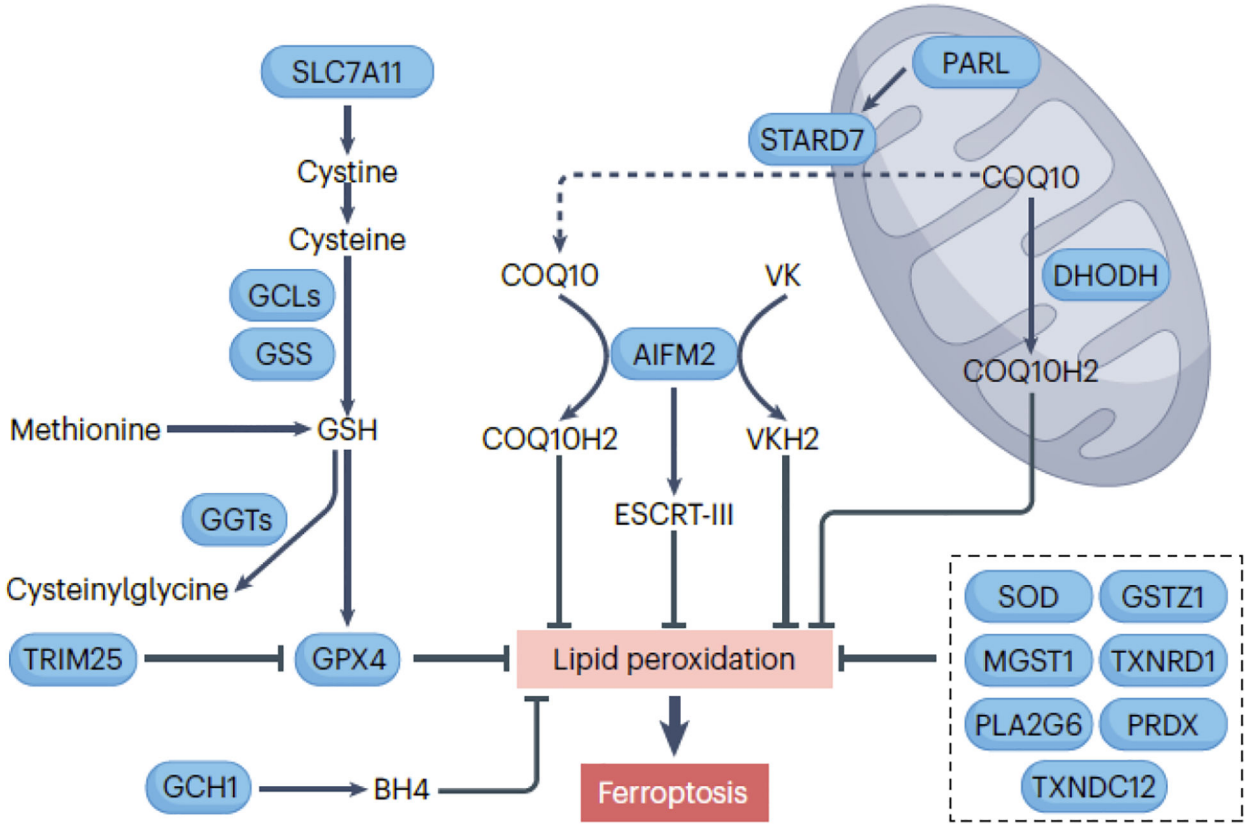

Figure 4. Enzymatic antioxidants in ferroptosis.

The main enzyme central to the antioxidant defense against ferroptosis is GPX4 (glutathione peroxidase 4), which requires the tripeptide cofactor glutathione (GSH), composed of glutamate, cysteine, and glycine. SLC7A11 (solute carrier family 7 member 11) is a key component of the cystine/glutamate antiporter system x_c^-, responsible for allowing the uptake of cystine, which is then reduced to cysteine within the cells. The synthesis of the majority of cellular GSH involves the rate-limiting substrate cysteine, catalyzed by GCLC (glutamate-cysteine ligase catalytic subunit) and GSS (glutathione synthetase). Cysteine can also be derived from the metabolism of methionine. A family of enzymes called GGT (gamma-glutamyltransferase) catalyze the breakdown of GSH into cysteinylglycine and free amino acids. AIFM2 (apoptosis inducing factor mitochondria associated 2) and DHODH dihydroorotate dehydrogenase (quinone)) play pivotal roles in the reduction of COQ10 (coenzyme Q10) to its antioxidant form, COQ10H2, in the plasma membrane/cytoplasm and mitochondria, respectively. The cleavage of STARD7 (StAR related lipid transfer domain containing 7) by the rhomboid protease PARL (presenilin associated rhomboid like) is essential for the synthesis and transport of COQ10 to the plasma membrane/cytoplasm, thereby inhibiting ferroptosis. Furthermore, AIFM2-mediated membrane repair and vitamin K (VK) reduction also contribute to its antiferroptotic activity. GCH1 (GTP cyclohydrolase 1) participates in the biosynthesis of tetrahydrobiopterin (BH4), a cofactor that helps maintain cellular redox balance and antioxidant defenses, thereby inhibiting susceptibility to ferroptotic cell death. Several other enzymes, such as SOD2 (superoxide dismutase) family, MGST1 (microsomal glutathione S-transferase 1), GSTZ1 (glutathione S-transferase zeta

1), TXNRD1 (thioredoxin reductase 1), PLA2G6 (phospholipase A2 group VI), and PRDX (peroxiredoxin) family inhibit ferroptosis in some cases.