Daolin Tang *Editor*

Ferroptosis in Health and Disease

Second Edition

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Preface

The second edition of "Ferroptosis in Health and Disease" represents a comprehensive update to the first edition, which was published several years ago. Since then, there have been numerous exciting developments in our understanding of the molecular mechanisms and physiological implications of ferroptosis, a form of regulated cell death that is dependent on iron and lipid peroxidation.

In this updated edition, I have brought together leading experts in the field to provide a detailed overview of the latest findings in ferroptosis research. The book covers a wide range of topics, including the molecular mechanisms of ferroptosis, its role in various physiological and pathological contexts, and the potential therapeutic approaches to modulate ferroptosis for the treatment of human diseases. The book begins with an introduction to ferroptosis, including its definition, history, and key features. The molecular mechanisms of ferroptosis are then discussed in detail, including the roles of iron, lipid peroxidation, and redox signaling in ferroptotic cell death. The book also covers the regulation of ferroptosis, including the involvement of key signaling pathways, such as the NRF2 and GPX4 pathways.

The role of ferroptosis in various physiological and pathological contexts is also examined, including its involvement in neurodegenerative diseases, cancer, and ischemia-reperfusion injury. The book concludes with a discussion of potential therapeutic approaches to modulate ferroptosis, including the use of antioxidants, iron chelators, and small molecule inhibitors of ferroptosis.

I hope that this book will be a valuable resource for researchers, clinicians, and students who are interested in understanding the complex interplay between iron metabolism, lipid peroxidation, and cell death. I believe that the insights presented in this book will stimulate new ideas and approaches for the development of novel therapeutic strategies targeting ferroptosis in human diseases.

I would like to express my sincere gratitude to all the contributors to this book for their insightful and thought-provoking chapters. Their expertise and dedication have made this updated edition possible. I would also like to thank the editorial and production staff at our publisher for their support and hard work in bringing this edition to fruition.

Dallas, TX, USA — Daolin Tang

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Lipid Metabolism and Homeostasis in Ferroptosis

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Daolin Tang and Rui Kang

Abstract

Lipids are a diverse group of molecules that are crucial for cell structure, energy storage, and cell signaling. Maintaining lipid homeostasis involves a complex network of processes that regulate the synthesis, uptake, transport, storage, and breakdown of lipids. The disruption of lipid homeostasis can result in cell death, particularly through ferroptosis, which is an oxidative cell death driven by uncontrolled lipid peroxidation of polyunsaturated fatty acids and subsequent membrane rupture. Ferroptosis regulation occurs at multiple levels, involving nuclear transcription factors, protein degradation machinery (such as autophagy), and membrane repair pathways. Impaired ferroptosis is implicated in several diseases, including metabolic disorders like diabetes and obesity, as well as cardiovascular disease, neurodegenerative disorders, and cancer. In this chapter, we will provide an overview of lipid metabolism steps and their underlying molecular pathways, and how they affect ferroptosis sensitivity. Understanding the mechanisms that regulate lipid homeostasis during ferroptosis is crucial to develop new therapies for pathological conditions.

1.1 Introduction

Lipids, essential components of all cells, perform a variety of important functions. They are critical constituents of various cellular compartments, including the plasma membrane and organelle membranes, such as the nuclear, endoplasmic reticulum (ER), Golgi apparatus, and transportation vesicles like endosomes and lysosomes (Natesan and Kim 2021). Mammalian cells express tens of thousands of distinct lipid

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species and rely on hundreds of proteins to synthesize, metabolize, and transport them, thus maintaining lipid homeostasis. Despite being almost as complex and diverse as proteins, the functions of lipids in cell survival and cell death are context-dependent (Flores-Romero et al. 2020).

Ferroptosis is a regulated form of cell death that occurs when the accumulation of iron-dependent reactive oxygen species (ROS) leads to lipid peroxidation and insufficient capacity to eliminate lipid peroxides (Tang and Kroemer 2020a). Ferroptosis is distinguishable from apoptosis based on its unique morphological, biochemical, and genetic characteristics (Galluzzi et al. 2018). Morphologically, ferroptotic cells display smaller mitochondria, a decrease or disappearance of mitochondrial cristae, and condensed mitochondrial membrane densities, while their nuclear structure remains intact without karyorrhexis or margination of chromatin, as seen in apoptosis (Xie et al. 2016). Ferroptosis can be induced by small molecules, such as erastin, RSL3, and related compounds, while lipid peroxidation inhibitors, such as vitamin E, ferrostatin-1, and liproxstatin-1, as well as iron chelators (e.g., deferoxamine mesylate) can block it (Stockwell et al. 2017). Notably, ferroptosis does not require the caspase family, which are key mediators of apoptosis (Tang et al. 2019). Identifying the molecular mechanisms and signaling pathways of ferroptosis is crucial for developing new diagnostic and therapeutic approaches in human disease (Tang et al. 2021). Growing evidence suggests that lipid metabolism plays a crucial role in determining cellular sensitivity to ferroptosis (Lin et al. 2021; Carbone and Melino 2019). This chapter outlines the types and dynamics of lipid metabolism in regulating ferroptosis (Fig. 1.1).

1.2 Lipid Resource

In recent years, several studies have aimed to identify the lipid species that are oxidized during ferroptosis and are functionally relevant to the process. Lipidomics assays conducted in human fibrosarcoma cells treated with erastin or phosphatidylethanolamines (PE) have revealed that polyunsaturated fatty acids (PUFAs) are the most susceptible lipids to peroxidation during ferroptosis (Yang et al. 2016). Cells supplemented with PUFAs promote ferroptosis, while exchanging PUFAs with deuterated PUFAs that are less susceptible to oxidation, or deleting genes required for the activation and incorporation of PUFAs into phospholipids, inhibit ferroptosis (Doll et al. 2017; Kagan et al. 2017; Yang et al. 2016). Thus, supplementing the culture media with PUFAs can induce a ferroptosis-susceptible state in a melanoma cell line that is inherently resistant to ferroptosis, presumably due to increased PUFA incorporation into phospholipid membranes (Zou et al. 2020b).

PUFAs are highly susceptible to lipid peroxidation in the presence of oxygen, and this peroxidation reaction is accelerated by divalent metals, especially Fe (II) (Pratt et al. 2011). Cell types that contain relatively high levels of PUFAs are more sensitive to lethal oxidative stress (Alvarez and Storey 1989; Robison et al. 1982). Unsaturated organic molecules with weak C–H bonds, particularly those with

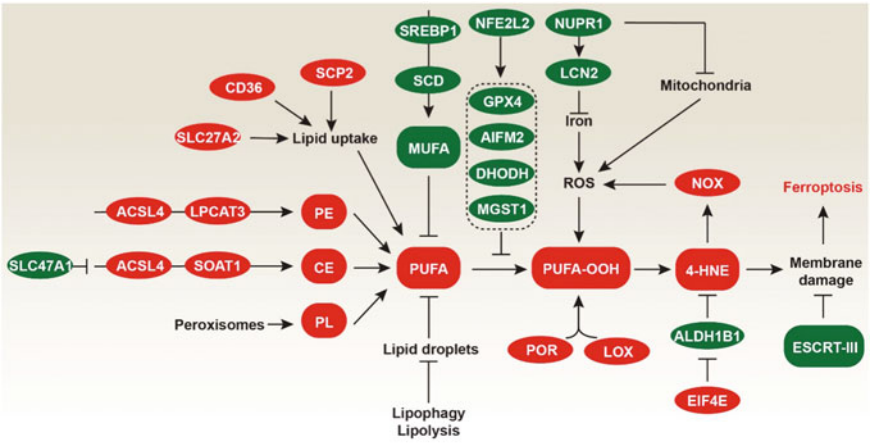

Fig. 1.1 Lipid metabolism in ferroptosis. Ferroptosis is primarily caused by the excessive oxidation of polyunsaturated fatty acids (PUFAs) on membrane structures, including the plasma membrane and organelle membrane. Three sources of PUFAs are generated by acyl-CoA synthase 4 (ACSL4)-lysophosphatidylcholine acyltransferase 3 (LPCAT3)-mediated phospholipids (PE), ACSL4-sterol O-acyltransferase 1 (SOAT1)-mediated cholesteryl ester (CE), and peroxisome-mediated plasmalogens (PL). Increased lipid uptake by CD36, sterol carrier protein 2 (SCP2), or solute carrier family 27 member 2 (SLC27A2) maintains PUFA levels, whereas lipid droplet formation reduces this process. Conversely, lipophagy and lipolysis can degrade lipid droplets to release fatty acids for ferroptosis. Sterol regulatory element binding transcription factor 1 (SREBP1)-dependent stearoyl-CoA desaturase (SCD) expression promotes monounsaturated fatty acid (MUFA) production, thereby inhibiting PUFA oxidation to PUFA-OOH. The generation of reactive oxygen species (ROS) for lipid peroxidation mainly occurs from iron-dependent Fenton reaction, mitochondrial respiration, or membrane NADPH oxidase (NOX) family. The POR and LOX families are key mediators of lipid peroxidation. Several antioxidant enzymes, such as glutathione peroxidase 4 (GPX4), apoptosis-inducing factor mitochondria-associated 2 (AIFM2), dihydroorotate dehydrogenase (DHODH), and microsomal glutathione S-transferase 1 (MGST1), inhibit lipid peroxidation at different subcellular locations. The master transcription factor, NFE2 like BZIP transcription factor 2 (NEF2L2), increases its protein stability during oxidative stress, leading to the expression of antioxidant genes to suppress ferroptosis. Nuclear protein-1 (NUPR1) is another important stress protein that leads to ferroptosis resistance by upregulating lipocalin-2 (LCN2) to limit iron accumulation or block mitochondrial oxidative damage. The end product of lipid peroxidation, 4-hydroxynonenal (4-HNE), can activate NOX on the membrane, thereby intensifying lipid peroxidation and cell membrane damage. In contrast, aldehyde dehydrogenase 1B1 (ALDH1B1) can scavenge 4-HNE, and this process is inhibited by the formation of the ALDH1B1-eukaryotic translation initiation factor 4E (EIF4E) complex. Finally, membrane damage can be repaired through the recruitment and activation of the endosomal sorting complexes required for transport-III (ESCRT-III) machinery

double bonds adjacent to methylene groups in PUFAs, are prone to undergo autoxidation, which weakens the hydrogen bond energy of the bis-allylic methylene groups, making them more susceptible to hydrogen abstraction and consequent oxygenation. Thus, the membrane phospholipids containing high levels of PUFAs are particularly sensitive to damage by ROS (Barrera 2012). Furthermore, PUFAs themselves shift into reactive free radicals after oxidizing with free radicals, which

can propagate lipid peroxidation chain reactions. Accordingly, the capability of cells to undergo ferroptosis generally depends on the abundance and localization of PUFA within the context of phospholipid bilayers (Conrad et al. 2018).

Using quantitative redox lipidomics, reverse genetics, bioinformatics, and systems biology, only one class of phospholipids, PE, has been found to be involved in RSL3 or deletion of glutathione peroxidase 4 (GPX4)-induced ferroptosis of mouse fibroblasts. These phospholipids undergo oxidation in the ER-associated compartments with specificity toward two fatty acyls – arachidonoyl (AA) and adrenoyl (AdA) (Kagan et al. 2017). The suppression of AA or AdA esterification into PE by genetic or pharmacological inhibition of acyl-CoA synthase 4 (ACSL4) appears to be a specific anti-ferroptotic rescue pathway (Kagan et al. 2017). Exogenous AA/AdA-PE-OOH, but not free AA-OOH or AdA-OOH, causes ferroptotic cell death. Peroxisome-mediated plasmalogen (PL) synthesis also contributes to ferroptosis by synthesizing polyunsaturated ether phospholipids (PUFA-PLs), which act as substrates for lipid peroxidation that, sequentially, leads to the induction of ferroptosis in neoplastic cells, neurons, and cardiomyocytes (Zou et al. 2020a). These works emphasize that PUFA-PLs are a distinct functional lipid class that is necessary and sufficient to induce sensitivity to ferroptosis.

Our study shows that PUFA-esterified cholesteryl esters (CEs) and lipid droplet degradation also promote ferroptosis in some contexts (Lin et al. 2022; Bai et al. 2019). In contrast, exogenous monounsaturated fatty acids (MUFAs), such as oleic acid and palmitoleic acid, inhibit ferroptosis induced by erastin and RSL3 by preventing plasma membrane lipid peroxidation (Magtanong et al. 2019). MUFAs inhibit the accumulation of lipid ROS at the plasma membrane and displace PUFAs from this location in the cell. Blocking MUFA uptake, activation, or de novo synthesis may help overcome the ferroptosis-resistant state and increase the effectiveness of current pro-ferroptotic agents. Overall, while different types of PUFAs promote ferroptosis, MUFAs inhibit it, although most of these conclusions have been drawn from studies in cancer cells.

1.3 Lipid Synthesis

The diversity of fatty acyl moieties in membrane phospholipids affects the biophysical properties of cell membranes, including their fluidity, curvature, and subdomain architecture, which, in turn, determine cell sensitivity to ferroptosis (Wang and Tontonoz 2019). The abundance and localization of PUFAs play a crucial role in the development of ferroptotic signals, which require the esterification and oxidation of free PUFAs in membrane phospholipids. To produce these signals, coenzyme-A-derivatives of PUFAs must be formed and inserted into phospholipids. The conversion of long-chain fatty acid stoacyl-CoA by acyl-CoA synthetase and reacylation by lysophospholipid acyltransferases are necessary for the incorporation of de novo synthesized fatty acids into phospholipids (Mashima et al. 2009; Hishikawa et al. 2008, 2014). The lipid composition of cells can profoundly affect their sensitivity to

ferroptosis-inducing agents due to the central role of lipid peroxidation in the ferroptosis process (Chen et al. 2021b, 2021c).

1.3.1 The ACSL4-LPCAT3-PUFA-PE Pathway

The ACSL family plays a critical role in lipid metabolism by converting long-chain fatty acids (LCFAs) to their CoA thioesters, which are used in various metabolic processes (Kuwata and Hara 2019). Among the five isoforms of the ACSL family, ACSL4 is particularly important for ferroptosis and regulating inflammation and has been implicated in diseases, such as cancer (Yuan et al. 2016; Li et al. 2019; Cheng et al. 2020). Other members of the ACSL family, such as ACSL1 and ACSL3, may play a context-dependent role in either promoting or inhibiting ferroptosis (Beatty et al. 2021; Klasson et al. 2022; Magtanong et al. 2019).

Recent studies using genetic screening systems and microarray analysis have identified ACSL4 as an essential component for ferroptosis execution, as it accumulates oxidized cellular membrane phospholipids (Dixon et al. 2015). The inhibition or knockout of ACSL4 suppresses ferroptosis, and the supplementation with certain fatty acids rescues sensitivity to ferroptosis in ACSL4-deficient cells (Doll et al. 2017). ACSL4 has a preference for long PUFAs, such as AA and AdA, which enrich cellular membranes and contribute to the production of lipid peroxide. This lipid peroxide further stimulates ferroptosis and provides mechanistic insights into why metastasis-prone cancer cells are highly sensitive to ferroptosis. ACSL4 expression is a good candidate predictive biomarker for ferroptosis sensitivity in hepatocellular carcinoma and for predicting sorafenib sensitivity (Feng et al. 2021; Yuan et al. 2016).

ACSL4 activation can activate lysophosphatidylcholine acyltransferases (LPCATs), a group of enzymes responsible for phospholipid remodeling. LPCATs catalyze the incorporation of fatty acyl chains into the sn-2 site of phosphatidylcholine (PC), thereby modulating the fatty acyl composition of phospholipids (Wang and Tontonoz 2019; Rong et al. 2013). Once ligated with coenzyme A through ACSL4, long-chain PUFAs are used by LPCAT3 to esterify lysophospholipids. These PUFAs may undergo peroxidation once seeded into membranes (Conrad and Pratt 2019). LPCAT3 plays a crucial role in maintaining systemic lipid homeostasis by regulating lipid absorption, lipoprotein secretion, and de novo lipogenesis (Wang and Tontonoz 2019). In addition, LPCAT3 is tightly involved in ferroptosis, as demonstrated by bulky insertional mutagenesis of RSL3 and ML162-resistant clone cells (Dixon et al. 2015). Mechanistically, LPCAT3 is thought to incorporate arachidonic acid into membranes, and the lack of LPCAT3 leads to extreme reductions in membrane arachidonate levels (Hashidate-Yoshida et al. 2015). Therefore, LPCAT3 is also an essential factor in ferroptosis induction, most likely by modulating arachidonic acid metabolism. LPCAT3 esterifies these derivatives into phosphatidylethanolamines (AA-PE and AdA-PE), which are primarily present in the ER. Although it has been shown to be involved in PC synthesis, the specific role of LPCAT3 in different tissues and cell types is not clear.

1.3.2 The ACSL4-SOAT1-PUFA-CE Pathway

The delicate balance between cell survival and death is heavily influenced by the physical and chemical properties of cell membranes. The susceptibility to ferroptosis is determined by lipid remodeling, which provides substrates for this process. The phospholipids PE and PC are key players in mediating ferroptosis. Solute carrier family 47 member 1 (SLC47A1, also known as MATE-1), a membrane transporter protein primarily expressed in the liver and kidneys, plays a vital role in eliminating various drugs and toxins from the body by transporting organic cations across cell membranes (Freitas-Lima et al. 2020; Estrela et al. 2017; Wittwer et al. 2013). Mutations in the SLC47A1 gene have been linked to altered drug response and toxicity in some individuals. Recent studies indicate that SLC47A1 acts as an endogenous repressor of ferroptosis, likely by influencing the concentrations and metabolism of PUFAs rather than eliminating ferroptosis activators (Lin et al. 2022). Direct or indirect inhibition of the function of lipid flippase of SLC47A1 may sensitize cancer cells to pharmacological ferroptosis induction (Lin et al. 2022).

Peroxisome proliferator activated receptor alpha (PPARA) is a transcription factor and nuclear receptor protein that regulates the expression of genes involved in cellular processes such as lipid metabolism, glucose homeostasis, and inflammation (Wagner and Wagner 2022). PPARA-mediated expression of SLC47A1 on cell membranes is a key regulator of lipid remodeling and cell survival during ferroptosis (Lin et al. 2022). In wild type cells, the ACSL4-LPCAT3 pathway-mediated PUFA-PE production favors ferroptosis. However, in SLC47A1 deficient cells, the ACSL4-sterol O-acyltransferase 1 (SOAT1) pathway-mediated PUFA-CE production promotes ferroptosis (Lin et al. 2022). SOAT1 is an enzyme primarily involved in esterifying cholesterol in the liver and intestine. Thus, the ACSL4-SOAT1 pathway is a potential target for treating ferroptosis-related diseases.

1.3.3 The Peroxisomes-PUFA-PL Pathway

Peroxisomes are organelles within eukaryotic cells that play a crucial role in a range of metabolic processes. Their enzymatic content facilitates the breakdown of fatty acids, amino acids, and other metabolites, as well as the generation and removal of hydrogen peroxide. Peroxisomes also contribute to lipid metabolism and biosynthesize PL, a crucial class of membrane lipids. Notably, peroxisomes synthesize PUFA-PL, which has been found to promote ferroptosis in cancer cells (Zou et al. 2020a; Cui et al. 2021). Through whole-genome CRISPR-Cas9 screens with GPX4 inhibitors, several peroxisomal and ether-lipid biosynthesis genes (e.g., peroxisomal biogenesis factor 3 [PEX3], PEX10, alkylglycerone phosphate synthase [AGPS], and fatty acyl-CoA reductase 1 [FAR1]) were identified as promoting resistance to ferroptosis when deleted (Zou et al. 2020a). This suggests that cells may downregulate PUFA-PL biosynthesis as a strategy to evade ferroptosis while upregulating it to promote sensitivity to this process by serving as substrates for lipid ROS propagation. These findings also suggest that PUFA-PL synthesized by

peroxisomes could represent a vulnerability that cells manipulate to evade or promote ferroptosis (Tang and Kroemer 2020b). Since peroxisomes have a dual role in oxidative stress, the underlying mechanisms and signals involved in different contexts have yet to be fully characterized.

1.3.4 The SREBP1-SCD Pathway

Stearoyl-CoA desaturase (SCD/SCD1) is an important lipid desaturase enzyme that catalyzes the rate-limiting step in the synthesis of MUFAs. Its upregulation is observed in various malignancies, including prostate, liver, and breast cancer, and it plays a crucial role in lipid metabolism. Inhibition of SCD can induce changes in cellular lipid content and lead to the depletion of endogenous membrane antioxidant CoQ10, which has been linked to ferroptosis (Tesfay et al. 2019). SCD inhibition can also decrease unsaturated fatty acyl chains in membrane phospholipids and increase long-chain saturated ceramides (Tesfay et al. 2019). Therefore, SCD is involved in the regulation network of various cancer cell lines and is a potential therapeutic target for ferroptosis-based cancer treatment.

Sterol regulatory element binding transcription factor 1 (SREBP1), a transcription factor, is a key regulator of lipid metabolism in the body, which is primarily responsible for regulating genes involved in fatty acid and cholesterol synthesis and uptake (Chen et al. 2021a). The dysregulation of SREBP1 is associated with metabolic disorders, such as obesity, diabetes, and atherosclerosis. SREBP1 activates the expression of SCD and promotes the production of MUFAs in ferroptosis (Yi et al. 2020; Zhao et al. 2020). The SREBP1-SCD pathway is a potential target for combined therapies in cancer treatment. Examining the effects of dietary factors, such as different types of fats and carbohydrates, on the SREBP1-SCD pathway and lipid metabolism poses a challenge for researchers.

1.4 Lipid Peroxidation

Redox equilibrium is a vital feature of biological systems, which enables them to balance oxidative and reducing reactions to achieve optimal conditions for life. However, in ferroptosis, this equilibrium is disrupted by the accumulation of oxidizing molecules caused by either overproduction or loss of cellular reducing ability. These accumulated oxidizing agents can oxidize various cellular components, including DNA, proteins, and lipids, thereby altering their structure, physical properties, and activity. One key downstream feature of ferroptosis is lipid peroxidation, with its products considered the proximate executioners of ferroptosis (Conrad et al. 2018). As such, compounds and molecules that impede lipid peroxidation may be useful in preventing ferroptosis (Kuang et al. 2020).

Lipid peroxidation is triggered by several steps, including hydrogen abstraction, double bond migration, the addition of triplet oxygen, and subsequent oxidation cascades, eventually leading to the breakage of the oxidized fatty acyl group and

production of by-products, such as 4-hydroxynonenal (4-HNE) and malondialdehyde (MDA) (Romero et al. 1998). This process primarily targets long-chain fatty acids with multiple double bonds, such as linoleic, arachidonic, and docosahexaenoic acids. Enzymatic and non-enzymatic processes can catalyze lipid peroxidation, with the substrate extent and typical mechanism being mostly similar in both cases.

Non-enzymatic lipid peroxidation is mediated by carbon- and oxygen-centered radicals known as autoxidation, with iron playing a critical role in initiating this process. While most iron is tightly bound in heme, FeS clusters, or ferritin, a small amount is loosely ligated (labile) and can participate in redox reactions. The labile iron pool can catalyze redox reactions via the "Fenton reaction" ($Fe(II) + H_2O_2 \rightarrow Fe(III) + HO\bullet + HO^-$), which leads to a free radical chain reaction that inserts a molecule of oxygen into the C–H bond of an organic substrate, ultimately generating a hydroperoxide (Winterbourn 1995). Consequently, non-enzymatic lipid peroxidation can be considered a chain reaction. Moreover, cellular enzymes, such as lipoxygenases (LOXs), Cytochrome P450 oxidoreductase (POR), and cyclooxygenases (COXs), can catalyze lipid peroxidation in a controlled manner, with LOXs and POR being involved in promoting ferroptosis.

LOXs are nonheme iron-containing dioxygenases that catalyze the stereospecific insertion of oxygen into PUFAs. In humans, there are six ALOX genes: ALOX5, ALOX12, ALOX12B, ALOX15, ALOX15B, and ALOXE3. LOXs are named and categorized based on the positional numbered carbon where they oxygenate their PUFA substrates. The role of LOXs in ferroptosis is tissue and cell specific (Wenzel et al. 2017; Mashima and Okuyama 2015; Liu et al. 2015; Li et al. 2021; Friedmann Angeli et al. 2014; Matsushita et al. 2015; Brutsch et al. 2015; Ou et al. 2016). LOXs catalyze the oxygenation of PUFAs to form lipid hydroperoxides, which can react further with iron to generate highly reactive lipid radicals. These radicals can then propagate lipid peroxidation and lead to the accumulation of lipid peroxides, ultimately triggering ferroptotic cell death. Inhibiting LOXs or their downstream signaling pathways has emerged as a potential therapeutic strategy for preventing ferroptosis in various diseases, including cancer, neurodegeneration, and ischemia–reperfusion injury.

POR is a flavoprotein that facilitates electron transfer to various enzymes, including cytochrome P450 monooxygenases involved in drug and xenobiotic metabolism, as well as in the biosynthesis of steroid hormones, bile acids, and vitamin D (Manikandan and Nagini 2018). In ferroptosis, POR plays a crucial role in promoting lipid peroxidation and cell death. Studies using CRISPR/Cas9-mediated suppressor screens identified POR as a contributor to ferroptotic cell death in clear-cell renal cell carcinoma and melanoma cell lines, demonstrating its ability to up-regulate the peroxidation of membrane polyunsaturated phospholipids (Zou et al. 2020b). Moreover, in HeLa cells, both POR and CYB5R1 promote ferroptosis by transferring electrons from NAD(P)H to oxygen to generate H_2O_2, which reacts with iron to produce hydroxyl radicals, leading to peroxidation of PUFA-containing membrane phospholipids in an ALOX-independent manner (Yan et al. 2020). Therefore,

inhibiting POR may represent a promising therapeutic target for the treatment of ferroptosis-related diseases.

1.5 Lipid Storage and Degradation

Lipid droplets are intracellular organelles found in most eukaryotic cells that are composed of a neutral lipid core (primarily triacylglycerol and cholesterol ester) surrounded by a phospholipid monolayer and associated proteins. They are dynamic structures that can grow or shrink in response to changes in cellular energy and nutrient availability, and they serve as a major storage site for lipids within cells. The formation of lipid droplets is primarily derived from the ER (Henne et al. 2018). These lipid droplets are metabolized to offer lipids for cellular metabolism and have a unique ability to consolidate various fatty acid fluxes and regulate their input into pathways required for membrane homeostasis (Petan 2020). In addition to their primary function, lipid droplets also play a crucial role in protecting the cell from lipotoxicity by regulating the (poly)unsaturated fatty acid trafficking (Herms et al. 2013; Herms et al. 2015). They modulate lipid peroxidation and ferroptosis sensitivity and act as antioxidant organelles by actively regulating the trafficking of PUFAs that block oxidative stress and cell death (Bai et al. 2019; Zou et al. 2019). There is a crosstalk between ferroptosis and lipid droplets. At the early stage of RSL3-induced ferroptosis, lipid droplet accumulation is increased, but it decreases at the later stage (Bai et al. 2019). Lipid droplet viscosity also increases in cancer cells during ferroptosis induction. Interestingly, molecules that promote lipid droplet formation negatively regulate ferroptosis, such as tumor protein D52 (TPD52), perilipin 2 (PLIN2), and squalene, which lead to ferroptosis resistance in certain cancer cells (Bai et al. 2019; Dong et al. 2020; Sun et al. 2020; Garcia-Bermudez et al. 2019). Lipid droplet degradation occurs via lipolysis and lipophagy, both of which exhibit a significant crosstalk and play a positive regulatory role in ferroptosis (Bai et al. 2019). Lipid droplets breakdown via lipophagy has been shown to promote GPX4 inhibition-induced ferroptosis in hepatocytes (Bai et al. 2019). PUFA-triacylglycerols stored within lipid droplets are drivers of ferroptotic sensitivity by providing PUFAs for phospholipid membrane synthesis (Bezawork-Geleta et al. 2022). Conversely, the presence of lipid droplets appears to inhibit ferroptosis in cells. The functional heterogeneity of lipid droplets and their roles in lipid metabolism, as well as their involvement in ferroptotic processes, still require further investigation.

1.6 Lipid Uptake and Utilization

The transfer of phospholipids from their sites of synthesis to organelle membranes is crucial for a variety of biological processes, including ferroptosis. Lipid transport proteins are responsible for lipid trafficking, and among these proteins, sterol carrier protein 2 (SCP2) is a type of lipid transport protein that facilitates the transport of

peroxidized lipids to mitochondria and promotes oxidative stress between compartments (Vila et al. 2004). SCP2-mediated trafficking of peroxidized lipids to mitochondria leads to GPX4 depletion-induced ferroptosis, which causes acute renal failure in mice (Friedmann Angeli et al. 2014). However, this effect is temporary and only slows down cell death kinetics, suggesting that other translocases may work in conjunction with SCP2.

In addition to de novo synthesis, cells can transport extracellular fatty acids using fatty acid transporters, such as CD36 (also known as fatty acid translocase [FAT]), solute carrier family 27 member 2 (SLC27A2, also known as fatty acid transporter 2 [FATP2], and fatty acid-binding proteins (FABPs). Cancer cells can also use exogenous lipids when de novo synthesis is inhibited. However, increased expression of CD36, which is involved in fatty acid intake, can promote tumor metastasis. Therefore, blocking CD36 can inhibit tumor growth and metastasis in prostate cancer models. In CD36-overexpressing cells (cancer or immune cells), fatty acids taken up via CD36 are mostly stored and not used for fatty acid oxidation, which can promote ferroptosis (Ma et al. 2021). CD36 may also suppress ferroptosis by exporting AA (Kuda et al. 2011). Loss of SLC27A2 results in impaired uptake of AA, leading to neutrophils resistant to ferroptosis in the tumor microenvironment (Veglia et al. 2019). In gastric cancer cells, the expression of SLC27A2 is decreased. However, cells can compensate for the reduction in SLC27A2 by enhancing the de novo synthesis of fatty acids to counteract the effect of AA deficiency, which enables cells to maintain sensitivity to ferroptosis (Lee et al. 2020).

Fatty acids are broken down in the mitochondria via β-oxidation, a biochemical process in which two carbon units are removed from the carboxyl end of a fatty acid molecule to produce acetyl-CoA. Acetyl-CoA carboxylase alpha (ACACA/ACC1) is a key enzyme involved in both fatty acid biosynthesis and β-oxidation and plays a context-dependent role in promoting ferroptosis (Shimada et al. 2016). Silencing of ACACA in HT1080 cells results in resistance to FIN56, but not RSL3 (Shimada et al. 2016). However, the inhibition of ACACA blocks both erastin- and RSL3-induced ferroptosis in MEFs (Li et al. 2020). 2,4-Dienoyl-CoA reductase 1 (DECR1), a rate-limiting enzyme that oxidizes PUFAs, induces lipid peroxidation and cellular ferroptosis (Nassar et al. 2020). In addition, reduced fatty acid metabolism resulting from VHL-mediated β-oxidation inhibition renders renal cancer cells highly sensitive to glutathione (GSH) inhibition-induced ferroptosis (Miess et al. 2018). Although inhibition of β-oxidation can restore tumor cells' sensitivity to ferroptosis, etomoxir, a potential inhibitor of β-oxidation of PUFA, stimulates rather than suppresses RSL3-induced cell death (Kagan et al. 2017). Gaining a better understanding of fatty acid turnover, including the uptake and utilization of fatty acids in cells, may help to unravel the complex regulation of ferroptosis by different lipids.

1.7 Defense Mechanism

Cells have developed various mechanisms to protect themselves against lipid oxidation damage. One of the most crucial strategies is the use of antioxidants, which are capable of neutralizing free radicals and preventing them from reacting with lipids. Antioxidants can either be obtained from the diet or produced within the body, and examples include vitamin E, vitamin C, glutathione (GSH), and superoxide dismutase. Additionally, cells have enzymes that can remove and replace oxidized lipids in cell membranes to aid in their repair and maintain membrane integrity. Furthermore, cells can regulate the composition of their membranes to make them less susceptible to oxidation, such as by increasing levels of saturated and monounsaturated fatty acids, which are more stable and less prone to oxidation than polyunsaturated fatty acids. Finally, cells have membrane repair mechanisms to defend against oxidative damage. Overall, these defense mechanisms against lipid oxidation damage work in concert to protect cells from the harmful effects of lipid oxidation and maintain their normal function during ferroptosis (Liu et al. 2021b). In the following sections, we discuss several important molecules involved in inhibiting ferroptosis-induced oxidation damage.

1.7.1 GPX4

There are numerous members of the GPXs family, including GPX1 to GPX8. However, GPX4 plays a more significant role in ferroptosis. GPX4, an enzyme that directly reduces lipid hydroperoxides in biomembranes, can convert GSH to oxidized glutathione disulfide (GSSG) and maintain cellular redox homeostasis (Yang et al. 2014). Both RSL3 and erastin are ferroptosis inducers that can cause an increase in lipid ROS. However, erastin reduces GSH content by inhibiting system xc^-, which hinders cystine absorption. On the other hand, RSL3 does not affect the GSH level when inducing BJeLR cell death. Therefore, it is speculated that RSL3 may induce ferroptosis through different mechanisms. Using fluorescein-labeled RSL3, BJeLR cells were screened, and GPX4 was identified as the target protein of RSL3. GPX4 is an inhibitory protein of the lipid peroxidation process that can degrade small molecular and relatively complex lipid peroxides (Yang et al. 2014). Conversely, upregulating GPX4 expression confers resistance to ferroptosis. Moreover, the mevalonate pathway (MVA pathway) can affect GPX4 by regulating the maturation of selenocysteine tRNA, which can cause ferroptosis in cells. Selenocysteine is one of the amino acids in the active center of GPX4, and its insertion into GPX4 requires a specific transporter, selenocysteine tRNA (Ingold et al. 2018; Warner et al. 2000). Isopentenyltransferase is required for the maturation of selenocysteine tRNA, which transfers the isopentenyl group in isopentenylpyrophosphate (IPP) to the selenocysteine tRNA precursor. IPP is a product of the MVA pathway; thus, the MVA pathway can downregulate IPP, affecting the synthesis of selenocysteine tRNA, interfering with the activity of GPX4, and causing ferroptosis.

1.7.2 AIFM2

CoQ10 is a lipid-soluble antioxidant that is endogenously produced and has been shown to block the harmful oxidation of proteins, lipids, and DNA. Recently, apoptosis-inducing factor mitochondria-associated 2 (AIFM2), also known as FSP1, has been identified as an antioxidant regulator in ferroptosis, regardless of its mitochondrial function (Bersuker et al. 2019; Doll et al. 2019). AIFM2 uses nicotinamide adenine dinucleotide phosphate (NADPH) to regenerate ubiquinol, the reduced form of CoQ10, which acts as a cellular intrinsic lipophilic radical-trapping antioxidant and prevents oxidative lipid damage in a wide range of cancer cell lines, including breast, lung, brain, colon, and liver cancer. N-myristoylation is required for the translocation of AIFM2 from mitochondria to the cell membrane where it catalyzes the regeneration of non-mitochondrial reduced CoQ10 using NADPH, thereby trapping lipid peroxides in a GPX4-independent manner. AIFM2 also inhibits ferroptosis by activating endosomal sorting complexes required for transport III (ESCRT-III)-dependent membrane repair instead of its oxidoreductase function (Dai et al. 2020c). AIFM2 activity in ferroptosis is specifically inhibited by a small molecule compound called iFSP1, although it was not toxic alone, unlike GPX4 inhibitors (Doll et al. 2019). AIFM2-mediated Vitamin K metabolism not only inhibits ferroptosis, but also highlights the multifaceted role of AIFM2 in controlling the antiferrptotic response.

1.7.3 DHODH

Dihydroorotate dehydrogenase (DHODH) is an enzyme involved in the biosynthesis of pyrimidines, essential building blocks of DNA and RNA. DHODH is located in the mitochondrial inner membrane and catalyzes the fourth step of the de novo synthesis of pyrimidines, converting dihydroorotate to orotate (Zhou et al. 2021). DHODH is also involved in the production of ubiquinone, an important component of the electron transport chain. Studies have shown that DHODH can affect ferroptosis by regulating the production of ubiquinone and the redox status of cells (Mao et al. 2021). DHODH inhibitors, such as brequinar and leflunomide, have been shown to induce ferroptosis in cancer cells by depleting ubiquinone levels and increasing oxidative stress. DHODH inhibition leads to a decrease in the activity of complex III of the electron transport chain, resulting in reduced production of the antioxidant ubiquinone and an increase in mitochondrial oxidative stress. This, in turn, leads to lipid peroxidation and ultimately ferroptosis (Mao et al. 2021). Overall, these findings establish a mitochondrial defense mechanism to inhibit ferroptosis. Given that DHODH is a synthetic lethal vulnerability in mutant *KRAS*-driven cancers (Koundinya et al. 2018), DHODH inhibition-induced ferroptosis may be a promising approach for targeted therapy in a wide range of cancers.

1.7.4 MGST1

Microsomal glutathione S-transferase 1 (MGST1) belongs to the glutathione S-transferase (GST) family of enzymes and is primarily found in the ER and the outer mitochondrial membrane of cells. MGST1 catalyzes the conjugation of the antioxidant molecule GSH to a variety of electrophilic compounds, including lipid peroxidation products, thereby preventing them from causing further damage to the cell. In ferroptosis, MGST1 plays a protective role by promoting the degradation of lipid peroxides (Kuang et al. 2021). Specifically, MGST1 can catalyze the conjugation of GSH to lipid peroxides, which generates a water-soluble product that can be excreted from the cell. This reduces the levels of lipid peroxides in the cell and helps to prevent the accumulation of lipid peroxides, which is a hallmark of ferroptosis. The overexpression of MGST1 has been shown to protect pancreatic cancer cells from ferroptosis, partially by binding to ALOX5 and reducing lipid peroxidation (Kuang et al. 2021). However, the expression of SLC7A11 and GPX4 remains unaffected by changes in MGST1. Taken together, these findings suggest that MGST1 may act as a ferroptosis suppressor alongside the GPX4 pathway, highlighting its potential as a novel therapeutic target for pancreatic tumors.

1.7.5 ALDH1B1

Aldehyde dehydrogenase 1 family member B1 (ALDH1B1) is a member of the aldehyde dehydrogenase enzyme family that catalyzes the oxidation of aldehydes to their corresponding carboxylic acids. ALDH1B1 is expressed in various tissues and plays a crucial role in cellular detoxification processes, protecting cancer cells against lipid peroxidation and ferroptosis by oxidizing lipid aldehydes generated during the lipid peroxidation process (Chen et al. 2022). Conversely, inhibiting ALDH1B1 activity has been shown to increase lipid peroxidation and ferroptosis in cancer cells, suggesting that it may be a potential therapeutic target for cancer treatment (Chen et al. 2022). Interestingly, eukaryotic initiation factor 4E (EIF4E) has a non-translational function of binding and inhibiting the anti-ferroptotic function of ALDH1B1 in the clearance of 4-HNE. Since EIF4E is usually overexpressed in cancer cells, inducing EIF4E-dependent ALDH1B1 inhibition and subsequent ferroptosis may be a safe anticancer strategy. Further studies are needed to determine the specificity and selectivity of the EIF4E–ALDH1B1–4-HNE pathway in tumor therapy.

1.7.6 NFE2L2

NFE2 like BZIP transcription factor 2 (NFE2L2, also known as NRF2) is a transcription factor that plays a critical role in the cellular defense against oxidative stress (Rojo de la Vega et al. 2018). Under normal conditions, NFE2L2 is kept in the cytoplasm by the Kelch-like ECH-associated protein 1 (KEAP1), an adaptor subunit

of Cullin 3-based E3 ubiquitin ligase. However, when cells are exposed to oxidative stress or electrophiles, NFE2L2 is released from KEAP1 and translocates to the nucleus, where it activates the expression of genes involved in antioxidant and detoxification pathways (Dai et al. 2020a). The degradation of KEAP1 by sequestosome 1 (SQSTM1) can increase the stability of the NFE2L2 protein and subsequently lead to increased gene transcription. These include genes encoding enzymes involved in GSH synthesis, NADPH regeneration, and phase II detoxification enzymes. Activation of NFE2L2 has been shown to protect cells against ferroptosis by increasing the expression of genes involved in iron metabolism (e.g., ferritin) and redox balance (e.g., MGST, NAD(P)H quinone dehydrogenase 1 [NQO1], GPX4, and metallothionein-1G [MT1G]), which are critical for ferroptosis suppression (Sun et al. 2016a; Sun et al. 2016b). On the other hand, inhibition of NFE2L2 has been shown to sensitize cells to ferroptosis by decreasing the expression of antioxidant and detoxification genes, and increasing the levels of lipid peroxidation products. Thus, NFE2L2 plays a central role in inhibiting ferroptosis by upregulating multiple genes involved in the antiferroptotic pathway.

1.7.7 NUPR1

Nuclear protein-1 (NUPR1; also known as p8) was initially discovered in 1997 as a small protein expressed in the rat and as a novel gene that is activated in the acute phase of induced pancreatitis and during pancreatic development (Liu and Costa 2022). NUPR1 is a transcription regulator protein that has been shown to promote cell survival in response to oxidative stress by enhancing the expression of antioxidant genes, such as NQO1, and inhibiting the production of ROS. NUPR1 regulates cellular damage and death in different forms, depending on the cell context and the types of stress induced. Regarding ferroptosis, NUPR1 has been identified as a negative regulator of ferroptosis in pancreatic cancer and liver cancer (Liu et al. 2021c; Huang et al. 2021). NUPR1 overexpression inhibits, while NUPR1 knockdown sensitizes cells to ferroptosis. NUPR1 inhibits ferroptosis by promoting LCN2 expression to limit iron accumulation or by inhibiting mitochondrial damage (Huang et al. 2021; Liu et al. 2021c). In addition to its nuclear function, NUPR1 is also expressed in the cytoplasm. Further studies are needed to define the cytosolic function of NUPR1 in ferroptosis.

1.7.8 ESCRT-III

The endosomal sorting complexes required for transport III (ESCRT-III) are a group of proteins that play a crucial role in several cellular processes, including membrane repair and programmed cell death. ESCRT-III is involved in multivesicular body (MVB) biogenesis, which is the process by which membrane proteins and lipids are sorted into intraluminal vesicles (ILVs) within the endosomal pathway (Lata et al. 2009). Additionally, ESCRT-III plays a key role in repairing damaged plasma

membranes by mediating the formation of a membrane repair complex at the site of injury (Liu et al. 2021a). In terms of ferroptosis, recent studies have suggested that ESCRT-III may play a role in protecting cells against ferroptosis-induced membrane damage (Dai et al. 2020b; Pedrera et al. 2021). ESCRT-III can facilitate the removal of oxidized phospholipids from the plasma membrane, which are a hallmark of ferroptosis. This process involves the recruitment of ESCRT-III to the damaged membrane, where it mediates the sequestration of oxidized phospholipids into ILVs, which are then trafficked to the lysosome for degradation. Collectively, these findings suggest that ESCRT-III plays a critical role in maintaining membrane integrity and protecting cells from oxidative damage, including that induced by ferroptosis.

1.8 Conclusion and Outlook

In conclusion, lipid metabolism and homeostasis play critical roles in the regulation of ferroptosis, a form of regulated cell death driven by lipid peroxidation. The accumulation of oxidized lipids, particularly PUFAs, in cell membranes triggers ferroptosis. To prevent this, cells have developed various mechanisms to regulate the composition of their membranes, repair damaged lipids, and neutralize free radicals that cause lipid peroxidation. In recent years, significant progress has been made in understanding the molecular mechanisms underlying ferroptosis and lipid metabolism. However, many questions remain unanswered, and further research is needed to fully elucidate the complex interplay between lipid metabolism, oxidative stress, and ferroptosis. This includes understanding the roles of different lipid species, identifying new regulators of lipid metabolism and ferroptosis, and developing new therapeutic strategies to target ferroptosis in various diseases.

In summary, the study of lipid metabolism and homeostasis in ferroptosis is a rapidly evolving field with great potential for improving our understanding of various diseases and developing new treatments.

Conflict of Interest The authors declare no conflicts of interest or financial interests.

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Iron Metabolism and Ferroptosis

2

Hao Zheng and Shinya Toyokuni

Abstract

No life on earth can stay alive without iron. Iron acquisition, transfer, storage, and its retrieval are explicitly regulated by various interacting mechanisms, including peptide hormones and transcriptional/posttranscriptional regulation. Mammals possess no physiological pathway to excrete iron outside of the body except for hemorrhage. Iron deficiency may cause anemia and muscle weakness. However, excess iron is a risk for cancer due to resultant oxidative stress. The word "ferroptosis" consists of Fe(II) and "falling down," respecting the Fenton reaction. Cancer cells accommodate higher catalytic Fe(II) in general but with more resistance to oxidative stress in comparison to the non-tumorous counterpart cells. Therefore, carcinogenesis can be a genetic evolutionary process to obtain ferroptosis-resistance, in which small molecules, such as erastin, can demolish. Recently, low-temperature plasma has become available with the development of engineering, which can precisely load oxidative stress to the preferred surface locations. This novel strategy may specifically kill certain cancer cells or even germs by targeting higher catalytic Fe(II) to result in ferroptosis and is expected to work as an additional medical intervention.

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2.1 Introduction

Iron is abundant in space based on the fact that many meteors, in which the major component is iron, have hit the earth thus far (Ruzicka et al. 2017). The earth also contains a large amount of iron (Ohta et al. 2016), and it is established by geological studies that the ancient sea of a few billion years ago harbored a high concentration of Fe(II) (Olson and Straub 2016) when the first life appeared on earth. Thus, it is not surprising that no independent life on the earth can survive without iron. The great oxygen event occurred approximately 2.5 billion years ago with the sedimentation of iron ore from soluble Fe(II). Then there was a period of relatively high concentration of hydrogen sulfur, and the age of oxygen started from 0.6 billion years ago (Olson and Straub 2016). Higher species, including mammals, have chosen iron to transport oxygen in the body during the evolutionary process. Molecular oxygen, abundant over the present earth, is versatile in the transfer of 1–4 electrons, thus ensuring constant electron flow. Oxygen is transported in mammals via heme in hemoglobin in red blood cells (Wriggleworth and Baum 1980).

2.2 Iron Metabolism in Circulation

Iron is the most abundant heavy metal in humans and the males have ~4 grams and the females have ~2.5 grams, and as much as 60% of iron is in hemoglobin (Wriggleworth and Baum 1980). Iron is a transition metal and its solubility is much higher as Fe(II), and Fe(III) is almost insoluble at neutral pH (Toyokuni 1996; Koppenol and Hider 2019). Apparently, iron has been extremely precious as a nutrient to all lives on the earth, so each species has a tactic to obtain iron efficiently. For example, bacteria secrete siderophore, a small molecule with high iron affinity, to adsorb iron from the outer environments (Miethke and Marahiel 2007). Mammals lack pathways to discharge iron to the outside of the body except for hemorrhage or phlebotomy (Toyokuni 2009b).

Iron solubility is greatly increased after acidification, which is the reason why iron is absorbed through duodenal villous epithelial cells from the diet because the pH of normal gastric juice is 1–2. Fe(III) is always reduced to Fe(II) when passing through the double-layered lipid membrane, including the plasma membrane. Two distinct iron transporters, DMT1 (Slc11A2) (Andrews 1999) and ferroportin (Slc40A1) (Donovan et al. 2000), are essential to absorb iron from the duodenum; namely, DMT1 to take Fe(II) into the cytoplasm and ferroportin to take Fe(II) from the cytoplasm to the portal circulation (Toyokuni 2009b). Secreted Fe(II) is immediately oxidized to Fe(III) by hephaestin (Vulpe et al. 1999) and captured by serum transferrin (Fig. 2.1). Portal vein pours into the liver, which is the major iron reservoir (hepatocytes) in our body. Curiously enough hepatocytes secrete a peptide hormone called hepcidin (Ganz and Nemeth 2012), which as a ligand for ferroportin decreases intestinal iron absorption as well as available iron in circulation by the degradation of ferroportin through the ubiquitin proteasome system (Fig. 2.2).

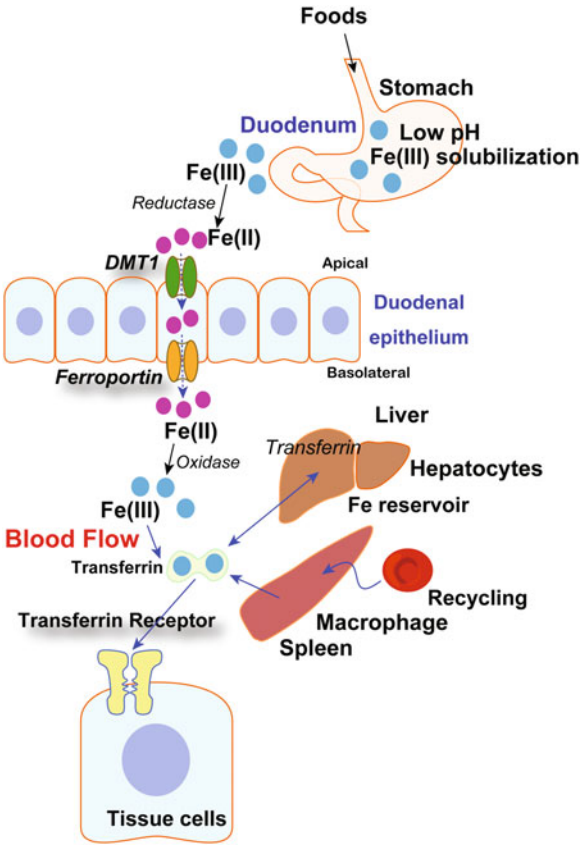

Fig. 2.1 Summary of iron acquisition by mammals. How iron is absorbed from the diet in the duodenum after solubilization with low pH in the stomach and delivered to each cell is schematically shown. Blue filled circles indicate Fe(III) (ferric iron) and red filled circles correspond to Fe(II) (ferrous iron). Note that iron ions are always reduced to Fe(II) form when they cross the double-layered lipid membrane through the iron transporters (DMT1 and ferroportin). Duodenal iron reductase is Duodenal Cytochrome b (DCYTB; also as CYBRD1) and duodenal iron oxidase is membrane-bound hephaestin. As soon as iron as Fe(III) is released to circulation (portal vein) from the basolateral membrane of duodenal enterocytes, transferrin captures Fe(III), which is transported to all sorts of cells via blood stream. Cellular iron metabolism will be described in Fig. 2.2. There is no active pathway to excrete iron. Refer to text for details

As already described, erythropoiesis is the largest consumer of iron. The major erythroid regulator of hepcidin is erythroferrone, which is synthesized and secreted by erythroblasts in the bone marrow (Ganz 2019). Erythropoietin produced by the renal interstitial cells induces the production of erythroferrone (Fig. 2.2).

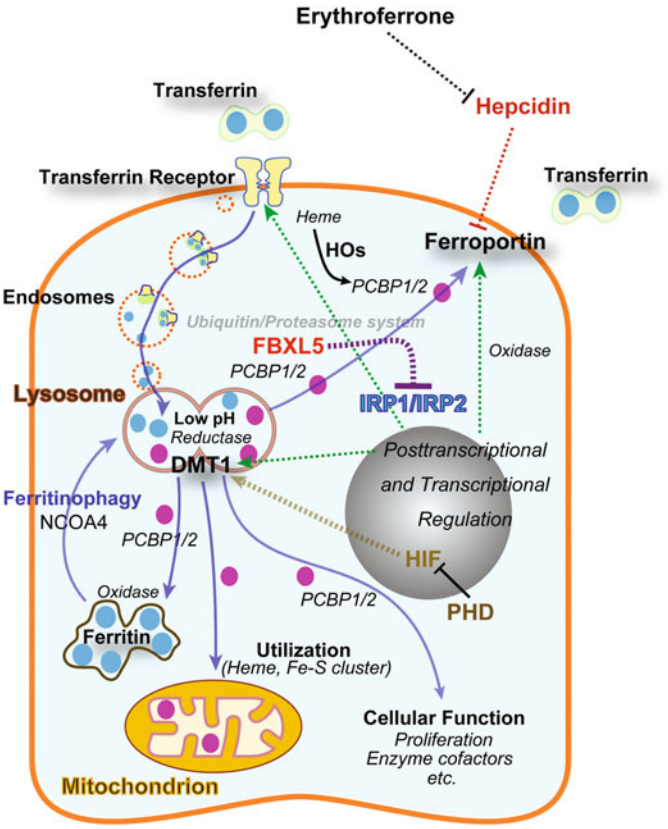

Fig. 2.2 Cellular regulation of iron metabolism. Major iron receiver in cells is the transferrin receptor where certain cells may also have DMT1 on the plasma membrane. Transferrin receptor after binding with transferrin is sorted to endosomes and lysosomes, where the binding of transferrin and transferrin receptor as well as that of iron and transferrin are dissolved in acidic environment. Here again Fe(III) in endosomes and lysosomes is reduced by six-transmembrane epithelial antigen of prostate 3 (STEAP3) prior to be released to cytosol via DMT1, where Fe(II) is captured by PCBP1/2 (iron chaperone) and sorted to its destination for functional use or storage in ferritin. Ferritin heavy chain works as oxidase to accommodate iron as Fe(III). Stored Fe(III) in ferritin can be retrieved via NCOA4-mediated ferritinophagy, and heme can be degraded by heme oxygenases (HOs) to recover Fe(II). Surplus iron may be secreted from the cell to circulation via ferroportin, which can transport Fe(II) through the plasma membrane from Fe(II) bound to PCBP1/2. Cellular iron status is finely regulated through multiple mechanisms, both transcriptional and post-transcriptional mechanisms. Of iron regulatory proteins 1 and 2 (IRP1/IRP2), IRP2 can be degraded by FBXL5 through the ubiquitin-proteasome system. Prolyl hydroxylase (PDH), a sensor for iron and oxygen, is the key enzyme for the degradation of hypoxia-inducible factor (HIF)-2α, which works as a transcription factor for DMT1 and CYBRD1. Furthermore, hepcidin, a peptide hormone secreted by hepatocytes, is a ligand for ferroportin, which is degraded after binding via proteasome. Hepcidin transcription is inhibited by erythroferrone secreted by erythroblasts. Blue filled circle, Fe (III) (ferric iron) and red filled circle, Fe(II) (ferrous iron). Refer to text for details

2.3 Iron Metabolism in Cells

Iron is a major cofactor for various enzymes either as Fe(II), heme, or the Fe-S cluster. These enzymes include ribonucleotide reductase for synthesizing DNA (Bollinger et al. 1991), cytochrome oxidases for energy production and metabolizing xenobiotics (Powers et al. 1981; de Ungria et al. 2000), and nitric oxide synthases for regulating vascular tones and epithelial secretory activities (McMillan et al. 1992). Therefore, all kinds of cells in our body require iron, which is delivered mainly by serum transferrin (Figs. 2.1 and 2.2). Of note, humans also possess possible siderophores (Devireddy et al. 2010) and siderophore-binding proteins, including lipocalin 2 (Flo et al. 2004; Rodvold et al. 2012), and some of the cells other than duodenal villous epithelia have DMT1 on the plasma membrane, which may be released via membrane budding as extracellular vesicles (Mackenzie et al. 2016). However, the human siderophore system has not been well established yet.

Iron metabolism is precisely regulated via iron regulatory proteins (IRPs; IRP1, and IRP2) and iron-responsive element (IRE) (Hentze and Kuhn 1996). In the case of iron deficiency, the number of transferrin receptor per cell is increased via increasing the lifespan of message of the transferrin receptor with binding of IRP to IREs present at the 3′ end of the message, whereas ferritin, an iron storage protein, is reduced by the translation block with binding of IRP to IREs present at the 5′ end of the message. Indeed, these are the posttranscriptional regulation mechanisms. Furthermore, F-box/LRR-repeat protein 5 (FBXL5) can target IRP2 for its degradation under iron repletion (Moroishi et al. 2011; Iwai 2018) (Fig. 2.2). We recently found that a typical exosome marker CD63 is under the regulation of the IRE/IRP posttranscriptional system. Loading of iron significantly increased CD63 protein with the IRE-IRP system and iron-loaded ferritin is secreted as extracellular vesicles (Yanatori et al. 2021; Toyokuni et al. 2021).

After binding with transferrin, transferrin receptor is sorted to endosomes and lysosomes, where Fe(III) is released at acidic pH. Here again DMT1 is used at the endosomal and lysosomal membranes after reduction to transport Fe(II) to the cytosolic fraction for use. Recently, poly(rC)-binding proteins 1 and 2 (PCBP1/2) were identified as major Fe(II) chaperones in the cytosol (Shi et al. 2008; Yanatori et al. 2014; Yanatori et al. 2017). Some of the Fe(II) is carried into mitochondria via mitoferrin for the synthesis of heme and the Fe-S cluster (Paradkar et al. 2009). Surplus Fe(II) is stored in the ferritin core, consisting of ferritin heavy chain with oxidase activity of Fe(II) and light chain. Unnecessary Fe(II) may be excreted into extracellular circulation via the only iron exporter, ferroportin, in each cell (Yanatori et al. 2016; Yanatori and Kishi 2019) (Fig. 2.2).

2.4 Excess Iron and Cancer

Iron is essential for every kind of life on the earth as described in the previous sections. Thus, iron deficiency due to food intake insufficiency or iron loss due to continued hemorrhage causes anemia and muscle dysfunction. However, iron excess

Table 2.1 Epidemiological association between local excess iron and cancer in humans

Disease	Target cell	Cancer	Molecular mechanism(s)
Genetic (hereditary) hemochromatosis	Hepatocytes	Hepatocellular carcinoma	Genetic; type 1–5 (6 different types) affecting different single genes (directly associated with iron metabolism), ultimately leading to uncontrolled systemic iron loading
Viral hepatitis (hepatitis B and C virus)	Hepatocytes	Hepatocellular carcinoma	Infection; hepatocytes are chronically damaged by autoimmune process, which decreases secretion of hepcidin, leading to unregulated iron absorption from the duodenum
Environmental exposure to asbestos	Mesothelial cells; alveolar/ bronchial cells	Malignant mesothelioma, lung cancer	Environmental exposure; asbestos fibers reach pulmonary alveoli after inhalation; macrophages cannot cope with long and thin fibers, which have high affinity for hemoglobin and histones; fibers accumulate endogenous iron and provides oxidative damage to pulmonary epithelial cells and mesothelial cells
Ovarian endometriosis	Ovarian surface epithelial cells	Clear cell carcinoma, endometrioid adenocarcinoma	Ectopia/regurgitation of menstruation; monthly hemorrhage induces severe iron deposits in the ovary

is not good for health either but is closely associated with carcinogenesis (Toyokuni 2002, 2009b, 2014; Toyokuni et al. 2017). There are three distinct categories of data, supporting this fact; patient information on specific diseases, human population data based on epidemiological analyses, and the abundant data on animal studies. Table 2.1 summarizes human-specific diseases associated with local iron overload and carcinogenesis of the corresponding cells.

Phlebotomy or iron chelation therapy is the only available method to reduce iron from the body. Of note, there is an epidemiological study that regular phlebotomy (500-ml whole blood) twice a year for 5 years reduced not only the incidence but also the mortality of many visceral cancers (Zacharski et al. 2008).

On the other hand, there are many animal models established on the association of iron excess and carcinogenesis. In 1959 Richmond reported that an intramuscular injection of the iron dextran complex produced spindle-cell sarcoma to highly pleomorphic sarcoma at the site of injection (Richmond 1959). In 1982, Okada and Midorikawa reported that repeated intraperitoneal injections of an iron chelate, ferric nitrilotriacetate (Fe-NTA), induced renal adenocarcinoma with high incidence in rats and mice (Okada and Midorikawa 1982; Ebina et al. 1986; Li et al. 1987; Toyokuni et al. 1998). This model was found with serendipity and we have been studying this model for over 30 years (Toyokuni 2016a). Fe-NTA is soluble at

neutral pH and still works as a catalyst (Toyokuni and Sagripanti 1992; Toyokuni and Sagripanti 1993).

The molecular mechanisms of excess iron-associated carcinogenesis are in two directions; iron is an inorganic essential nutrient for cell proliferation and excess iron generates catalytic Fe(II) in the body, which causes the Fenton reaction [Fe (II) + $H_2O_2 \rightarrow$Fe(III) + $^{\bullet}OH$ + OH^-] (Fenton 1894; Koppenol and Hider 2019). The generated hydroxyl radical ($^{\bullet}OH$) is indeed the most reactive species in the biological system, inducing strand breaks, modifications, and cross-links in all kinds of biomolecules, including DNA (Toyokuni and Sagripanti 1996). These covalent molecular alterations are the causes of somatic mutations, leading to carcinogenesis.

In the Fe-NTA model, it was a mystery why intraperitoneal injections of Fe-NTA caused renal adenocarcinoma. We have thus far elucidated that absorbed Fe-NTA into the blood flow is filtered through the glomeruli of the kidney into the lumina of the proximal tubules of the nephron system, where the Fenton reaction is induced presumably by an abundant reductant *L*-cysteine (Hamazaki et al. 1985, 1986, 1988, 1989; Toyokuni et al. 1990; Okada et al. 1993). It is interesting that the genetic alterations observed in Fe-NTA-induced renal adenocarcinoma reveal massive chromosomal alterations, including homozygous deletion of *p16/p15* tumor suppressor genes and amplification of *c-Met* oncogene (Tanaka et al. 1999; Toyokuni 2011; Akatsuka et al. 2012). Notably, Fe-NTA-induced carcinogenesis occurs in *wild*-type animals in high incidence (~90% in rats) (Ebina et al. 1986; Toyokuni 2016a). This is the reason why we ourselves believe that iron excess is one of the major causes of human carcinogenesis as well (Toyokuni 2016b). Iron gradually becomes excess in our body with aging according with the decrease in metabolic rates (Toyokuni 2019).

Excess iron is also associated with foreign body-induced carcinogenesis. Typically, this occurs in asbestos-induced mesothelial carcinogenesis in humans (Oury et al. 2014; Toyokuni 2019). Humans always try to use new materials to improve the quality of our lives especially after industrial revolution, which has sometimes caused unexpected diseases. Asbestos is just one of them. Asbestos is a natural fibrous mineral, so is resistant to heat, acid, and friction, and used for various industrial purposes to increase the quality of the products, including masks, cloths, brakes, ships, locomotives, and houses. These extremely thin mineral fibers have been mined and processed economically and with versatility, so asbestos has been used in huge amounts worldwide. In the 1960s it became clear that asbestos causes various pulmonary diseases because it flies in the air and reaches human pulmonary alveoli due to its physical characteristics (IARC WHO 2012). Within the lung, macrophages and histiocytes try to dispose of these thin fibrous foreign materials. However, macrophages cannot digest asbestos fibers because they are mineral, which was generated on the earth, consuming billions of years. Indeed, thin (<0.25 μm) and long (<20 μm) fibers easily kill these scavenger cells (Jiang et al. 2008), stay in the lung for decades, and finally reach the pleural cavity due to their negative pressure. During these periods, asbestos causes not only asbestosis (pulmonary fibrosis due to foreign body) but also malignant mesothelioma and promote lung cancer (Toyokuni 2009a). Here asbestos fibers specifically adsorb hemoglobin

and histones, which accumulate iron on the surface of asbestos fibers and cause oxidative DNA damage in the mesothelial cells after decades because mesothelial cells are also phagocytic cells (Nagai et al. 2011a). Additionally, the asbestos body, a hallmark of asbestos exposure in the lung, is a mass of solidified iron, called hemosiderin (2014).

These processes can be reproduced in animals by intraperitoneal injection of asbestos fibers, which requires only 1.5 years to obtain malignant mesothelioma after an injection of 10 mg of asbestos in ~100% of *wild*-type rats (Jiang et al. 2012). Notably, >90% cases of malignant mesothelioma in rats harbor homozygous deletion of *p16/p15* tumor suppressor genes, which is one of the major targets in the Fe-NTA-induced renal carcinogenesis model as well as human malignant mesothelioma (Jiang et al. 2012). Multiwalled carbon nanotube of ~50 nm diameter presents a similar pathology at least in rat experiments (Nagai et al. 2011b; Toyokuni 2013), thus requiring extremely careful handling for the powder form of carbon nanotubes of this diameter (designated as Group 2B carcinogen from International Agency on Research on Cancer) (Grosse et al. 2014). Thus, animal experiments unequivocally demonstrate that iron excess is associated with carcinogenesis.

2.5 Ferroptosis in Iron Excess

The concept of ferroptosis was coined by Stockwell et al. in 2012 (Dixon et al. 2012; Hirschhorn and Stockwell 2019). Ferroptosis is defined as "iron-dependent regulated necrosis involving lipid peroxidation." Ferroptosis consists of Ferro as Fe(II) and ptosis as "falling down," thus respecting the Fenton reaction (Koppenol and Hider 2019). Thus far, ferroptosis has been observed in renal tubular cells (Angeli et al. 2014; Seibt et al. 2019), degenerated neurons (Masaldan et al. 2019), senescent cells (Coradduzza et al. 2023), and cancer cells, including carcinoma and sarcoma (Stockwell et al. 2017). Whereas cells in different categories may reveal ferroptosis in the future, our laboratory has been working on renal tubular cells and their carcinogenesis for decades.

This first started with serendipity when Shigeru Okada and Osamu Midorikawa discovered renal cell carcinoma in rats in high incidence in 1982 after repeated intraperitoneal administration of ferric nitrilotriacetate (Fe-NTA) (Okada and Midorikawa 1982), which was a recognized hemochromatosis model in the 1970s (Awai et al. 1979). Intraperitoneal administration of Fe-NTA was quite reproducible in inducing necrosis of renal proximal tubules, starting 30 min after the injection and lasting for ~24 h (Hamazaki et al. 1985). Soon we noticed that this necrosis was associated with lipid peroxidation, when measurements of thiobarbituric acid-reactive substances were a standard strategy in the 1980s (Hamazaki et al. 1988). Here tubular necrosis presented nuclear pyknosis and eosinophilic degenerated cytoplasm. Electronmicroscopic analyses revealed cytoplasmic vacuoles, mitochondrial swelling, and rupture of plasma membranes (Hamazaki et al. 1988). We now believe that this is ferroptosis.

The Fe-NTA-induced renal carcinogenesis model has taught us two messages from the viewpoint of ferroptosis. Firstly, the ratio of iron as Fe(II) to sulfur as

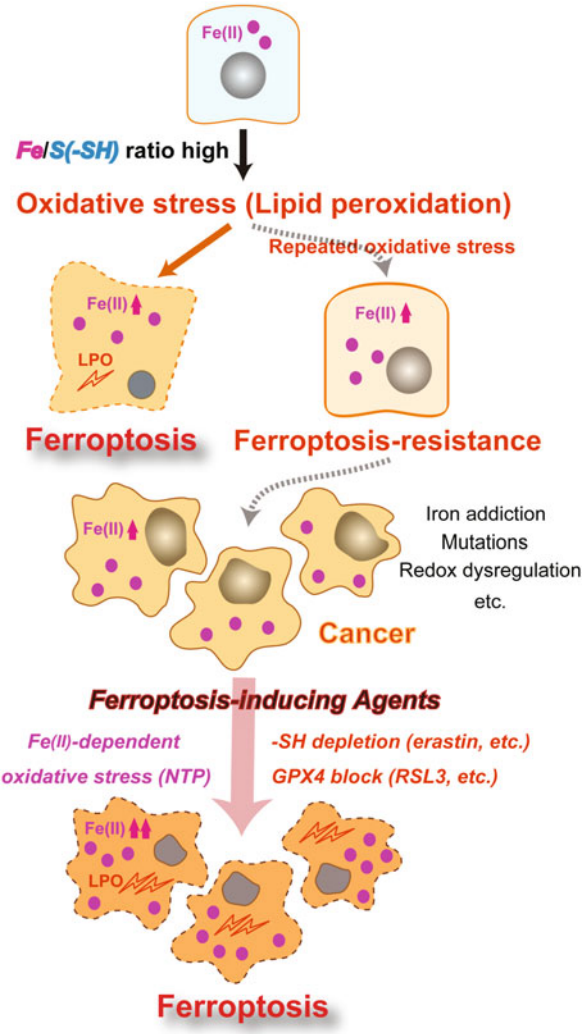

Fig. 2.3 Ferroptosis and carcinogenesis. Cellular imbalance between iron and sulfur (sulfhydryls, such as glutathione) in favor of iron causes ferroptosis. Thus, oxidative stress associated with local iron excess causes ferroptosis. Repeated oxidative stress induces cellular adaptation, ultimately leading to ferroptosis-resistance. Concomitant with iron addiction and acquired mutations via oxidative stress, ferroptosis-resistance cells become cancer cells. Cancer cells generally hold high amounts of catalytic Fe(II). Ferroptosis-inducing agents, such as glutathione peroxidase 4 (GPX4) blocker and NTP (non-thermal plasma), are novel strategies as cancer therapy. Refer to text for details

sulfhydryl (SH) is important when considering ferroptosis (Fig. 2.3). The original ferroptosis is induced by erastin in fibrosarcoma cells, where the squeezed cystine/glutamate antiporter caused depletion of glutathione, a major source of SH (Dixon et al. 2012). We think that iron excess can induce the same sequences of events. In the case of the Fe-NTA model, Fe-NTA is filtered, after absorption into the systemic blood flow from the portal vein, through the renal glomeruli into the lumina of the renal proximal tubules (Toyokuni et al. 1990), where Fe(II) is taken up through DMT1 in the brush border membrane and simultaneously initiates the Fenton reaction in vivo (Mukaide et al. 2014).

Secondly, we have recognized that one of the major processes of carcinogenesis is establishing ferroptosis-resistance (Fig. 2.3). In the Fe-NTA renal carcinogenesis

model, we noted no ferroptosis 3 weeks after the repeated intraperitoneal administration of Fe-NTA but observed scattered karyomegalic cells which may be already aneuploidy in certain portion of the genome (Hiroyasu et al. 2002). Moreover, A/J mice can maintain an appropriate level of oxidative stress to induce oxidative DNA damage after Fe-NTA exposure, without excessive ferroptosis at an early stage, eventually leading to a high incidence of renal cell carcinoma (Cheng et al. 2022). Of note, cancer cells harbor more catalytic Fe(II) in general (Ito et al. 2016; Schoenfeld et al. 2017), which confirms the presence of ferroptosis-resistance in cancer cells. For example, Nrf2 is a master transcription factor resisting oxidative stress in the cell (Suzuki and Yamamoto 2015), and Nrf2 is a modulator of ferroptosis to be preventive (Sun et al. 2016). The activation of Nrf2 is regulated by Keap1, which is a cytoplasmic sensor for oxidative stress, working as ubiquitin ligase for the steady disposal of Nrf2 in peaceful conditions (Suzuki and Yamamoto 2015). Further, Nrf2 is antagonized with Bach1 (Nishizawa et al. 2023). Many studies established that human cancer cells, especially, lung non-small cell cancers, exhibit mutations either in *Nrf2* or *Keap1* to cause persistent activation of Nrf2 (Ohta et al. 2008; Menegon et al. 2016). Recently, we observed that BRCA1 haploinsufficiency causes mitochondrial dysfunction, leading to cellular iron deposition under the Fe-NTA-induced renal carcinogenesis model. As mitochondria require sufficient amounts of iron for the biosynthesis of heme and the biogenesis of iron-sulfur cluster (Duan et al. 2023), we supposed that iron metabolism alteration via mitochondrial damage generated ferroptosis-resistance earlier in the *Brca1* mutants in comparison to the *wild-type* (Kong et al. 2022; Toyokuni et al. 2023). Similar results were obtained in that Brca1 haploinsufficiency in rats promoted chrysotile-induced mesothelial carcinogenesis via iron metabolism dysregulation and ferroptosis-resistance in males (Luo et al. 2023).

By the use of the Fe-NTA renal carcinogenesis model, we as pathologists are trying to find the best marker to detect ferroptosis in formalin-fixed paraffin-embedded (FFPE) sections because scientific history teaches us that morphological methods significantly broaden the research area for any novel biological phenomena, such as terminal deoxynucleotidyl transferase dUTP nick end labeling for apoptosis (Heatwole 1999) and 3-nitrotyrosine for peroxinitrite (Smith et al. 1997) though biochemical/molecular methods should be combined for confirmation. FFPE sections are indeed the treasure trove in that they can be preserved at room temperature for nearly 50 years for fine analytical use (Hiroyasu et al. 2004). In the 1990s we had performed comprehensive studies using mass spectrometry to find out the best markers for oxidative stress, using this oxidative renal damage model of Fe-NTA. Oxidative damage causes cellular lipid peroxidation, which ultimately generates aldehydes (Toyokuni 1999; Toyokuni and Akatsuka 2007). We found that 4-hydroxy-2-nonenal (HNE) is the most sensitive among the aldehydes we could measure (Toyokuni et al. 1997) and 8-hydroxy-2′-deoxyguanosine was the most sensitive among the oxidatively modified DNA bases (Toyokuni et al. 1994) in the rat kidney 6 h after single intraperitoneal injection of Fe-NTA. Based on these results, we developed five mouse monoclonal antibodies (HNEJ1–5) against the hemiacetal structure of Michael adducts in HNE-modified proteins (Toyokuni et al. 1995; Ozeki et al. 2005) and HNEJ-1 was recently found to work best for the

detection of ferroptosis in the FFPF specimens (Zheng et al. 2021). As we have been interested in the physiological meaning of ferroptosis, HNEJ-1 was applied on the FFPF specimens to define physiological ferroptosis. Ferroptotic cells increased with age in various organs, including kidney, spleen, ovary, and uterus, which was accompanied by substantial iron accumulation. The ferroptotic process was observed in the nucleated erythrocytes between E13.5 and E15.5, which disappeared in the enucleated erythrocytes (Zheng et al. 2021). These observations demonstrate the existence of physiological ferroptotic processes.

2.6 Ferroptosis and Ferritinophagy

Surplus iron is stored safely in the core of ferritin as insoluble Fe(III) in each cell. Ferritin is a huge protein of 450 kDa, consisting of 24 subunits of either heavy or light chain, of which heavy chain (FTH) reveals oxidase activity, changing from soluble Fe(II) to insoluble Fe(III) (Lawson et al. 1989). How each cell takes out the iron from holo-ferritin when necessary has been a question for a long time. Now it is recognized that ferritin-specific autophagy is present as ferritinophagy, mediated by nuclear receptor coactivator 4 (NCOA4) (Mancias et al. 2014), which specifically target ferritin for degradation in the autophagosome (Fig. 2.2). As expected, ferritinophagy via NCOA4 is required for erythropoiesis. Mancias et al. found that red blood cells do not develop properly in zebrafish which have low amounts of the NCOA4 protein (Mancias et al. 2015). NCOA4, coupled with iron charperone protein PCBP1, mediating the flux of iron out and into of ferritin respectively, is critical for regulating heme synthesis and further erythropoiesis differentiation (Ryu et al. 2017). NCOA4 and PCBP1 are implicated in mediating the flux of iron into and out of ferritin through a direct protein–protein interaction. The dynamic process of NCOA4-PCBP1 interaction is induced by iron deprivation and suppressed by iron excess (Ryu et al. 2018).

As expected, ferritinophagy and ferroptosis are significantly interrelated. Promotion of ferritinophagy is associated with ferroptosis (Gao et al. 2016; Hou et al. 2016; Stockwell et al. 2017), when the released Fe(II) is not immediately used for cellular functions, such as synthesizing proteins with iron cofactors (*ex.* hemoglobin). NCOA4 deficiency abolishes ferritinophagy and confers protection against erastin-induced ferroptosis, whereas its overexpression retards RAS-selective lethal 3 (RSL3)-triggered ferroptotic cell death (Gryzik et al. 2021). Moreover, PCBP1 was reported to negatively regulate ferritinophagy-mediated ferroptosis by destabilizing BECN1 mRNA (Lee et al. 2022). The DMT1-PCBP2 pathway was speculated to function at a final step of the iron-recycling pathway through ferritinophagy (Yanatori et al. 2020). Our laboratory reported that PCBP2 deficiency promoted erastin-induced ferroptosis via increased level of catalytic ferrous iron in human malignant mesothelioma cells (Yue 2022).

Cancer cells are generally under persistent oxidative stress while they continue proliferation and invasion (Toyokuni et al. 1995). Recently, our laboratory as well as other investigators established that low-temperature plasma (LTP)/non-thermal

plasma (NTP) specifically kills cancer cells in comparison to non-tumorous cells (Shi et al. 2017; Toyokuni et al. 2018). LTP is the fourth physical state accompanied by ionization over solid/liquid/gas and can supply various reactive species at human body temperature. LTP generation has become possible, depending on recent engineering technology since the 1990s. Whereas LTP generation uses high voltage electricity with ambient air, the essential components are electrons, free radicals, peroxides, UV lights, and their reaction products (Okazaki et al. 2014). Of note, LTP exposure kills malignant mesothelioma cells presumably via ferroptosis but not mesothelial cells (Fig. 2.3). Autophagy and endocytosis are promoted during this process (Shi et al. 2017). We also know by in vitro experiments that holo ferritin exposed to LTP produces catalytic Fe(II), indicating that the liberation of Fe(III) and reduction thereafter occur simultaneously (Furuta et al. 2018). We also observed iron-dependent lipid peroxidation and mitochondrial superoxide generation during the cell death of oral squamous cell carcinoma cells exposed to LTP, thus suggesting the presence of the ferroptotic process (Sato et al. 2019). Furthermore, it has been reported that culture media or solutions exposed to LTP show similar effects (Kurake et al. 2018). On application of plasma-activated lactate (PAL) to malignant mesothelioma cells, we observed that ferroptosis is a major death code. During this process, we observed nitric oxide-associated autophagy in the lysosomes, which eventually leads to ferroptosis (Jiang et al. 2021). Therefore, LTP exposure may be a handy method to induce ferroptosis in certain cancer cells.

2.7 Conclusion

Ferroptosis and iron metabolism are intimately associated. Excess iron is a major background for carcinogenesis and ferroptosis-resistance is one of the common characteristics of cancer cells. Conversely, small molecules, such as erastin, can regulate cellular signaling/metabolism toward iron-dependent oxidative stress in cancer cells. Non-thermal plasma may be a novel strategy to cause ferroptosis specifically in cancer cells.

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Targeting Epigenetic Regulation of Ferroptosis in Cancer Therapy

3

Zuli Wang, Tania Tao, and Yongguang Tao

Abstract

Ferroptosis is a regulated form of cell death characterized by iron-catalyzed and reactive oxygen species (ROS)-mediated lipid peroxidation. It plays a crucial role in tumorigenesis, tumor progression, and cancer therapy. Epigenetic regulation has emerged as a promising target for cancer treatment, but the regulatory mechanisms involved in ferroptosis are complex and diverse. In this chapter, we provide a comprehensive summary of the key mechanisms and regulators that are primarily associated with iron metabolism, ROS signaling, and lipid metabolism in ferroptosis. Additionally, we delve into the epigenetic aspects of ferroptosis regulation, including chromatin remodeling, DNA methylation, histone modifications, and non-coding RNAs, and their impact on ferroptosis-related factors. Furthermore, we highlight the growing interest in utilizing epigenetic drugs to target ferroptosis as a therapeutic strategy in cancer treatment. We emphasize the significance of understanding the role of epigenetic regulation in

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ferroptosis, as it holds the potential to enhance the effectiveness of cancer therapy by combining epigenetic inhibition with ferroptosis induction.

3.1 Background

Ferroptosis is a type of regulated cell death characterized by iron-dependent processes and driven by reactive oxygen species (ROS), resulting in the accumulation of lipid peroxidation. In certain cancers, particularly cancer stem cells (CSCs), there is a dependence on iron, making ferroptosis a vulnerability that can be targeted for therapeutic intervention (Rodriguez et al. 2022). The canonical antioxidant defense system involves the solute carrier family 7 member 11 (SLC7A11), which imports cystine for the production of glutathione peroxidase 4 (GPX4) to detoxify lipid peroxides through the consumption of glutathione (GSH). This system functions to inhibit ferroptosis. Studies have shown that targeting the SLC7A11-GSH-GPX4 axis not only increases the sensitivity of cancer cells to radiotherapy but also enhances the efficacy of immunotherapies, such as CD8+ T cell-based approaches, by promoting tumor lipid oxidation (Lang et al. 2019). The regulation of ferroptosis involves multiple and complex processes, including iron metabolism, ROS signaling, and lipid metabolism. Dr. Brent R Stockwell, a prominent researcher who defined the concept of ferroptosis, has emphasized the urgent need to uncover the underlying mechanisms of ferroptosis (Stockwell 2022). Therefore, gaining a deeper understanding of the regulation of ferroptosis is crucial for the development of effective ferroptosis-based cancer therapies (Chen et al. 2021a).

Cancer is a disease characterized by genetic and epigenetic dysregulation. Unlike genetic mutations, epigenetic modifications, including chromatin remodeling, DNA methylation, and histone modifications, are specifically controlled by epigenetic factors and can be targeted by small molecule compounds. These epigenetic modifications play a significant role in anticancer treatments and can enhance the sensitivity of cancer cells to therapy (Garcia-Martinez et al. 2021). For instance, azacytidine, approved by the Food and Drug Administration (FDA), is an inhibitor of DNA methyltransferases (DNMTs) and has shown great efficacy in treating acute myelogenous leukemia and other cancers (Sun et al. 2022). Additionally, non-coding RNAs (ncRNAs), such as circular RNAs (circRNAs), long non-coding RNAs (lncRNAs), and microRNAs (miRNAs), have emerged as important epigenetic determinants that serve as diagnostic biomarkers for cancer and provide valuable insights for therapeutic targeting (Slack and Chinnaiyan 2019). Epigenetic proteins and RNAs are closely involved in various cellular activities, including cell growth, differentiation, and fate determination. Abnormalities and uncontrolled actions of these cellular processes contribute to disease states and the initiation of cancer (Wimalasena et al. 2020). Consequently, targeting epigenetic regulators holds significant promise for cancer therapy.

Increasing evidence indicates that numerous epigenetic factors participate in both ferroptosis and tumorigenesis, opening up new avenues for therapeutic interventions (Wu et al. 2020). In this chapter, we first summarize the critical mechanisms and regulators of ferroptosis. Additionally, we extensively discuss the epigenetic

regulation of factors associated with ferroptosis. Finally, we highlight the importance of epigenetic drugs targeting ferroptosis in cancer therapy. Therefore, particular attention is given to the role of epigenetic regulation in ferroptosis, as it holds great potential for effective cancer treatments.

3.2 Mechanisms and Regulators of Ferroptosis

The regulation of ferroptosis involves three main metabolic pathways: iron metabolism, reactive oxygen species (ROS) control, and lipid metabolism, which collectively influence the peroxidation of polyunsaturated fatty acids (PUFAs), such as membrane phospholipids (PL). Additionally, other metabolic alterations, including coenzyme Q10, nicotinamide adenine dinucleotide phosphate (NADPH), and selenium, also play a role in modulating cellular sensitivity to ferroptosis (Stockwell et al. 2017). Furthermore, based on diverse subcellular localization, there are at least three systems involved in protecting cells against ferroptosis: GPX4 in the cytosol and mitochondria, FSP1 on the plasma membrane, and dihydroorotate dehydrogenase (DHODH) in mitochondria (Mao et al. 2021). While significant progress has been made in unraveling the mechanisms of ferroptosis, our understanding of this process still remains somewhat limited.

With regard to iron metabolism, Fe^{3+} is imported into cells by transferrin (Tf) through transferrin receptor protein (TfR) and metal transporter SLC39A14, whereas ferroportin is responsible for the mediation of iron export. Subsequently, free iron is used by iron regulatory protein (IRP) 1 and 2 to store in ferritin and iron-sulfur protein, thereby playing a role in systemic iron homeostasis and ferroptosis (Chen et al. 2020a, b). However, metalloreductase (STEAP3) and divalent metal transporter 1 (DMT1) can mediate the release of Fe^{2+}. Moreover, nuclear receptor coactivator 4 (NCOA4)-ferritin heavy chain (FTH) is able to degrade ferritin and produce Fe^{2+} (Hou et al. 2016). Hydroxyl radicals (•OH) generated by the Fenton reaction between Fe^{2+} and H_2O_2 initiate ferroptosis (Fuhrmann and Brune 2022). Given the strong iron demand of tumor cells particularly CSCs (El Hout et al. 2018), targeting iron status determines the cellular sensitivity toward ferroptosis and becomes a vulnerability in cancer.

As the intermediate regulator of ferroptosis, the interconversion of ROS is necessary for ferroptosis. It is reported that iron-catalyzed Fenton reactions produce HO• from O^{2-} and H_2O_2, which are generated from nicotinamide adenine dinucleotide phosphate (NADPH) oxidases (NOX) through mitochondrial oxidative phosphorylation and peroxisomes. Moreover, arachidonate lipoxygenases (ALOXs) and cytochrome P450 reductase (POR) regulate mitochondrial respiratory and promote lipid peroxidation. In contrast, after superoxide dismutase (SOD) transforms O^{2-} into H_2O_2, the catalase (CAT) and peroxiredoxin (PRDX) have the ability to eliminate H_2O_2, resulting in the inhibition of ferroptosis (Benfeitas et al. 2017; Li et al. 2019; Lei et al. 2022). In addition, ferroptosis suppressor protein 1 (FSP1), an important oxidoreductase parallel to GPX4, functions as a lipophilic radical-trapping antioxidant by reducing coenzyme Q10 (CoQ10) (Bersuker et al. 2019). Moreover,

same to the radical-trapping function of FSP1, DHODH represses ferroptosis via coordinating with GPX4 (Wang and Min 2021). Recently, a study has reported that guanosine triphosphate cyclohydrolase 1 (GCH1) is used to synthesize tetrahydrobiopterin [BH4], which promotes the production of CoQ10 and halts lipid peroxidation (Zheng and Conrad 2020). The levels of ROS in cancer cells are higher than in normal cells, which are the characteristics of several malignant progression (Cheung and Vousden 2022), indicating that ROS control is conducive to inhibiting malignancy and curing cancer.

Compared to normal cells, cancer cells are preferable to lipid metabolism for their development and therapy resistance. Acyl-coenzyme A (CoA) synthetase long-chain family member 4 (ACSL4) and lysophosphatidylcholine acyltransferase 3 (LPCAT3), the key activators of PUFA, are responsible for integrating PUFA into PL and accelerating the PUFA peroxidation and ferroptosis. Interestingly, the interaction between monounsaturated fatty acids (MUFA) and PL catalyzed by ACSL3 is capable of preventing the peroxidation of PUFA (Li and Li 2020; Stockwell 2022). In addition, cysteine is imported by cellular cystine-glutamate antiporter (system x_c^-) and is, in turn, used for GSH biosynthesis. Subsequently, GPX4 transforms reactive PUFA phospholipid hydroperoxides (PUFA-PL-OOH) into non-reactive and non-lethal PUFA phospholipid alcohols (PUFA-PL-OH) through consumption of GSH (Jiang et al. 2021). Moreover, as a unit of system x_c^-, cystine/glutamate antiporter SLC7A11 is promoted by nuclear factor e2-related factor (NRF2) and is repressed by P53 via transcription, participating in nutrient dependency and ferroptosis of tumors (Koppula et al. 2021). These alterations that occurred in lipid metabolism during ferroptosis offer several potential strategies for cancer therapy.

Above all, the ferroptotic metabolism routes, such as iron, ROS, and lipid, are various, and their regulators are complex (Fig. 3.1). Based on our understanding of ferroptosis, treatment of cancer is hard by regulating these mediators. In recent years, their upstream monitors, such as chromatin remodeling, DNA methylation, histone modifications, and non-coding RNAs, influence cellular susceptibility to ferroptosis (Wang et al. 2021a). Therefore, the identification of epigenetic factors is important for ferroptosis control in response to changes in the cellular environment.

3.3 Epigenetic Regulation in Ferroptosis

3.3.1 Chromatin Remodeling

Chromatin remodeling is a dynamic modification that mobilizes nucleosomes and makes the concentrated genomic DNA accessible to the transcription machinery. The AT-rich interactive domain-containing protein 1A (ARID1A), a switch/sucrose nonfermenting (SWI/SNF) chromatin-remodeling factor, facilitates the NRF2-mediated transcriptional activation of SLC7A11. Thus, the ARID1A-deficient cancer cells are vulnerable to inhibition of the antioxidant GSH and targeting of glutamate-cysteine ligase catalytic (Ogiwara et al. 2019). As a chromatin remodeling

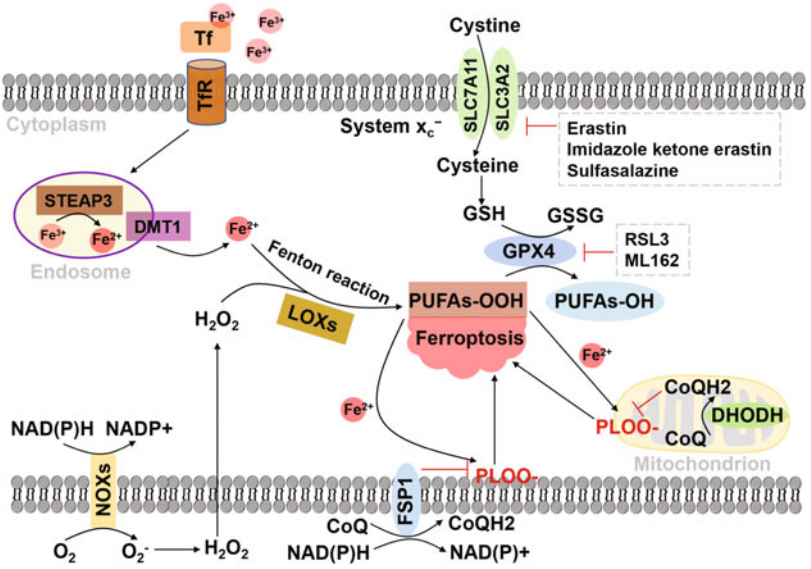

Fig. 3.1 The core induction mechanisms and inhibition systems of ferroptosis. Fe^{3+} is imported into cells by Tf through its receptor TfR. Then, STEAP3 is able to transform Fe^{3+}, and DMT1 can release Fe^{2+} from the endosome. Cellular O^{2-} can be generated from NOXs. The Fenton reaction between Fe^{2+} and H_2O_2 induces the production of PUFAs-OOH mediated by LOXs, resulting in ferroptosis. GPX4 utilizes SL7A11-transported cystine to inhibit ferroptosis by converting PUFAs-OOH into PUFAs-OH. The SLC7A11 inhibitor (erastin, imidazole ketone erastin, and sulfasalazine) and GPX4 inhibitor (RSL3 and ML162) play an important role in the induction of ferroptosis. Moreover, FSP1 in the cell membrane and DHODH in the mitochondrion are capable of producing CoQH2 and subsequent ferroptosis suppression through decreasing the accumulation of PLOO-

ATPases, lymphoid-specific helicase (LSH) belonging to the sucrose nonfermenting (SNF) family promotes the transcription of ferroptosis repressors, such as fatty acid desaturases 2 (FADS2) and stearoyl-CoA desaturase 1 (SCD1). LSH is negatively regulated by HIF1A, indicating that it can respond quickly to hypoxic environments and dynamically regulate target genes (Tang et al. 2021; Wang et al. 2021a). Moreover, a recent study has reported that LSH is able to enhance the expression of SLC7A11 by binding to its promoter region and play a critical role in tumor development through inhibiting ferroptosis (Wang et al. 2021d). In response to various stress, nuclear protein 1 (NUPR1) is highly expressed and transactivating the transcription of e iron exporter lipocalin 2 (LCN2), thereby leading to erastin- or RSL3-induced ferroptosis in pancreatic ductal adenocarcinoma cells (Chen et al. 2021b).

This finding proposes a promising strategy targeting metabolic properties for the development of anti-cancer therapeutics. However, other chromatin remodeling regulators, such as Imitation Switch (ISWI) and chromodomain helicase DNA (CHD) of ferroptosis, are required for further exploration.

3.3.2 DNA Methylation

Aberrant DNA methylation is existed in cancer cells and promotes cellular oncogenesis, which is mainly catalyzed by three DNA methyltransferases (DNMTs), including DNMT1, DNMT3A, and DNMT3B (Nishiyama and Nakanishi 2021). According to databases like The Cancer Genome Atlas (TCGA), it is found that most ferroptosis-related genes were aberrantly expressed in various tumors due to the great contribution of DNA methylation (Liu et al. 2020b). For the DNA hypermethylation at promoter/enhancer regions, elongation of very long-chain fatty acid protein 5 (ELOVL5) and fatty acid desaturase 1 (FADS1) related to PUFA biosynthesis is frequently silenced and render intestinal-type gastric cancer cells (GCs) resistant to ferroptosis, while the methylation regulators remain unknown (Lee et al. 2020b). In contrast, the promoter of the FSP1 gene is hypermethylated, resulting in the silencing of FSP1 expression and the dependence of acute lymphoblastic leukemia (ALL) cells on GSH-mediated ferroptosis defenses (Pontel et al. 2022).

Notably, the DNA methylation of four ferroptosis related genes, such as (zinc finger E-box binding homeobox 1) ZEB1 and CDGSH iron sulfur domain 1 (CISD1), is able to independently predict prognosis and probably guide the treatment in cutaneous melanoma (Wang et al. 2022a). Besides, ferroptosis-related genes AKR1C2 and SOCS1 are hypermethylated and are promising biomarkers for predicting prognosis in patients with AML (Zhou and Chen 2021). There is a study reported that homocysteine induces the DNA methylation of the GPX4 gene and then enhances ferroptosis sensitivity (Zhang et al. 2020). Regrettably, despite the methylation level of various ferroptosis-related genes that have been demonstrated, their methyltransferases remain largely unknown.

3.3.3 Histones Modification

Histones, particularly the histone amino-terminal tails modified by post-translational methylation and acetylation, regulate the transcription and expression of target genes (Stillman 2018). KDM3B, a histone H3 lysine 9 demethylase, synergizes with the transcription factor ATF4 to prevent ferroptosis via upregulating the levels of SLC7A11 (Logie et al. 2021). There is a study that demonstrated that methionine adenosyltransferase 2A (MAT2A) generates adenosylmethionine (SAM) and promotes the trimethylation of lysine-4 on histone H3 (H3K4me3) at the promoter region of ACSL3, resulting in resisting ferroptosis by increasing ACSL3 expression (Ma et al. 2022). Besides, histone methyltransferase MLL4 (KMT2D) is one of the most commonly mutated genes in various cancers and mediates tumor suppression through the markers (Alox12, Alox12b, and Aloxe3) of ferroptosis (Egolf et al. 2021). As a histone H3K9me3 methyltransferases, SUV39H1 inhibition upregulates DPP4 expression by reducing the H3K9me3, thereby inducing lipid peroxidation and ferroptosis (Wang et al. 2021b).

In response to ferroptosis stimulus, histone lysine acetyltransferase 2B (KAT2B) is hard to prevent HNF4A from binding to the ferroptosis downregulated genes, while KAT2B is recruited to the ferroptosis upregulated factors and facilitates the transcriptional role of HIC1 (Zhang et al. 2019a). Fatty acid metabolism-related genes (HADH, ACSL1, and ACAA2), whose expression is dependent on the activity of super-enhancers by the bromodomain-containing protein 4 (BRD4) and HMGB2, play a role in PUFAs synthesis. Namely, the loss of BRD4 protects cells from excessive lipid peroxides (Yang et al. 2022). Silencing of BRD4 downregulates some critical ferroptosis-associated genes (GPX4, SLC7A11, and SLC3A2) in breast and lung cancer cell lines (Sui et al. 2019). Additionally, histone deacetylase SIRT1 causes epigenetic reprogramming of epithelial-mesenchymal transition (EMT) and consequently promotes the susceptibility to ferroptosis in head and neck cancer (HNC) cells (Lee et al. 2020a).

Gan professor's team has found that a nuclear deubiquitinating enzyme BRCA1-associated protein 1 (BAP1) decreases histone 2A ubiquitination occupancy on the promoter of SLC7A11 and inhibits the expression of SLC7A11, leading to ferroptosis promotion (Zhang et al. 2018). Conversely, the main H2Aub ubiquitin ligase PRC1 increases SLC7A11 expression (Zhang et al. 2019b). Moreover, research proves that p53 decreases monoubiquitination of histone H2B on lysine 120 (H2Bub1) occupancy on the SLC7A11 gene by promoting the nuclear translocation of deubiquitinase USP7. This leads to the reduction of SLC7A11 expression during erastin treatment (Wang et al. 2019). Importantly, the global and detailed modifications, and the relationship between histone modifications and/or DNA methylation are required for further investigation.

3.3.4 Non-coding RNAs

MicroRNA (miRNA), a powerful 22 nucleotide noncoding RNA, not only participates in the progression of cancer but also participates in the development of drug resistance via interacting with a range of target genes. For example, miR-23a-3p is overexpressed in sorafenib-resistant hepatocellular carcinoma (HCC) cells and directly targeted the 3′-untranslated regions (UTR) of ACSL4, which suggests that miR-23a-3p inhibitor can improve sorafenib responsiveness via inducing ferroptosis (Diener et al. 2022; Lu et al. 2022). The number of miRNAs can decrease the expression of TfR-1, such as miR-210, miR-7-5p, and miR-320. Moreover, miR-638 and miR-200b have a significant inhibition role in ferritin expression (Wang et al. 2018). miRNA-137 targets SLC1A5 and induces ferroptosis by suppressing glutamine transportation.

Long noncoding RNAs (lncRNAs), defined as RNA transcripts beyond 200 nucleotides, have drawn our great attention for their transcription regulatory and emerging role in cancers. As a competitive endogenous RNA, lncRNA LINC00336 competes against miR-6852 to interact with cystathionine-β-synthase (CBS), thereby triggering the ferroptosis process. In addition, lncRNA PVT1 can enhance ferroptosis by reducing the binding of miR-214 to TP53 3′UTR (Zhang

et al. 2022; Wang et al. 2020). A cytosolic lncRNA P53RRA displaces P53 to bind G3BP1, leading to the retention of P53 in the nucleus and triggering cell ferroptosis and apoptosis (Mao et al. 2018). Interestingly, when cancer cells are exposed to erastin, lncRNA MT1DP increases intracellular ferrous iron concentration and reduces GSH levels via stabilizing miR-365a-3p and modulating NRF2 expression (Gai et al. 2020). Recently, the findings uncover that a nuclear LINC00618 decrease in human acute myeloid leukemia (AML) accelerates ferroptosis via decreasing the expression of SLC7A11 and promotes apoptosis by increasing the levels of cleaved caspase-3, demonstrating that ferroptosis is in a manner dependent on apoptosis (Wang et al. 2021d).

Regarding circular RNAs (circRNAs), they are novel and back-spliced from pre-mRNAs. Several circRNAs have been proven to compete with miRNAs targeting SLC7A11, such as circFNDC3B targeting miR-520d-5p, circ_0067934 targeting miR-545-3p, and circ_0097009 targeting miR-1261, and mainly play a role in inhibiting ferroptosis and promoting the development of various cancer (Zuo et al. 2022). CircRNA cIARS physically and directly interacted with RNA binding protein (RBP) ALKBH5 and suppressed the ALKBH5-mediated autophagy inhibition, which positively modulates sorafenib-induced ferroptosis via ferritinophagy (Liu et al. 2020a). Moreover, circABCB10 silencing inhibits cell ferroptosis and apoptosis by regulating the miR-326/CCL5 axis in rectal cancer (Xian et al. 2020).

Despite the non-coding RNAs are proven to participate in multiple ferroptosis mediators [56], the comprehensive network of non-coding RNAs needs to be excavated and built quickly.

3.3.5 Other Epigenetic Regulators

Selenium (Se), an important micronutrient, is associated with the health of human beings and can promote the transcription of GPX4 and other selenoproteins expression. Selenoproteins, including thioredoxin reductases (TrxR/TXNRD) and antioxidant GSH peroxidases (GPX), contain selenocysteine residues, which are able to counter the induction of ferroptosis (Wu et al. 2020). Iron metabolism-related genes, such as TFRC, SLC11A2, and FTH, are mediated by hypoxia-responsive elements binding to their promoter regions. Hypoxia-induced HIF-1α activation inhibits ferroptosis treated by RSL3 (Wang et al. 2021a; Li et al. 2020). Moreover, iron-responsive element-binding protein 2 (IREB2) stabilizes TFRC or DMT1 by directly binding to the 3′-untranslated region (UTR) of mRNA (Song et al. 2021). In hepatoblastoma, m6A methylase METTL3/IGF2BP1-mediated N6-methyladenosine (m6A) modification promotes SLC7A11 mRNA stability and upregulates its expression by inhibiting the deadenylation process, resulting in ferroptosis resistance (Liu et al. 2019). Researchers have identified that OTUB1 can directly interact with and stabilize SLC7A11, suggesting that OTUB1 is a key factor in regulating ferroptosis and provides a potential target in cancer therapy (Liu et al. 2019).

Currently, the epigenetic regulators of ferroptosis are largely uncovered, especially the epigenetic regulation of important ferroptosis factors like SLC7A11 (Table 3.1). Hence, future studies should shed light on post-transcriptional and post-translational regulation in ferroptosis.

3.4 Epigenetic Drugs in Cancer Therapy

Previous studies reported that 5-azacitidine specifically inhibits DNA methylation by trapping DNA methyltransferases like DNMT1 and decreases ferroptosis susceptibility by increasing the expression of E-cadherin and GPX4 (Lee et al. 2020a; Pei et al. 2022). Bromodomain protein BRD4 is capable of recognizing acetylation sites and recruiting transcription factors. BRD4 inhibitor (+)-JQ1 (JQ1) has been demonstrated to induce ferroptosis via ferritinophagy and decreases in GPX4 and SLC7A11. Moreover, ferroptosis inducers erastin, RSL3, and sorafenib can enhance the anticancer effect of JQ1 (Sui et al. 2019). Pharmacological inhibition of SIRT1 by histone deacetylase (HDAC) inhibitor EX-527 repressed ferroptosis, whereas SIRT inducers (resveratrol and SRT1720) enhanced ferroptosis, indicating that epigenetic reprogramming contributes to ferroptosis in HNC cells (Lee et al. 2020a).

The study reported that the miR-3587 inhibitor could attenuate ferroptosis through heme oxygenase-1 (HO-1) (Tao et al. 2021). Nanoparticle-based agents provide a new choice for ferroptosis induction or sensitization. The researchers construct an all-in-one nanoplatform with functions of tumor targeting, monitoring, and treatment by constructing a homotypic cancer cell membrane-camouflaged iron-small interfering RNA nanohybrid. The SLC7A11-targeted siRNA in the nanohybrid inhibits the biosynthesis of GSH by cutting off the supply of intracellular cystine, resulting in the accumulation of ROS, which further increases the accretion of lipid peroxides to enhance iron-induced ferroptosis (Huang et al. 2022; Wang et al. 2022b). However, the research and application of DNMT inhibitors and nanoparticle-based non-coding RNAs have a long way to go.

Hence, the epigenetic drugs of ferroptosis are significant for the inhibition of cancer progression but are required for further exploration (Fig. 3.2). Moreover, the combination of epigenetic drugs and ferroptosis inducers may have a promising therapeutic effect on various cancer.

3.5 Conclusions and Perspectives

Since the discovery of ferroptosis in 2012, it has been implicated in various diseases, including cancer, ischemia/reperfusion injury, and neurodegenerative disorders, such as Alzheimer's disease. This has provided novel targets for cancer therapy, and inhibitors of ferroptosis hold significant therapeutic potential in clinical settings (Yan et al. 2021). Importantly, ferroptosis plays a distinct role in cancer treatment compared to traditional radiotherapy and chemotherapy. For instance, platinum-tolerant cells rely on the Wnt receptor Frizzled-7 (FZD7)-β-catenin-Tp63-GPX4

Table 3.1 Epigenetic regulators in ferroptosis

Genes	Chromatin remodeling	DNA methylation	Histone modifications	Noncoding RNAs	Others
SLC7A11	ARID1A LSH		KDM3B BRD4 BAP1 PRC1 USP7	LINC00618 miR-520d-5p miR-545-3p miR-1261 circFNDC3B circ_0067934 circ_0097009	OTUB1 METTL3/ IGF2BP1 Nanoparticle
FADS2	LSH				
SCD1	LSH				
LCN2	NUPR1				
GPX4		Homocysteine	BRD4	miRNA-17-92	Se
ELOVL5		Unknown			
FADS1		Unknown			
FSP1		Unknown			
CISD1		Unknown			
AKR1C2		Unknown			
SOCS1		Unknown			
ACSL3			MAT2A		
Alox12			KMT2D		
DPP4			SUV39H1		
SLC3A2			BRD4		
HADH			BRD4		
ACSL1			BRD4		
ACSL4				miR-23a-3p miRNA-17-92	
TfR-1				miR-210 miR-7-5p miR-320	
Ferritin				miR-638 miR-200b	
SLC1A5				miRNA-137	
CBS				miR-6852 LINC00336	
TP53				miR-214 lncRNA PVT1	
P53				P53RRA	
NRF2				miR-365a-3p MT1DP	
ALKBH5				circRNA cIARS	
TFRC					HIF-1α IREB2
DMT1					IREB2

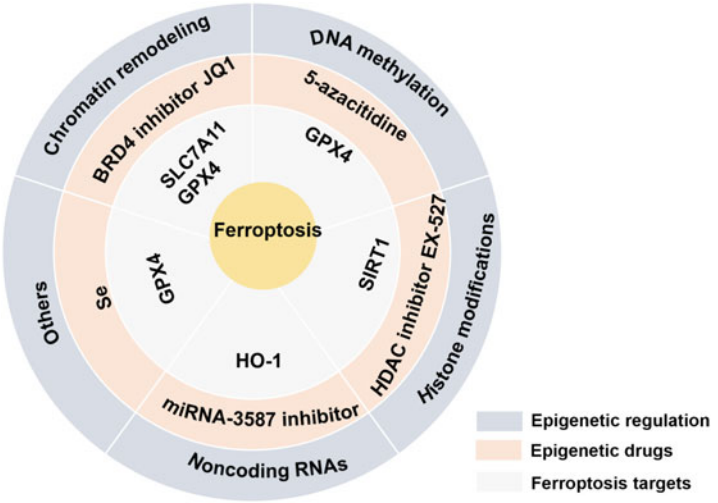

Fig. 3.2 Epigenetic drugs and their targets in cancer therapy. Chromatin remodeling factors BRD4 are inhibited by JQ1, thereby increasing the sensitivity of cells to ferroptosis through repressing the expression of SLC7A11. 5-azacitidine, a DNA methylation inhibitor, is able to promote GPX4 expression and then resist ferroptotic induction. HDAC inhibitor EX-527 targeting SIRT1 induces cell ferroptosis through epigenetic reprogramming of EMT. MiR-3587 inhibitor could attenuate ferroptosis through heme oxygenase-1 (HO-1). Moreover, Se is necessary for GPX4-inhibited ferroptosis

pathway for survival, and they exhibit increased sensitivity to ferroptosis upon treatment with GPX4 inhibitors (Wang et al. 2021c). Similarly, colorectal cancer stem cells (CSCs) have lower levels of ROS, making them vulnerable to the SLC7A11 inhibitor erastin (Xu et al. 2020). The addiction of cancer cells, particularly CSCs, to iron and the susceptibility of CSCs with low ROS levels to ferroptosis highlight the potential of ferroptosis in targeting drug-resistant cancer cells and CSCs (El Hout et al. 2018). Therefore, it is crucial to unravel the specific mechanisms and regulators of ferroptosis to further advance our understanding of this process.

The control of ferroptosis involves key processes, such as iron metabolism (TFR1 and NCOA4), ROS control (FSP1, DHODH, and ALOX), and lipid metabolism (ACSL4, SLC7A11, and GPX4). Perturbations in the execution of these factors can modulate the susceptibility to ferroptosis. While the core factors of ferroptosis have been extensively studied, the upstream regulators responsible for controlling these factors remain largely unknown (Stockwell 2022). It is well established that ferroptosis-related genes, as well as lipid and ROS metabolism, are complex and diverse. In response to both internal and external stimuli triggering ferroptosis, epigenetic modifications serve as a bridge connecting these stimuli to the execution factors. Furthermore, epigenetic regulators have a significant impact on tumorigenesis and cancer progression, making them promising targets for cancer treatment (Hogg et al. 2020). Therefore, the epigenetic regulation of ferroptosis

deserves particular attention in understanding and targeting this process in cancer therapy.

This chapter focuses on summarizing the role of chromatin remodeling, DNA methylation, histone modifications, and non-coding RNAs in the control of ferroptosis (Tang et al. 2021). It has been found that LSH serves as an important chromatin remodeling factor in ferroptosis regulation, while the involvement of other remodeling complexes, such as ISWI and CHD, remains largely unexplored. Numerous studies have demonstrated the presence of methylation in various ferroptosis-related genes; however, the specific methyltransferases responsible for these modifications have yet to be identified. Additionally, the identified enzymes do not include critical methyltransferases like DNMT1. Although there have been some studies investigating histone modifications on ferroptosis factors, further research is needed to elucidate the comprehensive and detailed nature of these modifications and their relationship with DNA methylation. Non-coding RNAs have been shown to participate in the regulation of multiple ferroptosis mediators (Beermann et al. 2016); however, a comprehensive network of non-coding RNAs in ferroptosis is still to be established. Therefore, there is a need for further exploration and establishment of post-transcriptional and post-translational regulatory mechanisms in ferroptosis. Unfortunately, the research and application of DNMT inhibitors and nanoparticle-based non-coding RNAs in ferroptosis-targeted cancer therapy present significant challenges.

In summary, this chapter aims to provide insights into the epigenetic regulation of ferroptosis, which can help in understanding the cellular response to ferroptosis stimuli through epigenetic mechanisms. Importantly, the combination of epigenetic inhibition and ferroptosis induction represents an effective strategy for cancer therapy.

Conflict of Interest The authors declare no conflicts of interest or financial interests.

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The Role of Autophagy in Ferroptosis

4

Rui Kang and Daolin Tang

Abstract

Autophagy is a self-degrading process that helps determine the fate of cells in response to stress. Although the role of autophagy in promoting cell survival is well understood, the connection between autophagy and cell death is still not entirely clear. Ferroptosis is a type of cell death that is driven by iron-mediated lipid peroxidation and plasma membrane rupture. Recent studies suggest that the level of autophagy in a cell can impact its sensitivity to ferroptosis. The selective destruction of certain proteins or organelles through autophagy can trigger ferroptosis by promoting iron accumulation and lipid peroxidation. Some of the genes and proteins involved in regulating autophagy-dependent ferroptosis may differ from those involved in starvation-induced autophagy. To develop effective treatments for related diseases, a deeper understanding of the mechanisms and regulation of autophagy in ferroptosis is essential.

4.1 Introduction

Autophagy plays a critical role in maintaining cellular homeostasis by breaking down and disposing of intracellular waste, including unused proteins and damaged organelles (Dikic and Elazar 2018). There are different types of autophagy, including microautophagy, chaperone-mediated autophagy (CMA), and macroautophagy, which can be further classified into bulk and selective autophagy. By degrading these waste products, autophagy provides cells with the resources they need to perform their biological functions and survive in the face of stressors, such as starvation, hypoxia, and drug toxicity (Kroemer et al. 2010). However, excessive

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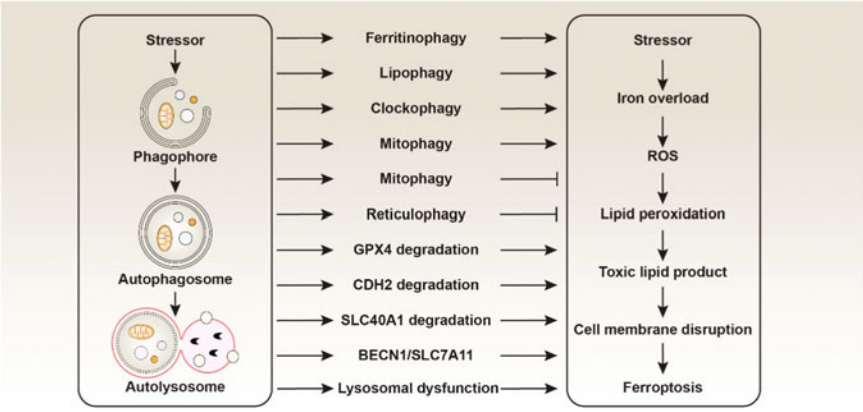

Fig. 4.1 Role of autophagy in ferroptosis. Autophagy is a type of cellular degradation that is dependent on the lysosome and can occur in both selective and non-selective forms. Ferroptosis, on the other hand, is a type of cell death that is driven by oxidative stress and is dependent on iron. This type of cell death is characterized by lipid peroxidation, which leads to the production of toxic lipids. The role of selective autophagy in ferroptosis is complex, as it can both enhance and inhibit the process through the degradation of various organelles or proteins. Moreover, the BECN1 protein has been found to bind directly to SLC7A11, thereby inhibiting cystine uptake and maintaining low levels of the antioxidant glutathione

autophagy can lead to cell damage or death, known as autophagy-dependent cell death (Tang et al. 2019). This process is regulated by the autophagy machinery, including autophagy-associated genes and protein complexes (Galluzzi et al. 2018; Xie et al. 2015). Autophagy-dependent cell death has been linked to various diseases, including cancer (Kriel and Loos 2019; Li et al. 2021b). Despite recent advancements, the underlying signaling pathways and regulatory networks that govern different types of selective autophagy-mediated cell death remain to be fully understood.

Ferroptosis is a regulated form of cell death that is triggered by oxidative damage from iron accumulation, leading to lipid peroxidation (Tang and Kroemer 2020). Despite its historical classification as a type of autophagy-independent cell death due to the inability of the autophagy inhibitor chloroquine to block the activity of ferroptosis activators in Rat sarcoma (RAS)-mutated cancer cells (Dixon et al. 2012), recent genetic evidence suggests that autophagy plays a role in the initiation and execution of ferroptosis through the triggering of iron accumulation and lipid peroxidation (Zhou et al. 2020; Liu et al. 2020a; Zhang et al. 2022; Xie et al. 2020a). The depletion of key components of the autophagy machinery (such as ATG5, ATG7, or BECN1) decreases the sensitivity of cells to ferroptosis, while the activation of autophagy-related factors accelerates ferroptosis in both cancer and non-cancer cells (Liu et al. 2020a; Chen et al. 2021a).

In this chapter, we present the fundamental mechanisms behind autophagy and ferroptosis. Our focus is on how dysregulated autophagy impacts ferroptosis

(Fig. 4.1). This understanding may offer valuable insights into the possibility of targeting autophagy-dependent ferroptosis in diseases (Chen et al. 2023).

4.2 Mechanism of Autophagy

Autophagy is a complex, lysosome-dependent cellular degradation process that involves the formation of intracellular membrane structures, including phagophore, autophagosome, and autolysosome. On the other hand, other processes like CMA and microautophagy have different mechanisms. CMA specifically degrades proteins with the KFERQ motif, which is recognized by the heat shock protein 70 (HSP70) and regulated by lysosome-associated membrane protein 2 (LAMP2A) in lysosomes (Kaushik and Cuervo 2018). Microautophagy involves the direct phagocytosis of degraded cargo through lysosomes or late endosomes (Wang et al. 2022). This section provides a detailed description of the dynamic process and regulation of macroautophagy (hereinafter referred to as autophagy) in mammalian cells.

4.2.1 Phagophore

Autophagy is activated by various external (such as starvation, hypoxia, and pH changes) and internal factors (such as metabolic stress, aging, accumulation of misfolded proteins, and DNA damage). The activation of autophagy is triggered by the activation or impairment of multiple signaling pathways, such as the mitogen-activated protein kinase (MAPK) pathway, the phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit delta (PI3K) pathway, the adenosine monophosphate (AMP)-activated protein kinase (AMPK) pathway, and the mammalian target of rapamycin (MTOR) pathway (Xie et al. 2015; Lahiri et al. 2021). These signals cause the formation of a cup-shaped phagophore structure, which is formed by membranes from various sources (such as the endoplasmic reticulum [ER], mitochondria, Golgi apparatus, and plasma membrane), and engulfs cytoplasmic molecules.

The formation of the phagophore structure is facilitated by two protein complexes, namely the unc-51-like autophagy-activating kinase (ULK) complex and the class III phosphatidylinositol 3-kinase (PtdIns3K) complex (Mizushima 2010). The ULK complex includes the core component ULK1 (a homolog of yeast Atg1), ATG13, and the scaffolding protein RB1-inducible coiled-coil 1 (RB1CC1, also known as FIP200, a homolog of yeast Atg17). The PtdIns3K complex consists of phosphatidylinositol 3-kinase catalytic subunit type 3 (PIK3C3, an ortholog of yeast Vps34), BECN1 (a mammalian homolog of yeast Vps30/Atg6), and phosphoinositide-3-kinase regulatory subunit 4 (PIK3R4, a mammalian homolog of yeast Vps15).

The ULK1 complex integrates signals from two key regulators of nutrient and energy levels, MTOR and AMPK, to initiate autophagy in response to changes in

intracellular energy and nutrient levels. ULK1 kinase activity then activates the PtdIns3K complex, which is responsible for the formation of phagophore assembly sites, membrane nucleation, membrane elongation, and the formation of autophagosomes (Mizushima 2010). BECN1 is a key player in the regulation of autophagy, as it binds to many proteins and serves as a hub to control the induction of autophagy and phagophore formation (Kang et al. 2011).

4.2.2 Autophagosome

Autophagosomes, double-membrane structures derived from phagophore elongation, are formed by two ubiquitin-like conjugation systems. The first system involves the covalent conjugation of ATG12 and ATG5, which is catalyzed by ATG7 and ATG10. This conjugation forms a complex with ATG16L, which acts as an E3 enzyme to facilitate the conjugation of ubiquitin-like microtubule-associated protein 1 light chain 3 (MAP1LC3/LC3)-family proteins (Scherz-Shouval et al. 2019; Nakatogawa 2013).

The second system involves the processing and lipidation of pro-MAP1LC3. The C-terminal polypeptide is cleaved by ATG4, a cysteine protease, to form MAP1LC3-I (Scherz-Shouval et al. 2007). This protein is then covalently associated with phosphatidylethanolamine (PE) in the presence of ATG7 and ATG3, resulting in the formation of MAP1LC3-II, the membrane-associated protein that is essential for autophagosome formation and degradation of cargo proteins. MAP1LC3-II serves not only as a marker for autophagosomes but also as a bridge between degraded substrates and autophagy receptors like sequestosome 1 (SQSTM1/p62). In addition to these processes, ATG9A-containing vesicles also contribute to starvation-induced autophagosome formation but not ferroptosis-associated autophagosome formation (Liu et al. 2022a). Therefore, different stimulatory signals and altered membrane sources are involved in the formation of autophagosomes.

4.2.3 Autolysosome

Autophagosomes fuse with lysosomes to form autolysosomes, although the exact molecular mechanism in mammalian cells remains unclear. Several lysosomal and membrane proteins have been implicated in this process, including lysosome-associated membrane protein 2 (LAMP2), the GTPase-activating protein RAB7A, the homotypic fusion and protein sorting (HOPS) complex, the soluble N-ethylmaleimide–sensitive fusion factor attachment protein receptor (SNARE) family, integration of autophagosomal syntaxin 17 (STX17), and lysosomal vesicle-associated membrane protein 8 (VAMP8) (Chi et al. 2019; Shen et al. 2021; Tian et al. 2020, 2021; Bernard and Klionsky 2015). The inner membrane of autophagosomes, including MAP1LC3-II, is also degraded by lysosomal enzymes. This degradation of MAP1LC3-II does not necessarily indicate inhibition of autophagy, and measuring autophagic flux in conjunction with late-stage autophagy inhibitors is a reliable method for assessing autophagic activity.

The cargo in lysosomes is typically degraded by lysosomal acid enzymes, such as cathepsin B (CTSB), cathepsin D (CTSD), cathepsin L (CTSL), acid phosphatases, and lipases. The selectivity of the cargo degradation remains unknown. The degraded cargo can be recycled to construct cellular components for self-renewal. However, excessive lysosomal degradation can be harmful to cellular function, so maintaining a healthy balance of degradation is essential for maintaining normal cellular homeostasis.

4.3 Selective Autophagy

Compared to bulk autophagy, selective autophagy is a targeted form of autophagy where specific cellular components, such as damaged organelles, misfolded proteins, or invading pathogens, are selectively degraded. Selective autophagy relies on specific autophagy receptors, which recognize and bind to these components to deliver them to autophagosomes for degradation (Vargas et al. 2022). The specificity of selective autophagy allows cells to maintain cellular homeostasis and regulate the quality control of cellular components. Autophagy receptors can be divided into two categories: ubiquitin-binding receptors, which recognize ubiquitinated cargoes, and cargo-localizing receptors, which bind directly to the degraded cargoes. Selective autophagy receptors contain a motif known as the Atg8-interacting motif or LC3-interacting region (AIM/LIR) to bind to the LC3/GABARAP/Atg8 family of lipidated proteins.

In yeast, the first identified form of selective autophagy was the cytoplasm-to-vacuole transport, which involved Atg11- and Atg19-dependent processing of aminopeptidase 1, aspartate aminopeptidase, and α-mannosidase (Baba et al. 1997). In mammalian cells, the receptors involved in selective autophagy are more diverse, and over 30 have been studied. Mitophagy, the selective removal of mitochondria, is the most studied form of autophagy receptor in mammals and involves the recognition of ubiquitinated mitochondrial proteins (Xie et al. 2020b, 2021). PTEN-induced putative kinase 1 (PINK1) and Parkin RBR E3 ubiquitin protein ligase (Parkin) proteins play a key role in this process, with PINK1 being a mitochondrial serine/threonine kinase and Parkin being an E3 ubiquitin ligase (Lazarou et al. 2015).

LC3 proteins or LC3 receptors can induce autophagy through a direct association with mitochondrial proteins in a ubiquitin-independent mechanism. Different receptors are involved in different types of selective autophagy and play a role in regulating cellular homeostasis. The binding of cargo-containing autophagy receptors to the Atg8/LC3 family of proteins ensures selectivity. In mammalian cells, there are two Atg8 protein families: MAP1LC3 and gamma-aminobutyric acid (GABA) type A receptor-associated protein (GABARAP), which are further divided into seven isoforms (Schaaf et al. 2016). Selective autophagy receptors bind LC3/GABARAP proteins through conserved LIR and GABARAP-interacting motifs, with the core motif being [W/F/Y]-XX-[L/V/I]. LIRs also contain residues that can be phosphorylated to increase interaction with the Atg8/LC3 family (Rogov et al. 2017).

4.4 Mechanism of Ferroptosis

Ferroptosis is a type of iron-dependent oxidative cell death that is largely regulated by the body's antioxidant defenses (Kuang et al. 2020). However, other signals or molecules also play a role in determining a cell's sensitivity to ferroptosis. In the following section, we will provide an overview of the key mechanisms and antioxidant systems that govern ferroptosis (Tang et al. 2021; Liu et al. 2021b).

4.4.1 Iron Toxicity

Iron is a vital trace element for all living organisms that exist in two redox states: ferrous (Fe^{2+}) and ferric (Fe^{3+}). The balance of iron in the body is crucial for proper functioning, including oxygen transport, electron transfer, and DNA synthesis. This balance is achieved through the processes of uptake, utilization, storage, and export of iron (Torti et al. 2018; Crielaard et al. 2017).

Cells acquire Fe^{3+} through transferrin receptor (TFRC)-mediated endocytosis, which is bound to transferrin (TF). Fe^{3+} is then reduced to Fe^{2+} by the STEAP3 metalloreductase within the endosome and released into the cytosol via the solute carrier family 11 member 2 (SLC11A2) pathway. Fe^{2+} can be stored in ferritin, including the ferritin heavy polypeptide 1 (FTH1) and ferritin light polypeptide 1 (FTL1) subunits.

Fe^{2+} also plays a role in oxygen transport and the creation of mitochondrial iron–sulfur clusters. Excess Fe^{2+} can be exported from the cell by solute carrier family 40 member 1 (SLC40A1), which converts Fe^{2+} to Fe^{3+}. As such, any disruptions in iron metabolism may impact ferroptosis sensitivity (Chen et al. 2021c).

Iron contributes to ferroptosis not only by creating reactive oxygen species (ROS) through a non-enzymatic Fenton reaction but also by serving as a co-factor for metabolic enzymes, such as arachidonate lipoxygenase (ALOX) and cytochrome P450 oxidoreductase (POR), which can induce lipid peroxidation (Zou et al. 2020; Li et al. 2021a). This is why an increase in intracellular iron can trigger ferroptosis (Ajoolabady et al. 2021).

For instance, the degradation of ferritin through ferritinophagy, which is facilitated by nuclear receptor coactivator 4 (NCOA4), leads to iron release from ferritin and ferroptosis in pancreatic cancer cells (Hou et al. 2016). Conversely, glutamate oxaloacetate transaminase 1 (GOT1) can inhibit ferroptosis by inhibiting ferritinophagy (Kremer et al. 2021). Overall, the regulation of iron homeostasis in relation to ferroptosis is complex and delicate.

4.4.2 Lipid Toxicity

Ferroptosis is characterized by excessive lipid peroxidation and the formation of toxic lipid metabolites (Lin et al. 2021). The lipids most susceptible to peroxidation, arachidonic acid (AA) and adrenic acid (AdA), can lead to cell membrane rupture.

The synthesis of polyunsaturated fatty acid phospholipids (PUFA-PLs) is governed by two key mediators, acyl-CoA synthetase long-chain family member 4 (ACSL4) and lysophosphatidylcholine acyltransferase 3 (LPCAT3) (Yuan et al. 2016; Dixon et al. 2015). ACSL4 links PUFAs (AA or AdA) to CoA to form PUFA-CoAs, which are then esterified with PLs by LPCAT3. The carboxylation of acetyl CoA to malonyl-CoA by acetyl CoA carboxylase (ACC) is also needed for some PUFA synthesis. Inhibition of ACSL4 or LPCAT3 can, therefore, prevent or reduce sensitivity to ferroptosis. The lipid flippase solute carrier family 47 member 1 (SLC47A1) deficiency can also use the ACSL4-sterol O-acyltransferase 1 (SOAT1) pathway instead of the ACSL4-LPCAT3 pathway to produce PUFA-containing cholesterol esters and promote ferroptosis (Lin et al. 2022).

The role of lipoxygenases (ALOXs), a non-heme iron-dependent enzyme that can directly oxidize PUFA-containing lipids in membranes, in ferroptosis is content-dependent. For example, ALOX12 and ALOX15 are not essential for ferroptosis in mice, suggesting that other regulators, such as cytochrome P450 oxidoreductase (POR), contribute to lipid peroxidation during ferroptosis (Zou et al. 2020). Mono-unsaturated fatty acids (MUFAs) produced by stearoyl-CoA desaturase (SCD) can completely inhibit PUFA-mediated ferroptosis (Magtanong et al. 2019). Additionally, 4-hydroxy-2-nonenal (4-HNE), a byproduct of lipid peroxidation, has recently been implicated as a direct mediator of ferroptosis (Chen et al. 2022b), highlighting that the effector of ferroptosis may not necessarily be a protein.

4.4.3 GPX4 Antioxidant System

Glutathione peroxidase 4 (GPX4) is the sole GPX member found in mammalian cells, and it converts phospholipid (PL) hydroperoxides into alcohols. Studies have revealed that cytosolic GPX4 plays a crucial role in inhibiting ferroptosis (Yang et al. 2014), as evidenced by the fact that re-expression of cytosolic GPX4 rescues *Gpx4* deletion-induced ferroptosis in mouse embryonic fibroblasts (Yant et al. 2003). In certain cases, mitochondrial GPX4 also helps in blocking mitochondrial oxidative damage-induced ferroptosis in cancer cells (Mao et al. 2021b).

The anti-ferroptotic effects of GPX4 are dependent on reduced glutathione (GSH), a tripeptide derived from glycine, glutamate, and cysteine, with cysteine being the rate-limiting precursor. Most cells obtain cysteine through the system xc^--mediated uptake and subsequent transformation of cystine, which involves solute carrier family 7 member 11 (SLC7A11) and solute carrier family 3 member 2 (SLC3A2). Deprivation of cysteine through dietary means or pharmacological inhibition of the SLC7A11-GPX4 pathway (using erastin or RSL3) can trigger ferroptosis in various cells. While GPX4 is considered a key suppressor of ferroptosis, the conditional inactivation of GPX4 does not always result in ferroptosis (Viswanathan et al. 2017; Kang et al. 2018a), pointing to the presence of a GPX4-independent pathway.

4.4.4 AIFM2 Antioxidant System

Screening with CRISPR-Cas9 has identified apoptosis-inducing factor mitochondria-associated 2 (AIFM2, also known as FSP1) as a defense protein against ferroptosis, which operates in parallel to the GPX4 system (Doll et al. 2019). The mechanism involves N-myristoylation, which is necessary for the translocation of AIFM2 from mitochondria to the plasma membrane, thereby reducing lipid peroxidation and ferroptosis. This is achieved by reducing ubiquinone to ubiquinol (CoQH2) through its NADH:ubiquinone oxidoreductase activity, which traps lipid peroxyl radicals and prevents lipid autoxidation or indirectly oxidized α-tocopheryl radicals (vitamin E, a lipid antioxidant) (Doll et al. 2019). As a result, AIFM2 functions as a non-mitochondrial electron transport chain.

AIFM2 also serves as a vitamin K reductase, converting vitamin K into hydroquinone (VKH2) and thereby protecting GPX4-depleted cells from harmful lipid peroxidation and ferroptosis (Mishima et al. 2022). In addition to its reported enzymatic functions, AIFM2 can activate endosomal sorting complexes required for transport (ESCRT)-III–dependent membrane repair mechanisms to inhibit ferroptosis (Dai et al. 2020c).

In conclusion, these findings underscore the presence of three different molecular and cellular mechanisms through which AIFM2 protects against ferroptosis.

4.4.5 DHODH Antioxidant System

Mitochondria play a crucial role in regulating cellular metabolism and death, and they generate a substantial amount of ROS during oxidative phosphorylation. An imbalance in the mitochondrial antioxidant system can lead to lipid peroxidation. Dihydroorotate dehydrogenase (DHODH) is an enzyme that plays a crucial role in the de novo synthesis of pyrimidine nucleotides, which are the building blocks of DNA and RNA (Zhou et al. 2021). DHODH catalyzes the oxidation of dihydroorotate to orotate, which is an essential step in the formation of uracil and cytosine, two of the four nucleotides that make up the DNA molecule. In ferroptosis, DHODH controls pyrimidine synthesis in the inner mitochondrial membrane and can transfer electrons to CoQ to reduce it to CoQH2 (Mao et al. 2021a). An increase in DHODH-mediated CoQH2 production compensates for lipid peroxidation caused by the inactivation of GPX4. This mechanism can only be rescued by mitochondrial GPX4 or DHODH, not cytosolic GPX4. This highlights the existence of a mitochondrial antioxidant defense mechanism that helps prevent ferroptosis.

4.4.6 NFE2L2 Antioxidant System

NFE2L2, also known as NRF2, is a crucial transcription factor in the cap'n'collar (CNC) family (Dai et al. 2020a). It plays a vital role in maintaining redox homeostasis and regulating antioxidant genes by binding to antioxidant response elements

(AREs). Under normal circumstances, the activity of NFE2L2 is suppressed as it is bound by Kelch-like ECH-associated protein 1 (KEAP1) in the cytoplasm, leading to its degradation. However, under oxidative and electrophilic stress, NFE2L2 is dissociated from KEAP1 and travels to the nucleus, where it partners with small v-maf musculoaponeurotic fibrosarcoma oncogene homolog (sMaf) proteins to activate the expression of ARE-dependent target genes.

In addition, NFE2L2 also provides cellular protection against ferroptosis activators like erastin and sorafenib (Sun et al. 2016b). It increases the stability of the NFE2L2 protein by suppressing the formation of the NFE2L2-KEAP1 complex. This process is regulated by SQSTM1, an autophagy receptor, which activates NFE2L2 expression through the inactivation of KEAP1. The activation of the SQSTM1-KEAP1-NFE2L2 pathway, therefore, blocks ferroptosis induced by erastin and sorafenib in cancer cells by upregulating multiple target genes (Sun et al. 2016b). Along with the well-known NFE2L2-targeted genes, such as nicotinamide adenine dinucleotide phosphate hydrogen (NADPH) quinone dehydrogenase 1 (NQO1), GPX4, SLC7A11, and ferritin heavy chain 1 (FTH1), the cysteine-rich metallothionein 1G (MT1G) protein has been identified as a novel NFE2L2-targeted gene that provides resistance to ferroptosis in vitro and in xenograft mouse models (Sun et al. 2016a; Houessinon et al. 2016).

4.4.7 Membrane Repair System

The plasma membrane's integrity and function are essential for cell survival and communication, and the membrane repair system helps preserve them. This system includes regrouping lipids, forming lipid rafts, and involving proteins, such as phospholipases, lipases, and flippases. A lipid flippase, SLC47A1, can prevent ferroptosis in cancer cells by restructuring lipids and has been observed at elevated levels in cancers (Wittwer et al. 2013). Targeting SLC47A1 may overcome resistance to ferroptosis in cancer cells.

The ESCRT complexes play a crucial role in repairing the plasma membrane and preventing various forms of regulated necrosis, including ferroptosis (Liu et al. 2021a). Inhibiting charged multivesicular body protein 5 (CHMP5) or CHMP6, components of ESCRT-III, increases ferroptosis susceptibility in HCC cells (Dai et al. 2020b). Calcium signaling and endoplasmic reticulum stress regulate ferroptosis through the activation of ESCRT-III on cell membranes.

4.5 Autophagy in Ferroptosis

Autophagy can either inhibit or promote cell death depending on the context. This section discusses the mechanisms by which autophagy regulates ferroptosis in cancer cells (Xie et al. 2016).

4.5.1 Ferritinophagy

Ferritinophagy is a type of autophagy, which is a process where cells break down and recycle their own components (Ajoolabady et al. 2021). Ferritinophagy specifically degrades ferritin, a complex made up of two proteins, FTH1 and FTL1. A cytosolic autophagy receptor called NCOA4 is involved in mediating ferritin degradation and promoting ferroptosis. Ferroptosis is a type of programmed cell death that occurs when cells accumulate toxic levels of iron.

The expression of NCOA4 can be regulated by different signaling pathways. For example, O-GlcNAcylation of FTH1 can inhibit its binding to NCOA4 and thus decrease ferritinophagy. On the other hand, inhibiting O-GlcNAcylation can enhance ferritinophagy (Yu et al. 2022). Another example is poly(rC) binding protein 1 (PCBP1), which can suppress autophagy and thus inhibit ferritinophagy-dependent ferroptosis (Lee et al. 2022). In contrast, ELAV-like RNA binding protein 1 (ELAVL1/HuR) can regulate ferritinophagy-dependent ferroptosis (Zhang et al. 2018).

Different factors can also affect ferritinophagy and ferroptosis. For example, hypoxia downregulates NCOA4 and increases FTH/FTL protein levels, which can inhibit ferritinophagy. On the other hand, transmembrane protein 164 (TMEM164), but not ATG9A, can selectively promote autophagosome formation to degrade ferritin during ferroptosis (Liu et al. 2022a). In addition, inhibiting the lysosomal V-ATPase with bafilomycin A1 can decrease ferritin degradation and ferroptosis (Gao et al. 2018), whereas dihydroartemisinin can induce ferritinophagy-dependent ferroptosis (Lin et al. 2016).

Overall, ferritinophagy plays a major role in promoting ferroptosis by increasing the accumulation of toxic iron. However, since NCOA4 is widely expressed, targeting NCOA4-dependent ferroptosis in cancer therapy may require careful monitoring of toxicity.

4.5.2 Lipophagy

Fatty acids are converted into triglycerides and cholesterol esters inside cells and stored in a spherical organelle called lipid droplets (LDs) (Maan et al. 2018). During times of stress, such as starvation or RSL3, LDs can be engulfed by autophagosomes for destruction in the lysosome, releasing free fatty acids. This process, called lipophagy, promotes RSL3-induced ferroptosis in primary mouse hepatocytes and the human liver cancer cell line HepG2. The regulation of this process is controlled by the LD cargo receptor RAB7A (Bai et al. 2019; Schroeder et al. 2015).

RAB7A selectively recognizes LDs and triggers the ATG5-dependent formation of autophagosomes (Bai et al. 2019). If ATG5 or RAB7A is knocked down, RSL3-induced ferroptosis is prevented both in vitro and in vivo (Bai et al. 2019). Similarly, knocking down the tumor protein D52 (TPD52), which regulates LD formation, increases RSL3-induced ferroptosis. On the other hand, overexpressing TPD52 limits ferroptosis (Bai et al. 2019). These findings suggest that increasing LD storage

can protect against lipid toxicity, whereas lipophagy makes cells more sensitive to ferroptosis by releasing more lipids for lipid peroxidation. Further research on the signals and mediators of lipophagy is crucial for developing ferroptosis-related therapies in cancers with high levels of LDs, such as liver cancer.

4.5.3 Clockophagy

The circadian rhythm is an internal mechanism that is controlled by circadian clock proteins, such as aryl hydrocarbon receptor nuclear translocator-like protein (ARNTL/BMAL1) and CLOCK. These proteins regulate various cellular processes including iron and lipid metabolism. It has been found that ARNTL degradation can occur through an autophagy-dependent pathway called clockophagy (Liu et al. 2019), which specifically degrades ARNTL1 and promotes ferroptosis in cancer cells when exposed to GPX4 inhibitors (e.g., RSL3 and FIN56) but not when exposed to SLC7A11 inhibitors (e.g., erastin, sulfasalazine, and sorafenib) (Yang et al. 2019).

Mass spectrometry analysis showed that SQSTM1 acts as an autophagy receptor that binds ARNTL to ATG5- and ATG7-dependent autophagosomes for degradation in the lysosome (Yang et al. 2019). This process does not require ATG9A. The degradation of ARNTL represses the expression of the target gene EGLN2, which controls lipid metabolism through hypoxia-inducible factor 1-alpha (HIF1A) (Yang et al. 2019). Additionally, the knockdown of the circadian protein PER1 impedes autophagy in the hippocampus, which may increase susceptibility to ferroptosis during cerebral ischemia (Rami et al. 2017). The ferroptosis inhibitor liproxstatin-1 prevents acute pancreatitis in pancreatic *Arntl*-specific knockout mice induced by L-arginine (Liu et al. 2020b). Furthermore, extracellular SQSTM1 induces ACSL4 expression to enhance autophagy-dependent ferroptosis and subsequent pancreatitis (Yang et al. 2022), providing alternative strategies to mediate ferroptosis-induced sterile inflammation.

These findings show a relationship between circadian rhythms, autophagy, and ferroptosis. Further research is needed to determine if regulators of ferroptosis are influenced by circadian rhythms.

4.5.4 Mitophagy

Mitophagy, a mechanism for controlling the quality of mitochondria, regulates the production of mitochondrial ROS and the sensitivity to cell death (Jiao et al. 2021). PINK1-Parkin-dependent mitophagy is the central way of managing mitochondrial health. However, the connection between mitochondria and ferroptosis is unclear and can even seem paradoxical. For instance, the mitochondrial respiratory chain inhibitor BAY 87–2243 triggers excessive mitophagy and leads to an increase in mitochondrial ROS production and subsequent ferroptosis in BRAFV600E melanoma cell lines, but overexpression of GPX4 or administration of ferrostatin-1

reverses the ferroptosis induced by BAY 87–2243 (Basit et al. 2017). On the other hand, a loss of function in fumarate hydratase, a component of the mitochondrial tricarboxylic acid cycle, confers resistance to ferroptosis induced by cysteine deprivation. The compound WJ460, which interacts with myoferlin protein, promotes mitophagy and mitochondrial fission and heightens pancreatic cancer cell sensitivity to ferroptosis (Rademaker et al. 2022). Additionally, zalcitabine-induced stress on mitochondrial DNA triggers autophagy-dependent ferroptosis through the stimulator of interferon response CGAMP interactor 1 (STING1, also known as STING or TMEM173) pathway (Li et al. 2021a). STING1 also collaborates with MFN1/2 to enhance mitochondrial fusion during ferroptosis (Li et al. 2021a). These studies shed light on the intricate relationship between mitophagy, mitochondrial dynamics, and ferroptosis, yet the role of specific autophagy receptors in mediating mitophagy and ferroptosis remains to be investigated further.

4.5.5 Reticulophagy

The ER is a cell organelle responsible for making proteins and lipids. When there is an accumulation of unfolded proteins during protein synthesis, the cell activates the "unfolded protein response" (UPR) to prevent damage. Excess UPR can lead to cell death (Oakes and Papa 2015). Reticulophagy is a type of autophagy that helps to remove unnecessary parts of the ER or proteins that the UPR cannot process. Sorafenib, a type of drug, has been shown to limit ferroptosis in liver cancer cells by inducing reticulophagy through the reticulophagy regulator family member 3 (FAM134B) receptor (Liu et al. 2022b). The exact mechanism is still unknown, but it may involve the activation of FAM134B by the protein poly(A) binding protein cytoplasmic 1 (PABPC1) during ferroptosis (Liu et al. 2022b). These findings suggest that different types of selective autophagy can play a role in controlling ferroptosis.

4.5.6 GPX4 Degradation

Both CMA and autophagy play a role in the degradation of GPX4, leading to the enhancement of ferroptosis in cancer cells (Yang et al. 2020; Han et al. 2021). The autophagy inducer rapamycin and the ferroptosis inducer RSL3 can suppress MTOR and degrade GPX4, implying that ferroptosis might be regulated by GPX4 degradation through autophagy (Liu et al. 2021d). Exogenous copper leads to an increase in GPX4 ubiquitination, and the formation of GPX4 aggregates through direct binding to the GPX4 protein cysteines at positions 107 and 148. TAX1BP1, or Tax1 binding protein 1, functions as an autophagic receptor that mediates GPX4 degradation and initiates ferroptosis in response to copper stress (Xue et al. 2023). Heat shock protein 90 (HSP90) has been discovered as a molecular chaperone that interacts with the CMA cargo receptor LAMP2A, thereby mediating GPX4 degradation during ferroptosis (Wu et al. 2019). Conversely, inhibiting HSP90 stops CMA-induced

ferroptosis in the mouse neuronal cell line HT-22. On the other hand, HSPA5, an ER-associated molecular chaperone, prevents erastin-induced ferroptosis in human pancreatic cancer cell lines by inhibiting GPX4 protein degradation (Zhu et al. 2017). These findings indicate that different HSP family proteins exert diverse molecular chaperone functions in either enhancing or inhibiting GPX4 degradation.

4.5.7 CDH2 Degradation

The role of specific autophagy receptors in ferroptosis is still an unresolved question in the field. However, recent research has shed light on this topic. A study that combined membrane protein screening with functional analyses showed that hippocalcin like 1 (HPCAL1), a calcin-like protein found in the hippocampus, serves as a specific autophagy receptor that promotes ferroptosis in cancer cells (Chen et al. 2022c). It does so by directly mediating the degradation of the cadherin 2 (CDH2) protein but not the degradation of ferritin, SLC40A1, or GPX4. HPCAL1 is not necessary for starvation-induced autophagy or cancer cell apoptosis, and its known role as a Ca^{2+}-binding protein is not essential for its role in autophagy-dependent ferroptosis. The study also found that PRKCQ-mediated phosphorylation of Thr149 on HPCAL1 is necessary for its recognition and degradation of CDH2 (Chen et al. 2022c). This discovery provides new insights into the specific role of autophagy-dependent ferroptosis in cancer therapy.

4.5.8 SLC40A1 Degradation

SLC40A1 is found in cells that store and release iron, such as cells in the lining of the small intestine (enterocytes) and cells in the liver (hepatocytes). Mutations in the SLC40A1 gene have been linked to a type of iron overload disorder called hemochromatosis. This condition is characterized by the buildup of too much iron in the body, which can lead to damage to tissues and organs over time. Hemochromatosis has several forms, each with a different underlying cause, but mutations in the SLC40A1 gene are responsible for about 10% of cases. Autophagic degradation of SLC40A1 by SQSTM1 also increases iron accumulation and ferroptosis (Li et al. 2021c). It is necessary to further evaluate the relationship between autophagy, ferroptosis, and hemochromatosis in the future.

4.5.9 BECN1-Mediated System xc^- Inhibition

The multifaceted protein BECN1 plays a crucial role in the regulation of ferroptosis (Kang et al. 2018b). Direct binding of BECN1 to SLC7A11, in response to inhibitors, such as erastin, sulfasalazine, and sorafenib but not GPX4 inhibitors, such as RSL3 and FIN56, results in cysteine deprivation-induced ferroptosis. Phosphorylation of BECN1 at S90 and S93 by AMPK promotes the formation of the

BECN1-SLC7A11 complex, and mutations at these phosphorylation sites prevent ferroptosis. The BECN1 activator, Tat-beclin 1 protein peptide, boosts both autophagy and ferroptosis-induced tumor suppression in mice. However, the mechanism by which AMPK selectively activates BECN1 remains unclear.

The RNA binding protein ELAVL1/HuR can also mediate ferroptosis by promoting autophagy activation through binding to AU-rich elements within the 3′--untranslated region F3 of BECN1 mRNA in human hepatic stellate cells (Zhang et al. 2018). This process may be further enhanced by the exosome-mediated delivery of BECN1 secreted by human umbilical cord mesenchymal stem cells. The binding of BECN1 to ubiquitin-specific protease 11 (USP11) in spinal cord ischemia-reperfusion injury further demonstrates BECN1's role in mediating autophagy-dependent ferroptosis (Rong et al. 2021). Overall, BECN1 can regulate ferroptosis by either limiting SLC7A11 activity directly or by activating an autophagy-dependent pathway.

4.5.10 Lysosomal Membrane Permeabilization

The accumulation of iron in the lysosomal lumen can trigger a Fenton response that increases lysosomal membrane permeabilization (LMP) and ultimately leads to cell death. In contrast, lysosomal ferritin can mitigate oxidative stress. LMP results in the release of lysosomal contents, such as CTSB, CTSD, and iron, which, in turn, increase ROS production and initiate ferroptosis. This process may also be regulated by V-ATPase-mediated selective autophagy (Chen et al. 2022a). Ferroptosis can also be induced by erastin-mediated lysosomal cell death. The signal transducer and activator of transcription 3 (STAT3) regulates this process by inducing CTSB expression, which is required for lysosomal cell death (Gao et al. 2018). However, inhibiting STAT3 pharmacologically (using the cathepsin inhibitor CA-074Me) or genetically limits ferroptosis in cancer cells. In 5-FU-resistant gastric cancer, STAT3 plays a negative role in ferroptosis by binding to the promoters of genes, such as GPX4, SLC7A11, and FTH1, that negatively regulate ferroptosis (Ouyang et al. 2022). These findings suggest that STAT3 has a context-dependent role in ferroptosis, and the receptors and mechanisms involved in mediating STAT3 degradation remain to be investigated.

4.6 Conclusions and Perspectives

Ferroptosis has seen significant advancements in research in recent years (Stockwell et al. 2017; Chen et al. 2021c; Chen et al. 2021b). Our perception of ferroptosis and how it is controlled has shifted as it has become known to have close ties to autophagy. Elevated autophagic activity can drive ferroptosis by selectively eliminating antioxidant proteins and cellular components, which raises questions about the molecular and metabolic checkpoints that influence cell survival or death. Ferroptosis and autophagy are complex processes that involve oxidative stress and

membrane structural changes and are regulated by various mechanisms. However, more needs to be understood about the role of selective autophagy in regulating ferroptosis sensitivity.

Developing therapeutic strategies targeting cancer cells that involve autophagy or ferroptosis can be challenging as both processes can occur in normal cells and tissues. Further identification of biomarkers, such as damage-associated molecular patterns (DAMPs) (Zhang et al. 2013), high-mobility group protein 1 (HMGB1) (Wen et al. 2019), and decorin (DCN) (Liu et al. 2021c), is crucial for future clinical studies on ferroptosis. Moreover, gaining insight into the immune properties of ferroptotic cell death is crucial for the development of new immunotherapies in the fight against diseases (Chen et al. 2022c; Liu et al. 2021c, 2021d; Zhang et al. 2020; Yang et al. 2022).

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Heat Shock Proteins and HSF1 in Ferroptosis

5

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Abstract

When cells are exposed to various stressors, such as heat, toxins, or oxidative stress, heat shock transcription factor 1 (HSF1) becomes activated and triggers the expression of genes that encode heat shock proteins (HSPs), which play important roles in a variety of cellular processes, including protein folding, transport, and degradation, as well as protection against cell damage and death. Ferroptosis is a non-apoptotic form of cell death that is triggered by iron-dependent lipid peroxidation. This process involves imbalanced redox systems, iron accumulation, and lipid peroxidation, which can impact various intracellular organelles and proteins. As a result, maintaining the stability of intracellular proteins is important for regulating ferroptosis sensitivity. In this chapter, we summarized the mechanisms that regulate protein homeostasis and described the role of HSF1-mediated expression of HSPs in ferroptosis. A better understanding of the link between HSPs and ferroptosis may pave the way for the development of novel strategies in the treatment of various diseases.

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5.1 Introduction

Heat shock proteins (HSPs) are a group of highly conserved proteins found in plants, fungi, insects, and mammals (Tong et al. 2022). They play a crucial role in various cellular processes and assist in intracellular protein folding, processing, and translocation to maintain protein stability and prevent protein aggregation (Tutar and Tutar 2010; Hartl et al. 2011). HSPs can be divided into different categories based on their molecular weight, including small HSPs, HSP40, HSP60, HSP70, HSP90, and large HSPs, with heat shock transcription factor 1 (HSF1) serving as a key regulator of gene transcription in HSPs.

In response to stressors, such as oxidative damage, thermal damage, hypoxia, and accumulation of misfolded proteins, HSPs are activated in a transcription-dependent manner to maintain protein homeostasis in the cytoplasm, endoplasmic reticulum, mitochondria, and other cellular regions (Richter et al. 2010). They help refold misfolded proteins, but if they are unable to do so, the misfolded proteins are degraded through the proteasome pathway to alleviate proteotoxic stress. However, if misfolded proteins accumulate, they tend to initiate corresponding signaling pathways that can lead to cell death. The outcome depends on the interplay between complex signaling pathways, the type, intensity, and duration of the stress.

According to the Nomenclature Committee on Cell Death, there are two categories of cell death: accidental cell death (ACD) and regulated cell death (RCD). ACD is caused by physical, chemical, or mechanical conditions, while RCD depends on specific molecular mechanisms (Galluzzi et al. 2018; Tang et al. 2021a). Ferroptosis is a type of non-apoptotic RCD that is caused by iron-dependent lipid peroxidation and subsequent plasma membrane damage (Tang and Kroemer 2020). It involves a complex interplay of regulatory factors (Chen et al. 2021e).

In this chapter, we discuss how HSPs regulate ferroptosis from a functional perspective, with the aim of providing new insights into the mechanism and defense of ferroptosis.

5.2 HSPs in Protein Homeostasis

HSPs are commonly referred to as molecular chaperones due to their original perception of providing protective functions within the cell. They aid in the refolding of proteins and the restoration of protein function when the cell is exposed to stressors, such as high temperatures, low oxygen levels, heavy metals, drugs, or other agents that cause protein denaturation. Different HSPs are distributed in various organelles and contribute to local protein homeostasis in unique ways. HSPs primarily maintain protein homeostasis through the heat shock response (HSR, for cytoplasmic protein homeostasis), the unfolded protein response (UPR, for endoplasmic reticulum [ER] protein homeostasis), and the mitochondrial unfolded protein response (mtUPR, for mitochondrial protein homeostasis). In this section, we will outline the process of these three mechanisms and their involvement with HSPs.

5.2.1 Heat Shock Response

HSR was first observed in 1962 when Italian geneticists discovered that Drosophila larvae chromosomes puffed up upon exposure to high temperatures (Ritossa 1996). This puff was taken as a sign of gene expression and the rapid gene transcription was seen to occur just minutes after the heat exposure. A growing number of studies later showed that HSPs were predominantly induced during HSR (Fig. 5.1).

HSR not only increases the expression of protective HSPs but also leads to the downregulation of other protein expression (Tissières et al. 1974; Lewis et al. 1975;

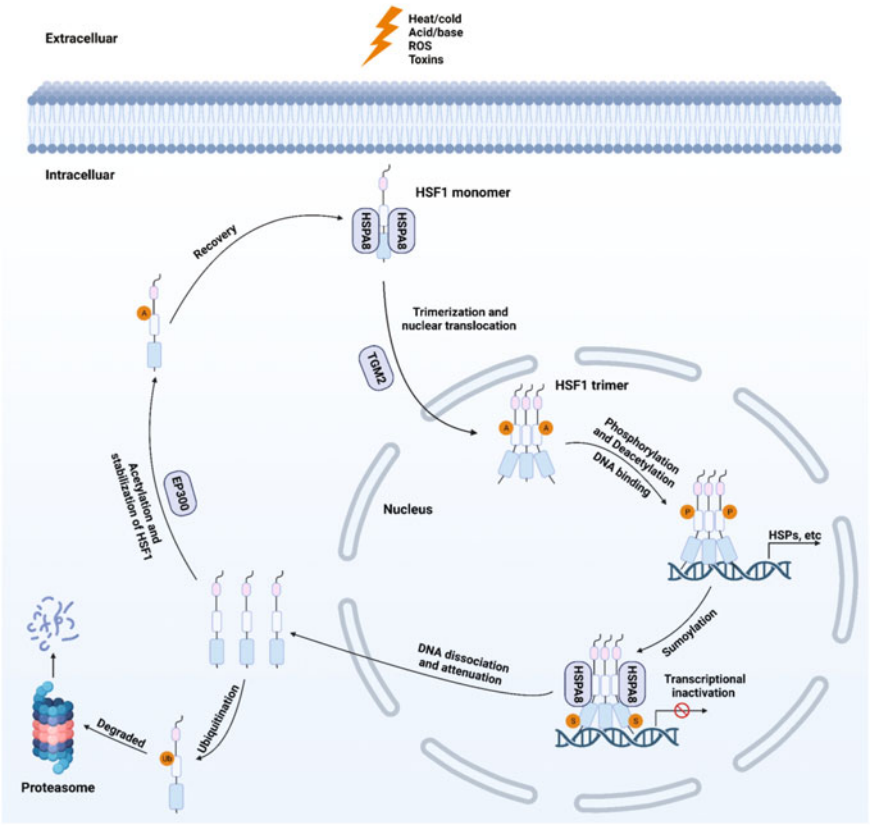

Fig. 5.1 Mechanism of activation of the heat shock response. The heat shock response is triggered by exposure to temperature changes, pH imbalances, elevated levels of ROS, and toxin accumulation. TGM2 catalyzes the trimerization and translocation of HSF1 to the nucleus. Once in the nucleus, HSF1 is phosphorylated and deacetylated, allowing it to bind to DNA and activate the transcription of target genes. HSPA8 can bind to HSF1 to prevent transactivation, while sumoylation of HSF1 recruits repressors. Over time, HSF1 loses its transcriptional activity and dissociates from DNA, leading to its ubiquitination and degradation by the proteasome. On the other hand, EP300 stabilizes HSF1 through acetylation, and HSF1 can then re-bind to HSP40 and HSPA8, maintaining an inactive state

Spradling et al. 1977; Vihervaara et al. 2018). This helps to restore the cell to homeostasis under proteotoxic conditions. HSR can be induced not just by high temperatures but also by various exogenous and endogenous stimuli, including cold stress and changes in pH (Colinet et al. 2010; Triandafillou et al. 2020; Petronini et al. 1995).

HSF1 is the main regulatory molecule of HSR (Pincus et al. 2018; Solís et al. 2018). In the absence of stress, HSF1 is maintained in an inactive monomeric form by molecular chaperones such as HSP70 and HSP40. When a proteotoxic stress occurs, the molecular chaperones are recruited to the misfolded protein, HSF1 is released and travels to the nucleus in the presence of transglutaminase 2 (TGM2) to form a homotrimer that binds to the heat shock element (HSE) and induces transcription of molecular chaperones and protein degradation-associated genes (Kmiecik and Mayer 2022).

To terminate HSR when the cells return to homeostasis, proper mechanisms are needed. During HSR recovery, the heat shock protein family A member 8 (HSPA8) binds to the trans-activation domain of HSF1 to initially attenuate HSR, and then high concentrations of HSPA8 induce the dissociation of HSF1 from DNA, leading to complete termination of HSR. The dissociated monomeric form of HSF1 is then transported to the ubiquitin-proteasome system (UPS) for degradation (Kmiecik et al. 2020; Connell et al. 2001).

HSP90AA1 (also known as HSP90) was also found to inhibit the transcriptional activity of HSF1 (Zou et al. 1998). These findings suggest that HSR is a crucial mechanism for maintaining cellular protein homeostasis and has negative feedback regulation to prevent excessive activation. Many of the genes regulated during HSR are not regulated by the HSF family, implying that the process also involves non-HSF-dependent mechanisms. For example, serum response factor (SRF) was found to rapidly mediate cytoskeletal gene expression in HSR of mouse fibroblasts, a process that does not require the involvement of the HSF family (Mahat et al. 2016).

5.2.2 Unfolded Protein Response

The ER is a central organelle that plays a crucial role in protein synthesis, folding, transport, and quality control (Kohli et al. 2021). Unlike the HSR, which reacts to proteotoxicity in the cytoplasm, the ER has a separate mechanism for maintaining protein homeostasis that involves molecular chaperones. The accumulation of misfolded proteins in the ER can result in ER stress, which is triggered by various factors, such as hypoxia, pH acidification, nutrient deficiencies, and calcium ion disruption (Kohli et al. 2021; Chen and Cubillos-Ruiz 2021). This stress activates an UPR to restore ER homeostasis.

One of the important molecular chaperones in the ER is heat shock protein family A member 5 (HSPA5), also known as 78-kDa glucose-regulated protein (GRP78) or binding immunoglobulin protein (BIP). HSPA5 is a member of the HSP70 family and has a nucleotide-binding domain (NBD) with ATPase activity and a substrate binding domain (SBD) that binds client proteins and hydrolyzes ATP to ADP with

the assistance of HSP40 (Pobre et al. 2019). HSPA5 helps ensure proper protein folding and transport in the ER, and in some cases, transports misfolded proteins to the ER membrane for degradation by the proteasome pathway (Kim et al. 2021; Ibrahim et al. 2019). The ER also has an ER-associated protein degradation (ERAD) mechanism that degrades proteins, whether misfolded or not, via synoviolin 1 (SYVN1)-mediated recognition and reverse transfer (Wei et al. 2018).

In addition to its chaperone role, HSPA5 also acts as a buffer for calcium ions in the ER to maintain calcium homeostasis (Prins and Michalak 2011). There is growing evidence that HSPA5 can migrate to the cell surface, where it functions as a receptor or binds to ligands (Van Krieken et al. 2021). For example, isthmin 1 (ISM1) regulates lung homeostasis by controlling alveolar macrophage populations and functional phenotypes through HSPA5-mediated apoptosis on the cell surface (Lam et al. 2022).

Three transmembrane proteins in the ER serve as pressure sensors for ER stress: activating transcription factor 6 (ATF6), eukaryotic translation initiation factor 2 alpha kinase 3 (EIF2AK3, also known as PERK), and endoplasmic reticulum to nucleus signaling 1 (ERN1, also known as IRE1α) (Hetz et al. 2020). When ER protein levels are stable, HSPA5 is associated with these three sensors to keep them in an inactive state. During ER stress, when HSPA5 has a higher affinity for unfolded or misfolded proteins, it dissociates from the sensors, activating the UPR (Wang et al. 2009; Walter and Ron 2011). The UPR is a conserved adaptive mechanism that has three branches to handle imbalances in protein homeostasis in the ER (Fig. 5.2) (Braakman and Bulleid 2011).

ERN1 functions as an ER transmembrane protein with both a cytoplasmic kinase structure and an RNase structural domain (Hetz and Glimcher 2009). Upon UPR activation, ERN1 oligomerizes and autophosphorylates, leading to the splicing of a 26-nucleotide fragment of X-box-binding protein 1 (XBP1) mRNA in the cytoplasm (Yoshida et al. 2001). The spliced XBP1 encodes a transcriptionally active X-box-binding protein, while unspliced XBP1 encodes a less active protein (Chen and Cubillos-Ruiz 2021). The spliced XBP1 induces HSPs and ERAD-related proteins (Calfon et al. 2002). The RNase domain of ERN1 also regulates protein synthesis in the ER through the regulated IRE1α-dependent decay pathway and regulates inflammation and cell survival through signaling pathways like nuclear factor kappa B (NF-κB) and mitogen-activated protein kinase (MAPK) (Hollien and Weissman 2006; Kang et al. 2022; Sozen et al. 2022; Liu et al. 2020a).

EIF2AK3 is a serine-threonine kinase that reduces protein synthesis by phosphorylating eukaryotic translation initiation factor 2A (EIF2A), inhibiting mRNA translation (Harding et al. 1999). The phosphorylation of EIF2A leads to the production of activating transcription factor 4 (ATF4), which regulates the expression of HSPs, genes involved in cell metabolism and cell death, and the DNA damage inducible transcript 3 (DDIT3, also known as CHOP) (Liu et al. 2015).

ATF6 translocates to the Golgi during UPR and is cleaved by membrane-bound transcription factor peptidase site 1 (MBTPS1, also known as S1P) and membrane-bound transcription factor peptidase site 2 (MBTPS2, also known as S2P), releasing

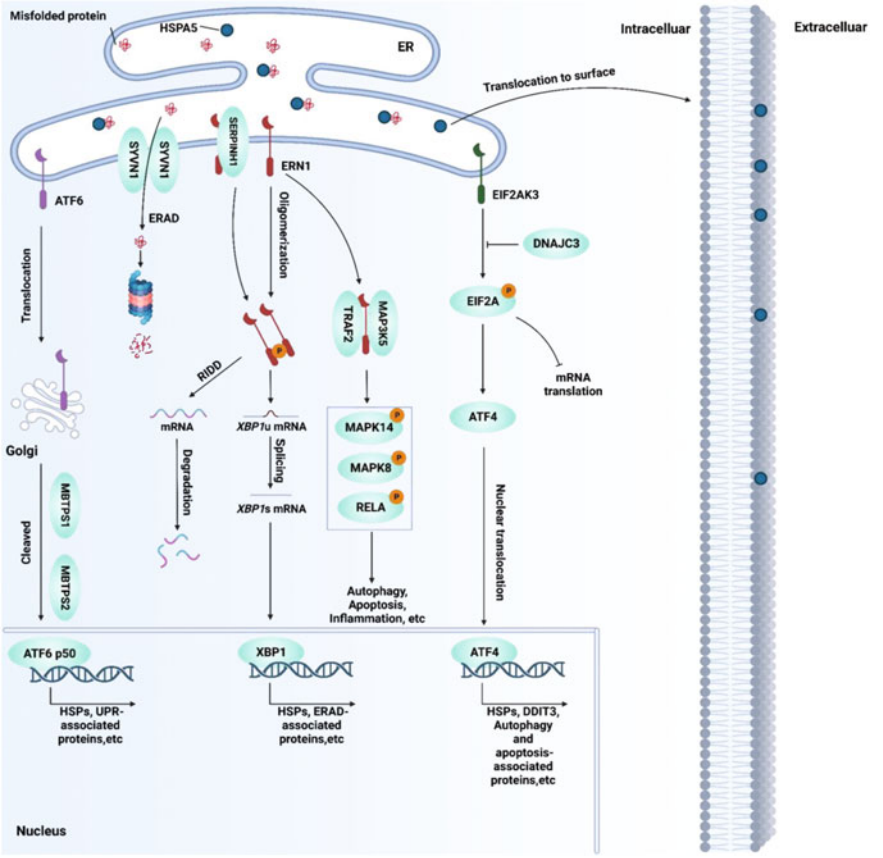

Fig. 5.2 The unfolded protein response in the endoplasmic reticulum. Increased levels of misfolded proteins in the endoplasmic reticulum trigger endoplasmic reticulum stress, leading to an unfolded protein response (UPR). The endoplasmic reticulum chaperone HSPA5 binds tightly to misfolded proteins, causing it to dissociate from the endoplasmic reticulum transmembrane sensors (ATF6, ERN1, and EIF2AK3) and activating them. HSPA5 helps to refold the misfolded protein, and if this is not possible, the protein is degraded by the proteasome through ERAD. Each of the three sensors activates downstream signaling pathways in its own unique way

the N-terminal portion of ATF6 (p50). The p50 enters the nucleus and induces the expression of HSPs and UPR-related genes (Haze et al. 1999; Hillary and FitzGerald 2018).

HSPA5 and serpin family H member 1 (SERPINH1, also known as HSP47) both play a role in regulating UPR activation. SERPINH1 binds to activate ERN1, enhancing XBP1 mRNA splicing and UPR-related gene transcription (Sepulveda et al. 2018). HSPA5 has a high affinity for misfolded proteins, and when misfolded proteins increase, SERPINH1 can bind to ERN1 to accelerate activation and enhance the UPR process. DnaJ heat shock protein family member B9 (DNAJB9), a co-chaperone of HSPA5, enhances ATP hydrolysis and HSPA5's binding efficiency

to ERN1, leading to ERN1 inactivation. XBP1 also transcribes DNAJB9, making ERN1-DNAJB9 a negative feedback mechanism (Lee et al. 2003).

Other ER chaperones also play critical roles in maintaining protein homeostasis. For instance, transmembrane proteins like calnexin (CANX) and its soluble form, calreticulin (CALR), located in the ER, ensure proper folding of glycoproteins. N-linked glycoproteins are modified in the ER by the addition of one or more precursor glycans (Kozlov and Gehring 2020). Following the sequential cleavage of the two terminal glucose residues by glucosidases I and II, the peptide chain interacts with CANX/CALR. If the protein is still misfolded, it undergoes re-glycosylation and binding to CANX/CALR in a process known as the CANX/CALR folding cycle (Kohli et al. 2021). If the protein fails to fold correctly, it is degraded by ERAD. Additionally, CANX and CALR also regulate ER calcium levels (Prins and Michalak 2011). HSP90B1, also called 94-kDa glucose-regulated protein (GRP94), is a member of the HSP90 family and contributes to calcium buffering in the ER, directing misfolded proteins to ERAD (Christianson et al. 2008; Eletto et al. 2010; Marzec et al. 2012). DnaJ heat shock protein family member C3 (DNAJC3), a member of the HSP40 family and localized in the ER, binds to the N-terminus of misfolded proteins to help refold them by interacting with HSPA5 through its J structural domain (Rutkowski et al. 2007; Oyadomari et al. 2006). DNAJC3 also directly suppresses EIF2AK3 activation, restoring protein synthesis and allowing the ER to return to homeostasis during the UPR (Oyadomari et al. 2006). Thus, the UPR of the ER and the protein folding and degradation mediated by HSPs are crucial for maintaining protein homeostasis in the ER.

5.2.3 Mitochondrial Unfolded Protein Response

Mitochondria are membrane-bound organelles commonly found in eukaryotes, with a primary function of producing ATP through respiration, as well as regulating cellular metabolism and signaling (Attardi and Schatz 1988; Spinelli and Haigis 2018; Zhang et al. 2018a). These organelles also have robust mechanisms to maintain protein homeostasis. The mtUPR regulates the mitochondrial protein input, folding, and quality control (Pickles et al. 2018). This response is often considered to be a transcriptional one, which increases the expression of mitochondrial molecular chaperones in response to an excess of misfolded proteins (Martinus et al. 1996; Bahr et al. 2022).

Activating transcription factor 5 (ATF5) plays a crucial role in the regulation of the mtUPR. Under non-stress conditions, ATF5 is transported to the mitochondria via the TIM-TOM transporter and degraded by the lon peptidase 1 (LONP1) protease. However, under stress conditions, the pathway of ATF5 into the mitochondria is blocked, and it accumulates in the cytoplasm, eventually translocating to the nucleus to induce the transcription of mitochondrial chaperones, proteases, and respiration-related genes (Fiorese and Haynes 2017; Rath et al. 2018).

The accumulation of unfolded proteins in the intermembrane space of mitochondria (ISM) results in an increased accumulation of reactive oxygen species

(ROS), which activates the AKT pathway, leading to the activation of estrogen receptor 1 (ESR1). ESR1 then enters the nucleus to induce the transcription of nuclear respiratory factor 1 (NRF1) and HtrA serine peptidase 2 (HTRA2) (Papa and Germain 2011). NRF1 interacts with sirtuin 7 (SIRT7) to inhibit the transcription of the translational machinery portion of the mitochondria, thereby reducing mitochondrial biogenesis and oxidative metabolism (Mohrin et al. 2015).

In the mitochondrial matrix, the accumulation of unfolded proteins activates eukaryotic translation initiation factor 2 alpha kinase 2 (EIF2AK2, also known as PKR) and further activates AP-1 transcription factor subunit (JUN) via the mitogen-activated protein kinase 8 (MAPK8) pathway. Phosphorylated JUN can induce the transcription of DDIT3, which can bind to the mtUPR element (MURE) promoter to induce the transcription of mitochondrial chaperones (Arnould et al. 2015; Quirós et al. 2016). Furthermore, EIF2AK2 can induce the integrated stress response (ISR) through the phosphorylation of EIF2A in response to intra- and extracellular stress (Zhang et al. 2021a).

Mitochondrial heat shock proteins 60 (mtHSP60) and 70 (mtHSP70) play crucial roles in maintaining mitochondrial homeostasis (Fig. 5.3). HSPA9, the main mtHSP70, is a member of the HSP70 family and has close homology to the murine mortalin (Xie et al. 2000). The theory of mitochondrial symbiosis suggests that early eukaryotic cells evolved mitochondria by engulfing prokaryotes with unique genomes, leading to the transfer of a variety of mitochondrial protein genomes to nuclear chromosomes (Roger et al. 2017). This also led to the development of mechanisms for translocation of mitochondrial proteins from the cytoplasm to mitochondria. When active, the translocase of outer mitochondrial membrane 40 (TOMM40) and translocase of inner mitochondrial membrane 23 (TIMM23) form a supercomplex to facilitate translocation between the outer and inner mitochondrial membranes, with the help of the active presequence translocase-associated motor (PAM), a functional complex located on the matrix side of the endosomal membrane (Hartl 1996). The translocase of inner mitochondrial membrane 44 (TIM44) interacts with HSPA9, localizing it to the inner mitochondrial membrane. ATP hydrolysis, regulated by grpE like 1 (GRPEL1) and grpE like 2 (GRPEL2), drives the entry of precursor peptides into the mitochondrial matrix (Srivastava et al. 2017). After translocation, HSPA9 enters the matrix, binds to the precursor peptide, and works with mtHSP60 to fold the protein. The co-chaperone HSP40 assists HSPA9 in non-covalently interacting with misfolded proteins, facilitating their refolding or degradation if repair fails. HSPA9 also supports the function of the suv3 like RNA helicase (SUPV3L1), a component of the mitochondrial protein translation complex (Herrmann et al. 1994). During stress, HSPA9 facilitates crosstalk between mitochondria and ER by enabling interaction between voltage-dependent anion channel 1 (VDAC1) on the outer mitochondrial membrane and inositol 1,4,5-trisphosphate receptor type 1 (ITPR1) on the ER, leading to calcium influx from the ER into the mitochondrial matrix (Honrath et al. 2017).

Heat shock protein family D member 1 (HSPD1), a component of the mtHSP60 family, has a dynamic state that fluctuates among single toroidal, heptameric single toroidal, and tetrameric double toroidal forms (Levy-Rimler et al. 2001; Viitanen

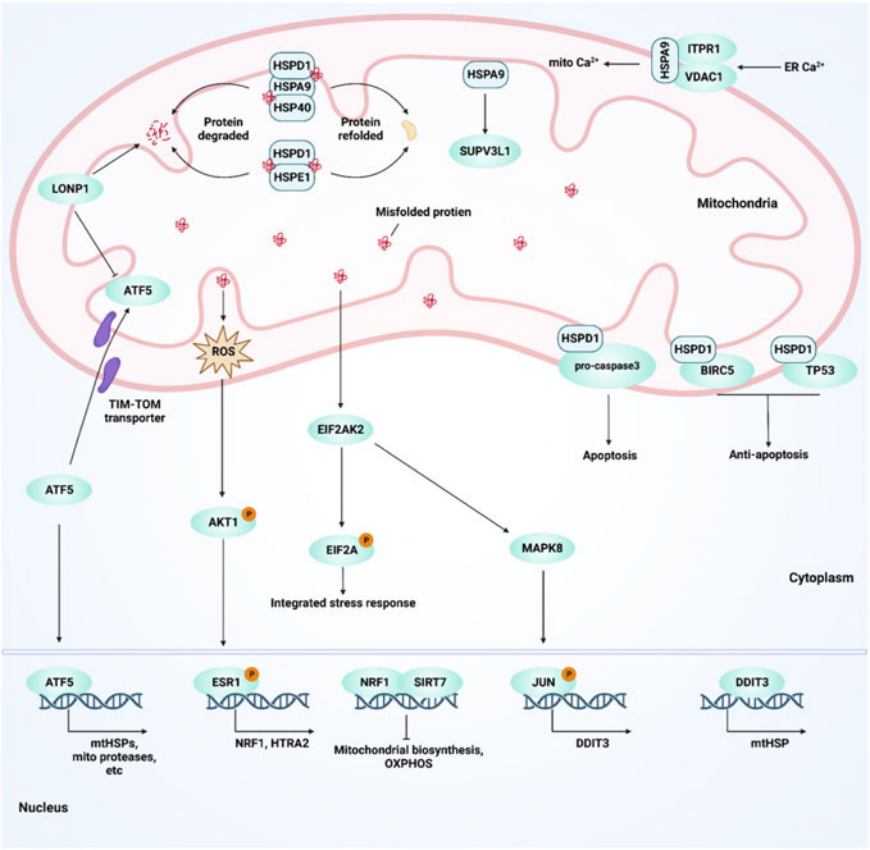

Fig. 5.3 Regulation of mitochondrial protein homeostasis and mtUPR. Misfolded proteins in the mitochondria are managed through the cooperation of co-chaperones, HSPD1, and HSPA9. HSPD1 interacts with pro-caspase3, BIRC5, and TP53 to regulate apoptosis, while HSPA9 interacts with SUPV3L1 to maintain the translation of mitochondrial proteins and to promote calcium influx into the mitochondria via interaction with ITPR1 from the endoplasmic reticulum and VDAC1 on the mitochondrial membrane. The presence of misfolded proteins in the mitochondria triggers the mtUPR response, activating various signaling pathways, inducing the production of mitochondrial heat shock proteins, and regulating mitochondrial biosynthesis and OXPHOS

et al. 1992). The toroids of HSPD1 are capable of binding to unfolded proteins and recruiting the co-chaperone heat shock protein family E member 1 (HSPE1) to bind to the heptameric single toroidal using ATP as a source of energy (Levy-Rimler et al. 2001). This helps to isolate the unfolded protein and support its proper folding, releasing the fully folded protein once ATP is hydrolyzed to ADP. HSPD1 can also fold proteins independently of HSPE1 (Dubaquié et al. 1998). In mitochondria, HSPD1 and HSPE1 form a complex with pro-caspase3 to increase the release of cytochrome C and activate pro-caspase3, resulting in ATP-dependent apoptosis (Samali et al. 1999). However, in cancer cells, HSPD1 stabilizes the anti-apoptotic

protein baculoviral IAP repeat containing 5 (BIRC5, also known as survivin) in mitochondria and forms a complex with tumor protein P53 (TP53) and BCL2 associated X (BAX) to prevent apoptosis initiation (Ghosh et al. 2008). This highlights that the role of HSPD1 in biology is largely dependent on the substrate protein it stabilizes.

5.3 Mechanism of Ferroptosis

Ferroptosis is a form of RCD that is driven by lipid peroxidation and dependent on iron (Xie et al. 2016). It is distinct from apoptosis driven by the caspase family. The small molecule erastin was originally discovered to induce a non-apoptotic type of cell death, and subsequent research revealed that the process is iron-dependent and involves interactions and regulation between multiple molecules (Dolma et al. 2003; Wolpaw et al. 2011). In recent years, it has become widely accepted that the mechanism of ferroptosis involves two initial signals (loss of antioxidant system and accumulation of iron), an intermediate event (lipid peroxidation), and ending events (Chen et al. 2021e; Liu et al. 2021a), as summarized in Fig. 5.4.

5.3.1 Antioxidant Pathways

Phospholipid hydroperoxide glutathione peroxidase 4 (GPX4) is an enzyme that prevents lipid peroxidation and depends on glutathione (GSH) and selenium for its function. GSH is a tripeptide with a cysteine component that acts as an intracellular antioxidant. The synthesis of GSH requires the uptake of cystine through the amino acid transporter system xc^-, followed by its reduction to cysteine (Dixon et al. 2012). The most common inducers of ferroptosis, erastin, and RSL3, work by inhibiting the uptake of cystine by system xc^- and directly inhibiting GPX4, respectively. System xc^- is composed of two subunits, solute carrier family 7 member 11 (SLC7A11) and solute carrier family 3 member 2 (SLC3A2), which are regulated by various intracellular molecules, transcription factors, and enzymes. For example, BECN1 can inhibit SLC7A11 activity (Song et al. 2018b), while OTU deubiquitinase ubiquitin aldehyde binding 1 (OTUB1) can stabilize SLC7A11 protein (Liu et al. 2019a). Transcription factors like NFE2 like BZIP transcription factor 2 (NFE2L2, also known as NRF2), TP53, and BRCA1 associated protein 1 (BAP1) also have a direct impact on the expression of SLC7A11 (Song et al. 2018a; Liu et al. 2019b; Kang et al. 2019; Feng et al. 2021; Zhang et al. 2018b).

Other pathways, such as ATP binding cassette subfamily B member 1 (ABCB1), increase susceptibility to ferroptosis by effluxing GSH from cells (Cao et al. 2019). Cysteine dioxygenase type 1 (CDO1) can sensitize cells to ferroptosis by depleting cysteine, reducing GSH synthesis. However, the knockdown of CDO1 maintains mitochondrial stability in erastin-treated cells (Hao et al. 2017). During cystine starvation, NFE2L2 prevents ferroptosis by regulating glutamate homeostasis and upregulating glutamate-cysteine ligase catalytic subunit (GCLC) (Kang et al. 2021).

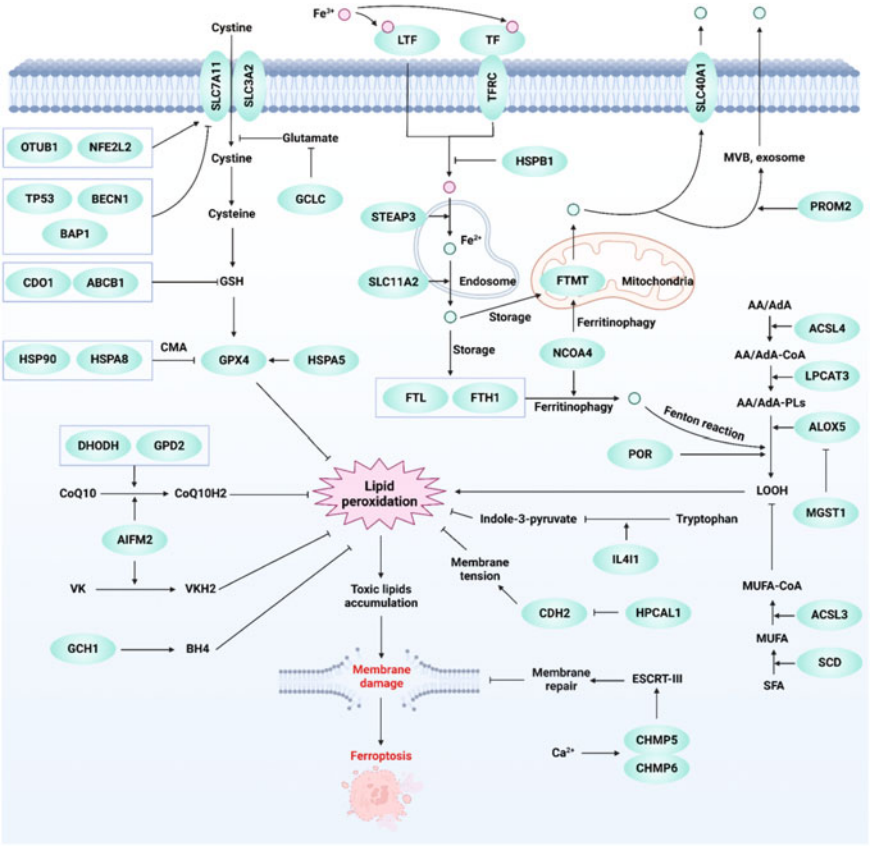

Fig. 5.4 The mechanism of ferroptosis. Ferroptosis is a type of cell death that occurs as a result of an imbalance between antioxidant pathways, iron accumulation, and lipid peroxidation. The core mechanism of ferroptosis is the excessive oxidation of cellular lipids, which results in membrane damage. The endosomal sorting complex required for transport III (ESCRT-III) plays a crucial role in regulating ferroptosis through its ability to repair damaged membranes

GPX4 is a central molecule in the negative regulation of ferroptosis, but it can also be degraded to induce ferroptosis through the action of RSL3 and FIN56 (Shimada et al. 2016). Erastin-induced ferroptosis leads to an increase in lysosomal associated membrane protein 2 (LAMP2A) and the activation of HSP90-dependent chaperone-mediated autophagy (CMA), resulting in GPX4 degradation (Wu et al. 2019b). However, erastin can also trigger ER stress in cells, leading to an increase in HSPA5 and interaction with GPX4, thereby protecting GPX4 from degradation and resisting ferroptosis (Zhu et al. 2017).

Cells cannot rely solely on GPX4 to protect against lipid peroxidation, as there are other mechanisms that respond to ferroptosis. Coenzyme Q10 (CoQ10), also known as ubiquinone-10, has been traditionally thought to play a role in transferring electrons for mitochondrial ATP production. However, recent research has shown

that in the presence of apoptosis inducing factor mitochondria associated 2 (AIFM2), CoQ10 can be reduced to ubiquinol (CoQ10H2) and trap lipid peroxyl radicals to prevent ferroptosis, a process that is independent of GPX4 (Doll et al. 2019; Bersuker et al. 2019). Additionally, the compound FIN56 not only induces GPX4 degradation, but also increases susceptibility to ferroptosis by depleting CoQ10 through the methoxypyruvate pathway. AIFM2 can also function independently of CoQ10, and vitamin K (VK) has redox activity and can be converted in the VK cycle by AIFM2 to vitamin K hydroquinone (VKH2), which can prevent lipid peroxidation (Mishima et al. 2022). More recently, it was found that dihydroorotic dehydrogenase (DHODH) and glycerol-3-phosphate dehydrogenase 2 (GPD2) can also reduce CoQ10 to CoQ10H2 and inhibit ferroptosis (Mao et al. 2021; Wu et al. 2022a), demonstrating the significant impact of AIFM2 and CoQ10 in the mechanism of ferroptosis without involving GPX4.

In addition, a CRISPR screen revealed that GTP cyclohydrolase-1 (GCH1) is a crucial factor in preventing ferroptosis (Kraft et al. 2020; Soula et al. 2020). GCH1 offers resistance through two methods: (1) the metabolic derivative of GCH1, tetrahydrobiopterin (BH_4), has its own ability to scavenge oxygen radicals, and (2) GCH1 can alter the lipid membrane environment, increase the presence of CoQ10H2, and decrease polyunsaturated fatty acid (PUFA) levels, thus inhibiting ferroptosis. Interleukin-4-induced-1 (IL4I1) is an amino acid oxidase produced by immune cells and its snake homologue L-amino acid oxidase (LAAO) causes oxidative stress and leads to cell death. However, in mammals, it generates indole-3-pyruvate (I3P) from tryptophan and offers resistance to ferroptosis through the free radical scavenging properties of I3P and the activation of antioxidant gene expression (Zeitler et al. 2021). This study highlights that proteins encoded by the same gene can act differently or even in opposing ways in different species, which suggests that the mechanisms involved in studying ferroptosis may vary among species (Kuang et al. 2020).

5.3.2 Iron Overload

Iron in cells can be divided into ferric ions (Fe^{3+}) and ferrous iron (Fe^{2+}). Food contains mainly insoluble Fe^{3+}, which needs to be reduced to Fe^{2+} for absorption. This reduction occurs when Fe^{3+} binds to transferrin (TF) and is recognized by the transferrin receptor (TFRC) on the cell membrane, allowing trivalent iron ions to enter the cell (Gao et al. 2015). Lactotransferrin (LTF) is also involved in the uptake of iron by cells (Wang et al. 2020). However, the protein kinase C-mediated phosphorylation of heat shock protein family B member 1 (HSPB1) can block iron uptake and thus limit ferroptosis (Sun et al. 2015).

Once inside the cell, Fe^{3+} is reduced to Fe^{2+} by six-transmembrane epithelial antigen of prostate 3 (STEAP3) in the endosome and transported to the cytoplasm by solute carrier family 11 member 2 (SLC11A2) (Fleming et al. 1997). Fe^{2+} can be stored in the cytoplasm in the form of ferritin, which includes ferritin light chain (FTL) and ferritin heavy chain 1 (FTH1). Nuclear receptor coactivator

4 (NCOA4)-mediated ferritinophagy leads to the release of Fe^{2+}, but we can limit ferroptosis by inhibiting ferritinophagy to increase iron storage (Hou et al. 2016). The overexpression of ferritin mitochondrial (FTMT) can also make cells resistant to ferroptosis (Wang et al. 2016). Ferritinophagy appears to be linked to cellular sensitivity to ferroptosis, but human macrophages can inhibit NCOA4-mediated FTMT degradation to limit ferroptosis under hypoxic conditions (Fuhrmann et al. 2020).

The inhibition of protein solute carrier family 40 member 1 (SLC40A1), which transports iron out of cells, leads to intracellular iron accumulation and induces ferroptosis (Ma et al. 2016). Prominin2 (PROM2), a pentraxin associated with the regulation of lipid dynamics, also inhibits ferroptosis by promoting the formation of multivesicular bodies (MVB) and exosomes that transport iron out of cells (Brown et al. 2019).

Intracellular Fe^{2+} is a crucial factor in the initiation of ferroptosis. Fe^{2+} can bind to GSH in the cell and be delivered to ferritin for storage via the chaperone poly(rC)-binding protein 1 (PCBP1). When GSH is depleted, it triggers the Fenton reaction with Fe^{2+}, producing ROS and increasing oxidative damage, as well as blocking iron storage and eventually leading to ferroptosis (Patel et al. 2021; Patel et al. 2019). Iron also increases the activity of arachidonate lipoxygenases (ALOXs) or hypoxia inducible factor prolyl-hydroxylases, which regulate lipid peroxidation and oxygen homeostasis, respectively (Chowdhury et al. 2013; Yang et al. 2016). Ferroptosis can be limited by the use of deferoxamine, an iron chelator (Chen et al. 2020).

5.3.3 Lipid Peroxidation

Lipid peroxidation of polyunsaturated fatty acids (PUFAs) plays a crucial role in the development of ferroptosis (Yang et al. 2016). Arachidonic acid (AA), a 20-carbon chain omega-6 fatty acid with 4 unsaturated bonds (20:4), is the most common PUFA in animal cells. AA and adrenic acid (AdA, 22:4) are sensitive to lipid peroxidation due to their two diallyl groups when located at the sn2 position of PUFA phospholipids (PLs) (Kagan et al. 2017).

Two key enzymes, acyl-CoA synthetase long chain family member 4 (ACSL4) and lysophosphatidylcholine acyltransferase 3 (LPCAT3), are involved in the production of AA/AdA derivatives and play an essential role in lipid-driven ferroptosis (Yuan et al. 2016; Doll et al. 2017). ACSL4 catalyzes the reaction of AA/AdA with CoA to form AA/AdA-CoA derivatives, which are then esterified to form phospholipids by LPCAT3. Studies have shown that the absence of ACSL4 can result in resistance to ferroptosis, and its re-expression can resensitize cells to ferroptosis (Doll et al. 2017). ACSL4 also participates in positive feedback regulation of ferroptosis through phosphorylation by protein kinase C beta (PRKCB) and regulation of E-cadherin expression through the merlin-Hippo-YAP pathway (Zhang et al. 2022b; Wu et al. 2019a).

However, it is important to note that the peroxidation of free fatty acids (FAs) is not critical for ferroptosis, only peroxidation of PLs. The use of the BODIPY

581/591 C11 fluorescent probe to measure PL peroxidation levels in ferroptosis-related experiments can impose limitations as it does not differentiate between PL and FA peroxidation (Liang et al. 2022).

PL peroxidation can occur through both enzymatic and non-enzymatic pathways. Non-enzymatic peroxidation of PL typically requires the involvement of redox metals, with iron being the most commonly observed. Intracellularly, iron is typically bound in complexes like ferritin, iron-sulfur clusters, and heme. The small amount of unbound iron is referred to as unstable iron pools and is highly reactive. Activated iron reacts with hydrogen peroxide in a Fenton reaction, resulting in the production of hydroxyl ions (OH^-) and hydroxyl radicals (OH) or a proton and peroxy radicals (OOH). Both hydroxyl and peroxyl radicals participate in lipid peroxidation. The ALOX family plays a significant role in the enzyme-dependent lipid peroxidation process. In H1299 cells, spermidine/spermine N1-acetyltransferase 1 (SAT1), which is a target gene of TP53, increases the expression of arachidonate 15-lipoxygenase (ALOX15) and is involved in TP53-mediated ferroptosis (Ou et al. 2016). In pancreatic cancer cells, microsomal glutathione S-transferase 1 (MGST1) binds to arachidonate 5-lipoxygenase (ALOX5), reduces lipid peroxidation, and inhibits ferroptosis (Kuang et al. 2021). However, it has been noted that the expression of ALOXs was not detected in many of the cell lines used for ferroptosis research (Ghandi et al. 2019), suggesting that ALOXs only act in ALOXs-expressing cell lines and are not the sole enzyme involved. Indeed, cytochrome P450 oxidoreductase (POR) in the ER has also been shown to play a role in lipid peroxidation (Yan et al. 2021; Zou et al. 2020). POR provides electrons to members of the CYP family, allowing them to oxidize and metabolize other molecules. POR induces ferroptosis in a CYP-independent manner through the production of hydrogen peroxide with cytochrome B5 reductase 1 (CYB5R1), which initiates lipid peroxidation through the Fenton reaction (Yan et al. 2021).

Compared to PUFA, monounsaturated fatty acids (MUFA) do not have a diallyl position and are therefore not prone to peroxidation. MUFAs inhibit ferroptosis. Exogenous MUFAs, after activation by acyl-CoA synthetase long chain family member 3 (ACSL3), reduce the sensitivity of the plasma membrane to oxidation and allow cells to resist ferroptosis (Magtanong et al. 2019). Stearoyl-CoA desaturase (SCD) protects ovarian cancer cells from ferroptosis by catalyzing the production of MUFAs (Tesfay et al. 2019). Of note, oleic acid, a MUFA, induces ferroptosis in lung-injured mice (Zhou et al. 2019), indicating that the mechanism by which MUFAs regulate ferroptosis can vary in different contexts. Overall, our current understanding of the modulation of lipid peroxidation in ferroptosis is just the tip of the iceberg (Chen et al. 2021d; Lin et al. 2021).

5.3.4 Membrane Damage and Repair

The final stage of the ferroptosis process has yet to be fully understood, despite the clear role of lipid reactive oxygen species and Fe^{2+} in the development of ferroptosis. The accumulation of toxic lipids, such as malondialdehyde (MDA) or

4-hydroxynonenal (4-HNE), causes oxidative damage to cell membranes and is a hallmark of ferroptosis. Exogenous 4-HNE can directly induce ferroptosis damage in a manner dependent on NOX (Chen et al. 2022c; Lin et al. 2022). The intracellular membrane repair mechanism, endosomal sorting complexes required for transport (ESCRT)-III, negatively regulates ferroptosis by preventing the accumulation of toxic lipids (Dai et al. 2020). The effect of traditional ferroptosis inducers, such as ER stress and calcium influx, leads to the buildup of the subunits charged multivesicular body protein 5 (CHMP5) and charged multivesicular body protein 6 (CHMP6) of ESCRT-III. A reduction in CHMP5 or CHMP6 levels makes cells more susceptible to ferroptosis. Recent research has also found that the autophagic receptor, hippocalcin like 1 (HPCAL1), selectively degrades cadherin-2 (CDH2) during ferroptosis, which reduces membrane tension and increases lipid peroxidation, making cells more susceptible to ferroptosis (Chen et al. 2022d). Thus, the regulation of membrane stability and membrane repair mechanisms may be an important area of investigation in ferroptosis research (Liu et al. 2020b).

5.4 The Function of HSPs in Ferroptosis

The process of ferroptosis involves the control of various key proteins related to redox systems, lipid metabolism, and iron balance. Here, we highlight the importance of several key HSPs in regulating these ferroptotic processes.

5.4.1 Small HSPs in Ferroptosis

Small heat shock proteins (sHSP) are widespread molecular chaperones made up of a highly conserved α-crystallin domain of around 90 amino acids, which is crucial for their function (Carra et al. 2013; Sun and MacRae 2005). In response to proteotoxic stress, sHSPs prevent the aggregation of misfolded proteins by binding to them in an ATP-independent manner, acting as the first line of defense against stress (Carra et al. 2012; Tedesco et al. 2022a). They also help HSP70 in refolding or degradation of misfolded proteins (Haslbeck and Vierling 2015). Although sHSPs exist as monomers, their function often requires the formation of high molecular weight multimers that maintain a balance between aggregation and dissociation (Lambert et al. 1999). The expression and distribution of different sHSPs vary greatly in tissues and cells, with HSPB1 being widely expressed (Tedesco et al. 2022b). HSPB1 is resistant to cellular oxidative stress due to phosphorylation of its dimer and is believed to have anti-apoptotic effects by binding to cytochrome C released from mitochondria, thus inhibiting caspase activation (Zavialov et al. 1998; Bruey et al. 2000).

HSPB1 plays an important role in regulating ferroptosis (Fig. 5.5a). The overexpression of HSPB1 reduces iron uptake in mice (Chen et al. 2006; Zhang et al. 2010). Erastin, a commonly used ferroptosis inducer, increases HSF1-dependent expression of HSPB1 in cells. Pre-treatment with heat shock or

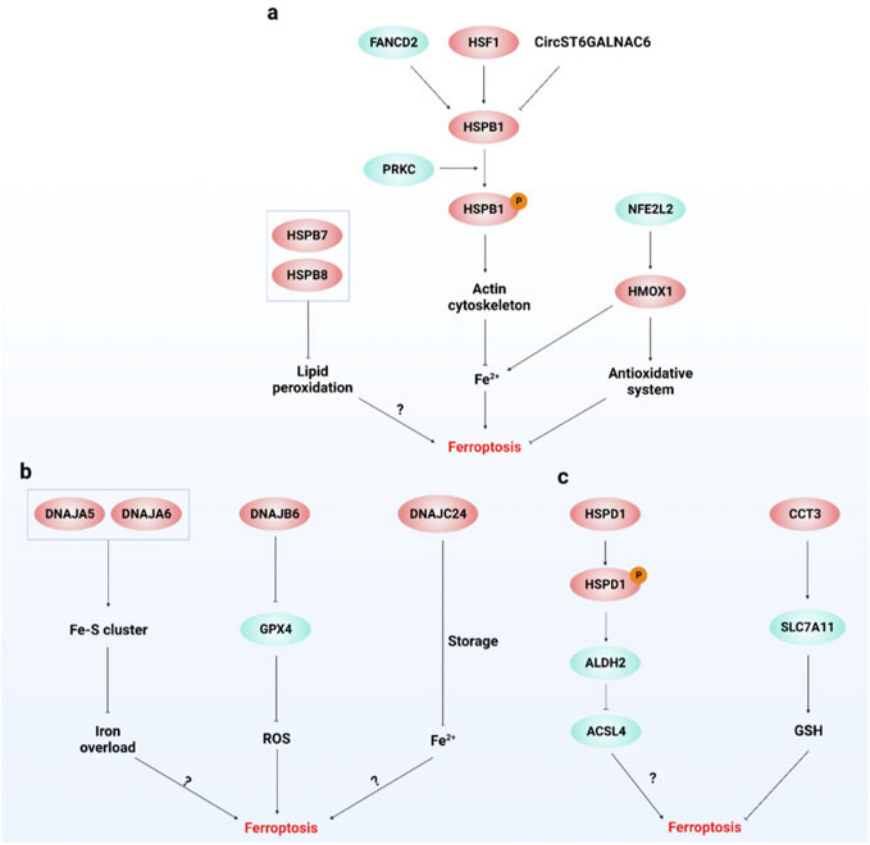

Fig. 5.5 Role of sHSP, HSP40, and HSP60 in ferroptosis. (**a**) HSPB1 plays a role in limiting ferroptosis by reducing iron uptake. On the other hand, HMOX1 promotes ferroptosis by catalyzing the breakdown of heme, leading to an increase in non-heme iron and inhibiting ferroptosis through its antioxidant pathway. The effects of HSPB7 and HSPB8 on ferroptosis are yet to be determined, but they are believed to protect against lipid peroxidation. (**b**) DNAJB6 induces ferroptosis by directly inhibiting GPX4. DNAJA5 and DNAJA6 promote the formation of iron-sulfur clusters, which helps to prevent iron overload. DNAJC24 serves as a storage protein for iron. (**c**) CCT3 works to inhibit ferroptosis by increasing the expression of SLC7A11. HSPD1 can aid in the proper folding of ALDH2 and stabilizing it, and ALDH2 can inhibit ferroptosis mediated by ACSL4. However, there is currently no evidence to suggest that HSPD1 directly inhibits ferroptosis

overexpression of HSPB1 makes cells more resistant to ferroptosis. Protein kinase C (PRKC)-mediated phosphorylation of the S15A/S86 site of HSPB1 also positively regulates actin polymerization and recombination, thus limiting iron uptake and ferroptosis (Sun et al. 2015). Other factors besides HSF1 and PRKC also affect HSPB1 expression and activity. The knockdown of the FA complementation group D2 (FANCD2) in bone marrow stromal cells leads to downregulation of HSPB1 and increased sensitivity to ferroptosis (Song et al. 2016). circST6GALNAC6, a bladder cancer tumor suppressor, blocks the phosphorylation of HSPB1 by erastin at the

Ser15 site, thus promoting ferroptosis (Wang et al. 2022b). HSPB1 has a high affinity for highly mobile membranes and is involved in regulating their physical properties, which may play a role in the regulation of ferroptosis (Csoboz et al. 2022). Ferroptosis typically results in plasma membrane damage, and the membrane regulation mechanism of HSPB1 may be involved in its regulation.

Heat shock protein 32 kDa (HSP32), also known as heme oxygenase 1 (HMOX1), is induced by oxidative stress or heme and to increase cellular sensitivity to hydrogen peroxide (Brand et al. 2010). HMOX1 is upregulated by doxorubicin in cardiomyocytes through the action of NFE2L2, which then catalyzes the degradation of heme iron and increases the levels of non-heme iron, contributing to the development of ferroptosis (Fig. 5.5a) (Fang et al. 2019). Similarly, tagitinin C, a sesquiterpene lactone, induces ER stress and ferroptosis in colorectal cancer cells. The EIF2AK3 pathway serves as a sensor for ER stress and mediates phosphorylation of NFE2L2, allowing it to enter the nucleus and induce HMOX1 expression, leading to ferroptosis (Wei et al. 2021).

It is important to note that the role of HMOX1 in ferroptosis is not always positive. For example, in renal proximal tubule cells, deletion of HMOX1 leads to increased sensitivity to erastin, which can be reversed by iron chelators (Adedoyin et al. 2018). In another study, aristolactam I induces mitochondrial iron accumulation in proximal renal tubular cells, inhibiting the NFE2L2-HMOX1 pathway and blocking antioxidant protection, resulting in ferroptosis (Deng et al. 2020).

These findings suggest that the regulation of HMOX1 and its role in ferroptosis are inconsistent across different tissues and cells, making it crucial to consider this when exploring the potential use of ferroptosis in diseases. For example, sickle cell disease is characterized by increased hemolysis and resulting heme overload in plasma. This can induce the upregulation of HMOX1, driving cardiomyopathy and ferroptosis, a process that can be prevented by inhibiting HMOX1 (Menon et al. 2022).

Additionally, heat shock protein family B member 8 (HSPB8) reduces elevated levels of lipid peroxidation during lung ischemia–reperfusion in mice (Yang et al. 2019). HSPB7 enhances the antioxidant capacity of cells through the NFE2L2-HMOX1 pathway in bovine adipocytes (Sun et al. 2019b). However, there is currently no direct evidence that HSPB7 and HSPB8 inhibit ferroptosis, and further research is needed to fully understand the relationship between small HSPs and ferroptosis.

5.4.2 HPS40 in Ferroptosis

HSP40 is the largest family of heat shock proteins, with over 50 members (Kampinga and Craig 2010). It is also known as the DNAJ protein and contains a highly conserved "J" domain, with the His-Pro-Asp (HPD) motif being essential for activating the ATPase activity of HSP70, making it a co-chaperone (Kityk et al. 2018). DNAJ is classified into three groups based on its structure: DNAJA, DNAJB, and DNAJC. DNAJA has an N-terminal "J" domain, a Gly-Phe-rich region, two

C-terminal β-barrel domains, a zinc finger structure, and a dimerization domain. DNAJB contains most of these structures, but not the zinc finger domain, while DNAJC usually only has the "J" domain (Kampinga and Craig 2010; Kaida and Iwakuma 2021). DNAJ regulates protein folding, translocation, and degradation by transporting substrate proteins to HSP70 and stimulating ATP hydrolysis, allowing stable binding to HSP70 (Hasegawa et al. 2017; Greene et al. 1998). Some DNAJ proteins also function independently of HSP70, such as DnaJ heat shock protein family member B8 (DNAJB8) which binds to polyglutamine proteins through its C-terminal serine-rich region and prevents aggregation through histone deacetylase 4 (HDAC4) (Hageman et al. 2010). DNAJ proteins have a dual role in regulating cell death. For example, DnaJ heat shock protein family member A3 (DNAJA3) acts as a mitochondrial chaperone in MCF-7 cells and interacts directly with TP53 to induce intrinsic apoptosis, while DnaJ heat shock protein family member B1 (DNAJB1) interacts with programmed cell death 5 (PDCD5) and degrades it, inhibiting TP53-mediated apoptosis in A549 cells (Trinh et al. 2010; Cui et al. 2015).

In the context of ferroptosis, DNAJ heat shock protein family member B6 (DNAJB6) inhibits the proliferation of esophageal squamous cell carcinoma (ESCC) by promoting ferroptosis (Fig. 5.5b). This overexpression of DNAJB6 leads to smaller mitochondria and a higher membrane density in ESCC cells, as well as loss of mitochondrial structural integrity, edema in the mitochondrial matrix, and increased levels of lipid peroxidation, resulting from the inhibition of GPX4 (Jiang et al. 2020). However, the specific mechanism by which DNAJB6 inhibits GPX4 is not yet clear. DNAJB6 inhibits tumor EMT and metastasis by upregulating the dickkopf Wnt signaling pathway inhibitor 1 (DKK1), which in turn inhibits the Wnt/β-catenin signaling pathway (Menezes et al. 2012). In gastric cancer, the activation of the Wnt/β-catenin pathway promotes the binding of the β-catenin/TCF4 transcriptional complex to the promoter region of GPX4, leading to increased expression of GPX4 and attenuated lipid ROS production, thereby inhibiting ferroptosis (Wang et al. 2022d). These findings suggest that DNAJB6 may play a role in regulating ferroptosis.

In Arabidopsis, two DNAJ proteins, DNA J protein A5 (DNAJA5) and DNA J protein A6 (DNAJA6), are capable of binding iron through their conserved cysteine residues, promoting iron incorporation into Fe-S clusters through the interaction of the "J" domain with the sulfur utilization factor (SUF). The deletion of DNAJA6 and DNAJA5 leads to defective Fe-S protein production in chloroplasts, photosynthetic dysfunction, and intracellular iron overload (Fig. 5.5b) (Zhang et al. 2021b). Among the DNAJC proteins, DNAJ heat shock protein family member C24 (DNAJC24) is composed of two domains: a conserved "J" domain and a CSL-type zinc finger domain linked by a flexible linker-helix (Sahi and Craig 2007). The flexible linker-helix structure allows for the regulation of the protein's function by adjusting the flexibility between the two domains. DNAJC24 is capable of binding iron through its CSL-domain and oligomerizing to act as a transient "iron storage protein," thereby regulating iron homeostasis (Fig. 5.5b). Jjj3 in yeast, a direct homolog of human DNAJC24, exhibits a similar iron-binding function, suggesting a conserved cross-species mechanism for iron chelation (Thakur et al. 2012).

5.4.3 HSP60 in Ferroptosis

HSP60, also known as HSPD1, has a mitochondrial targeting signal on its N-terminal side for import into the mitochondria (Vilasi et al. 2014). HSPD1 helps to maintain mitochondrial protein homeostasis by assisting in protein folding and interacting with HSPE1 (Levy-Rimler et al. 2001). It has three distinct domains: the apical, intermediate, and equatorial domains (Klebl et al. 2021). When unbound from ATP, the apical domain of HSPD1 is hydrophobic, allowing it to make contact with substrate proteins. When ATP binds to the equatorial domain, the HSPD1 forms a double ring structure in combination with HSPE1 and the apical domain, forming a "football complex." After ATP hydrolysis, the apical domain configuration of HSPD1 changes, causing the protein to fold and release (Tang et al. 2022).

HSPD1 is a crucial factor in the mtUPR and plays a role in regulating various stress response pathways to balance both intra- and extracellular stress. It generally acts as an anti-apoptotic factor, inhibiting TP53-dependent apoptosis by forming a complex with TP53 and blocking its activity (Ghosh et al. 2008) (Fig. 5.5c). HSPD1 also activates NF-κB-dependent BIRC5 expression to inhibit apoptosis (Chun et al. 2010; Huang et al. 2018). On the other hand, HSPD1 promotes apoptosis by forming a complex with procaspase 3 and activating caspase 3 via upstream caspase 9 (Xanthoudakis et al. 1999).

There are no reports indicating that HSPD1 regulates ferroptosis, but it may play a role in regulating lipid metabolism. HSPD1 helps to fold aldehyde dehydrogenase 2 family member (ALDH2) and acyl-CoA dehydrogenase short/branched chain (ACADSB) in ovarian cancer cells (Hu et al. 2021). Although ALDH2 inhibits ACSL4-mediated ferroptosis (Zhu et al. 2022), it is not clear if HSPD1 can limit ferroptosis by stabilizing ALDH2.

HSP60 belongs to the group I chaperonins, which are found in bacteria and organelles, and are distinct from group II chaperonins (CCT/TriC) that are present in the cytoplasm of archaea and eukaryotes (Archibald et al. 2000). Chaperonin containing TCP1 subunit 3 (CCT3) can inhibit ferroptosis in lung cancer cells by positively regulating the expression of SLC7A11 but not GPX4, as seen through transcription. The knockdown of CCT3 increases erastin toxicity, while ferrostatin-1 partially rescues cellular activity (Wang et al. 2022a). Alterations in CCT3 expression levels have been associated with various diseases, including cancer, neurodegenerative disorders, and ciliary dyskinesia. CCT3 is therefore an important target for understanding disease mechanisms and developing new therapeutic strategies.

5.4.4 HSP70 in Ferroptosis

HSP70 is a highly conserved protein that is composed of 13 HSPA family members and is involved in preventing the aggregation of misfolded proteins and promoting their refolding. It also solubilizes denatured proteins and degrades them in combination with the cellular degradation system. The interaction of HSP70 with a substrate protein alone only moderately stimulates ATPase activity, but the conversion to a

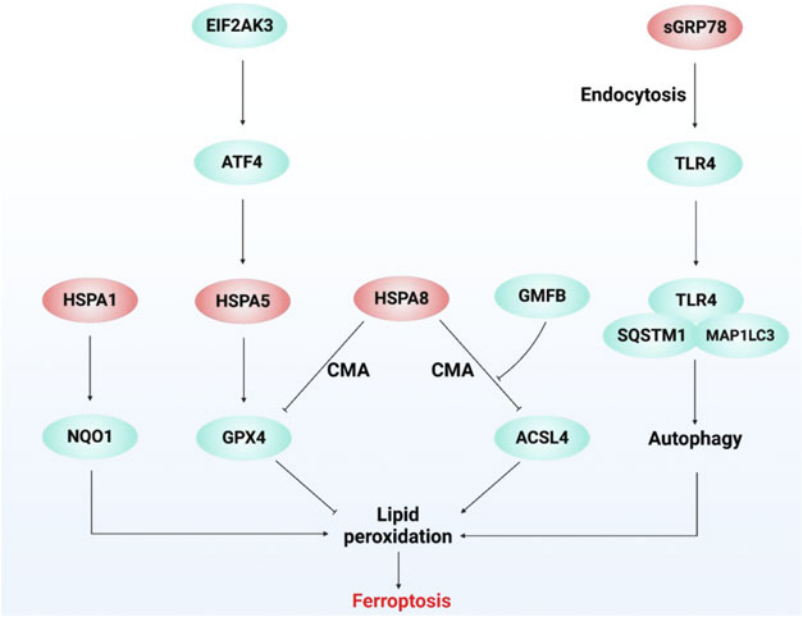

Fig. 5.6 Role of HSP70 in ferroptosis. The HSPA5 protein stabilizes GPX4, thereby limiting ferroptosis. The HSPA8 protein promotes ferroptosis by facilitating GPX4 degradation through the chaperone-mediated autophagy (CMA) pathway, but it can also inhibit ferroptosis by facilitating ACSL4 degradation through CMA. The HSPA1 protein contributes to ferroptosis by increasing lipid peroxidation through the upregulation of NQO1. Meanwhile, the sGRP78 protein mediates TLR4 endocytosis and triggers its degradation through autophagy, leading to ferroptosis

highly effective ATP-active state usually requires the involvement of the co-chaperone DNAJ protein (Rosenzweig et al. 2019). DNAJ proteins mediate the binding of substrate proteins to HSP70-ATP, promoting ATP hydrolysis and reducing the affinity of HSP70 for substrate proteins. HSPA1, HSPA8, HSPA9, and HSPA5 are the four most important and frequently studied members of the HSP70 family. HSPA1 inhibits apoptosis and regulates necrosis and autophagy (Gao et al. 2010; Nylandsted et al. 2004; Yaglom et al. 2003). The overexpression of HSPA8 promotes the proliferation, invasion, and migration of glioblastoma cells (Sun et al. 2019a). HSPA9 stabilizes the mitochondrial membrane permeability, promoting the survival of BRAF mutant tumor cells (Wu et al. 2020).

HSPA5, a member of the HSP70 family, could regulate ferroptosis in pancreatic cancer cells (Zhu et al. 2017). Erastin induces ATF4-dependent upregulation of HSPA5 in pancreatic cancer cells and to be more sensitive to ferroptosis after the knockdown of HSPA5, which acts as a negative regulator of ferroptosis by binding to GPX4 and avoiding its degradation (Fig. 5.6). The EIF2AK3-ATF4-HSPA5 pathway limits dihydroartemisinin (DHA)-induced ferroptosis in glioblastoma (Chen et al. 2019). Further research in other cell types, such as colorectal cancer cells, chondrocytes, and carp hepatocytes, showed that HSPA5 could also resist

ferroptosis through the same mechanism (Wang et al. 2022c; Lv et al. 2022; Cui et al. 2022). However, recent studies have identified the mechanism by which secreted HSPA5 induces ferroptosis. In cases of ER stress, HSPA5 expression is upregulated and can be expressed on the cell surface as well as secreted outside of the cell as secreted GRP78 (sGRP78) (Delpino and Castelli 2002). In myeloid cells, sGRP78 mediates toll-like receptor 4 (TLR4) endocytosis. Intracellular TLR4 forms a complex with MAP1LC3-SQSTM1 for lysosomal degradation, while sGRP78-enhanced autophagy-dependent TLR4 degradation induces both apoptosis and ferroptosis (Wu et al. 2022c). This highlights the dual role of HSPA5, which protects GPX4 from degradation and thus inhibits ferroptosis when acting as a molecular chaperone, and promotes ferroptosis through an autophagy-dependent pathway when secreted (Kang and Tang 2017). The next research direction for HSPA5 could be to investigate how its functions in ferroptosis are regulated in different cellular compartments and how these functions contribute to disease processes. Furthermore, in the field of nanomaterials, nanostructures loaded with Fe^{2+} and β-lapachone (LAP) easily trigger ferroptosis in tumor cells after treatment with NIR, and the process involved the upregulation of NAD(P)H dehydrogenase quinone 1 (NQO1), induced by HSPA1, leading to the promotion of iron-mediated lipid peroxidation (Xue et al. 2021).

HSPA8 plays a role in regulating ferroptosis through the process of chaperone-mediated autophagy (CMA). HSPA8 recognizes proteins with the KFERQ pentapeptide motif and transports them to LAMP2A at the lysosomal membrane for degradation. The drug NVP-AUY922 reduces the CMA-mediated degradation of GPX4, leading to the inhibition of ferroptosis in mouse lung epithelial cells (Li et al. 2022). On the other hand, glia maturation factor beta (GMFB) has the opposite effect by disrupting the lysosomal pH and reducing CMA-mediated degradation of ACSL4, thus promoting ferroptosis (Liu et al. 2022a). In conclusion, HSPA8 can either inhibit or promote ferroptosis, depending on the function of the protein it degrades through the CMA pathway. This makes it difficult to design specific therapeutic strategies that target HSPA8 without affecting its normal functions.

5.4.5 HSP90 in Ferroptosis

HSP90 is a highly abundant protein in the cytoplasm, making up 1-2% of total soluble proteins in stress-free conditions. It functions as an ATP-dependent molecular chaperone, regulating protein function through its interactions with substrate proteins (Schopf et al. 2017). The basic structure of HSP90 consists of three domains: the N-terminal domain (NTD), the middle domain (MD), and the C-terminal domain (CTD). The NTD contains an ATP-binding pocket known as the Bergerat fold, which is the binding site for nucleotides and drugs. The MD contains a catalytic loop, which binds to ATP and HSP90 client proteins, while the CTD facilitates dimerization and interaction with co-chaperones (Wu et al. 2017). It also has another ATP binding site. In mammals, there are two main isoforms of HSP90: inducible HSP90α and constitutive HSP90β, which are primarily located in

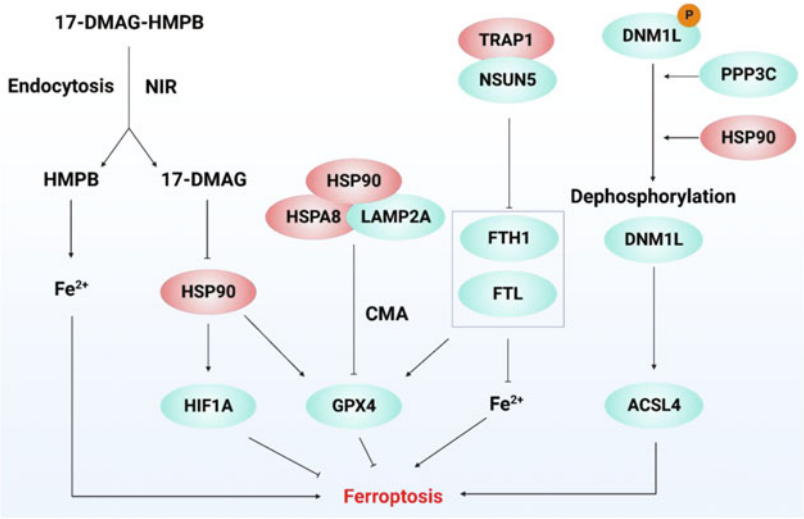

Fig. 5.7 Role of HSP90 in ferroptosis. The 17-DMAG-HMPB nanosystem is taken up by tumors via NIR endocytosis. The HMPB component promotes iron release, while 17-DMAG triggers ferroptosis by blocking the activity of HSP90. This leads to the inhibition of HIF1A and GPX4. HSP90 stimulates ferroptosis through its interaction with HSPA8, LAMP2A, and GPX4, promoting the degradation of GPX4 through the CMA pathway. HSP90 also causes dephosphorylation of DNM1L, which increases ACSL4-mediated ferroptosis. TRAP1 binding to NSUN5 results in the degradation of FTH1 and FTL, thereby promoting ferroptosis

the cytoplasm (Johnson 2012). Two additional isoforms, HSP90B1 located in the ER and TRAP1 in the mitochondria, also exist (Gupta 1995; Altieri 2013).

HSP90 is a crucial player in regulating ferroptosis (Fig. 5.7). The inhibitor of HSP90, 17-DMAG, when delivered to tumors using nanosystems and NIR, reduces the expression of HSP90 and induces ferroptosis by increasing Fe^{2+} release and decreasing GPX4 and hypoxia inducible factor 1 subunit alpha (HIF1A) expression (Lian et al. 2022). On the other hand, HSP90 also enhances ferroptosis through other pathways. For example, dephosphorylation of dynamin 1 like (DNM1L) by HSP90 and protein phosphatase 3 catalytic (PPP3C) at ser637 leads to altered mitochondrial morphology and increased lipid peroxidation through ACSL4, promoting ferroptosis (Miao et al. 2022). HSP90's involvement in CMA means it can also drive ferroptosis by degrading GPX4 through this pathway. Sb exposure in neurons results in HSPA8-HSP90-LAMP2A-GPX4 binding, forming a chaperone-GPX4 complex that accelerates GPX4 degradation and triggers ferroptosis (Yu et al. 2022). In addition to neuronal cells, ferroptosis can also be promoted in tumor cells and renal tubules with acute kidney injury through CMA-mediated GPX4 degradation induced by erastin and legumain, respectively (Chen et al. 2021a; Wu et al. 2019b). Furthermore, TRAP1, an isoform of HSP90, induces FTH1/FTL degradation in bone marrow mesenchymal stem cells (BMSCs) by binding to NOP2/Sun RNA methyltransferase 5 (NSUN5), resulting in Fe^{2+} accumulation and GPX4 reduction,

ultimately leading to ferroptosis (Liu et al. 2022b). The clinical development of HSP90 inhibitors has been challenging, in part because of their toxicity and off-target effects. HSP90 is also essential for the folding and stabilization of many normal proteins, and inhibition of HSP90 can lead to unintended consequences.

5.4.6 Ubiquitin in Ferroptosis

Ubiquitin, in addition to the several important heat shock protein families, also belongs to the heat shock proteins (Sharp et al. 1999). It is a small, 76-amino acid protein with a stable β-grasp fold structure that is highly conserved (Burroughs et al. 2007). The C-terminal glycine of ubiquitin is covalently attached to the substrate lysine through the action of three enzymes: E1 (ubiquitin activating enzyme), E2 (ubiquitin-binding enzyme), and E3 (ubiquitin ligase). The E1 enzyme activates the C-terminus of ubiquitin and transfers it to the E2 enzyme, which then transfers it to the substrate-binding E3 enzyme, forming a bond between the substrate and ubiquitin (Buetow and Huang 2016). The type of substrate that ubiquitin binds to primarily depends on the type of E3 enzyme, with over 600 E3 enzymes compared to only 2 E1 and 35 E2 enzymes (Deol et al. 2019).

Typical ubiquitination is the formation of an isopeptide bond between the lysine residue of the substrate protein and the C-terminal glycine of ubiquitin (Berndsen and Wolberger 2014). However, sometimes, it can also form ester or thioester bonds with serine/threonine or cysteine residues of the substrate (McDowell et al. 2010; Vosper et al. 2009). Substrate proteins can be labeled with a single ubiquitin molecule at one site, referred to as monoubiquitination. Ubiquitin contains eight potential ubiquitination sites, and further ubiquitination of these sites can result in the formation of ubiquitin chains. Finally, the ubiquitinated substrate proteins are degraded by the proteasome.

Several key proteins involved in ferroptosis can be regulated by ubiquitin, such as HECT, UBA, and WWE domain containing E3 ubiquitin protein ligase 1 (HUWE1) and NEDD4 like E3 ubiquitin protein ligase (NEDD4L) (Fig. 5.8). HUWE1 limits ferroptosis in acute liver injury by targeting the degradation of TFRC (Wu et al. 2022b). NEDD4L inhibits ferroptosis by binding to LTF to degrade it, reducing iron accumulation and lipid peroxidation in cancer cells (Wang et al. 2020).

Different E3 ubiquitin ligases regulate ferroptosis through multiple substrates. For example, NEDD4L binding to SLC7A11 is increased in breast cancer after ionizing radiation, leading to the ubiquitinated degradation of SLC7A11 and inducing ferroptosis (Liu et al. 2021d). Estrogen receptor 1 (ESR1) also inhibits ferroptosis by enhancing NEDD4L binding to TFRC, leading to its degradation during ionizing radiation-induced ferroptosis (Liu et al. 2022c). Other proteins, such as tripartite motif containing 26 (TRIM26), suppressor of cytokine signaling 2 (SOCS2), fascin actin-bundling protein 1 (FSCN1), and the lncRNA HEPFAL, can also promote SLC7A11 ubiquitination degradation to induce ferroptosis (Chen et al. 2022a; Zhu et al. 2021; Chen et al. 2022b; Zhang et al. 2022a). The mechanism by which ubiquitination and degradation of GPX4 induces ferroptosis has recently

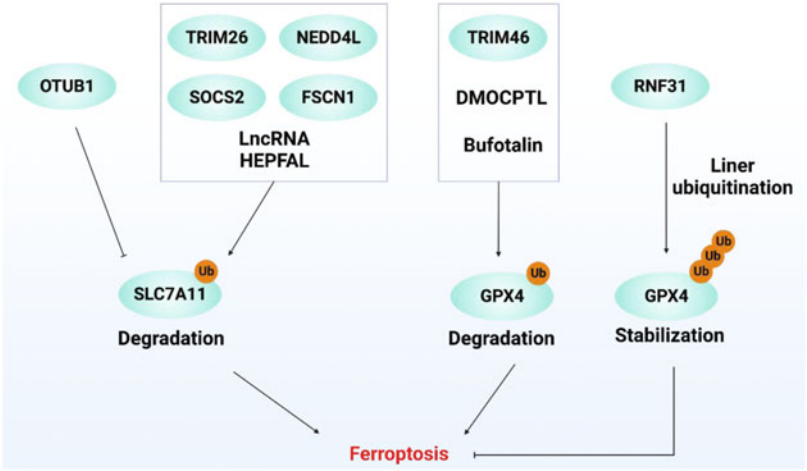

Fig. 5.8 Mechanism of SLC7A11 and GPX4 ubiquitination in ferroptosis. The E3 ubiquitin ligase targets SLC7A11 or GPX4 for degradation through ubiquitination, leading to ferroptosis. On the other hand, OTUB1 limits ferroptosis by stabilizing SLC7A11 through deubiquitination. Meanwhile, the linear ubiquitination of GPX4 by RNF31 stabilizes GPX4 and inhibits ferroptosis

been identified, with the discovery of DMOCPTL, a derivative of chamomile lactone, directly binding to GPX4 and promoting its ubiquitination and degradation (Ding et al. 2021). Bufotalin and the E3 ligase tripartite motif containing 46 (TRIM46) promote GPX4 ubiquitination to induce ferroptosis (Zhang et al. 2022c; Zhang et al. 2021c). GPX4 is also regulated in a novel approach, where ring finger protein 31 (RNF31) maintains GPX4 stability through linear ubiquitination of GPX4 (Dong et al. 2022).

Deubiquitination enzymes also play a role in regulating ferroptosis. The inhibition of ubiquitin specific peptidase 7 (USP7) triggers TP53 activation by blocking deubiquitination, leading to the downregulation of TFRC and the suppression of ferroptosis (Tang et al. 2021b). USP7 also stabilizes heterogeneous nuclear ribonucleoprotein A1 (HNRNPA1) through deubiquitination, and HNRNPA1 facilitates the packaging of miR-522 into exosomes. Exosomal miR-522 has the potential to inhibit ALOX15, and cancer-associated fibroblasts (CAFs) promote the secretion of miR-522 by chemotherapy drugs like cisplatin and paclitaxel, thus reducing the levels of lipid reactive oxygen species in cancer cells and enhancing chemosensitivity (Zhang et al. 2020). OTUB1 stabilizes SLC7A11 and suppresses ferroptosis by directly interacting with SLC7A11, a process that may involve OTUB1 persulfuration (Zhao et al. 2021; Chen et al. 2021b). These findings demonstrate the important role of deubiquitination enzymes in the regulation of ferroptosis.

Overall, there is a need to better understand the precise roles of the UPS pathway in ferroptosis-related diseases and to develop targeted therapies that exploit these pathways in a disease-specific manner. This requires a deeper understanding of the

molecular mechanisms underlying the UPS pathway and its interactions with ferroptotic processes.

5.4.7 HSF1 in Ferroptosis

HSF1 is a crucial transcription factor that regulates the expression of HSPs (Fig. 5.9). Within the HSF family, HSF1 has a significant role. When HSF1 is activated, it enters the nucleus and binds to the HSE of HSPs, a conserved sequence made up of three nGaan inverted repeats, located in the promoter region of the target gene, thereby initiating the transcription of HSPs.

The activation of HSF1 occurs in several stages: trimerization, nuclear translocation, DNA binding, transcriptional activation, and transcriptional inactivation and attenuation (Akerfelt et al. 2010). In a nonstressed state, HSF1 is in a monomeric form and bound to HSPs to prevent activation. Upon exposure to proteotoxic stress, HSPs have a higher affinity for misfolded proteins, causing them to separate from HSF1. The separation of HSPs allows HSF1 to trimerize and translocate to the nucleus with the help of TGM2 (Rossin et al. 2018). Once the trimeric HSF1 is phosphorylated, it can bind to the HSE of the target gene to regulate its transcription.

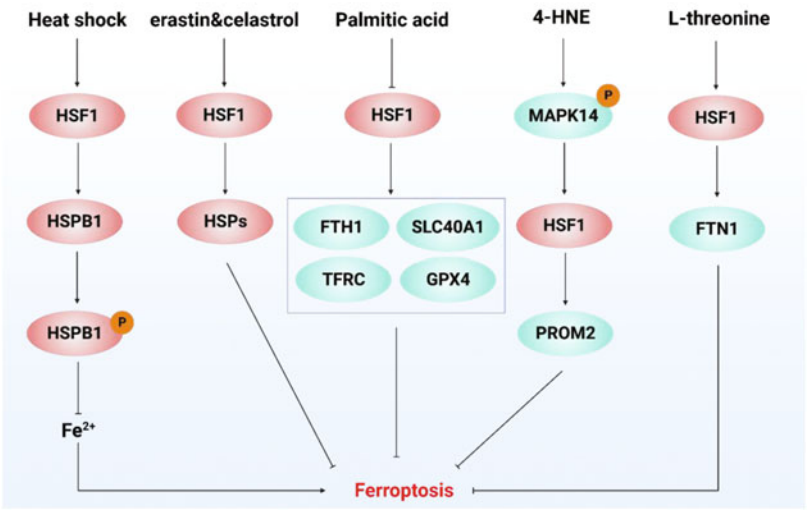

Fig. 5.9 Role of HSF1 in ferroptosis. Heat shock treatment helps cells build resistance to ferroptosis by increasing HSF1 and HSPB1 expression. Combining the treatment with erastin and celastrol can further enhance HSF1, leading to higher production of HSPs and resistance to ferroptosis. Conversely, palmitic acid suppresses HSF1, disrupting iron homeostasis and GPX4, thereby inducing ferroptosis. Ferroptosis can also be triggered by 4-HNE, which stimulates PROM2 transcription through a MAPK14-mediated activation of HSF1. On the other hand, L-threonine activates HSF1 and enhances FTN1 transcription, helping cells resist ferroptosis

The trimeric HSF1 in the nucleus can still interact with HSP70 or HSP40 to avoid transactivation (Shi et al. 1998).

HSPB1 is phosphorylated by PRKC, which reduces iron uptake and lipid peroxidation by stabilizing the cytoskeleton and inhibiting iron uptake. Erastin induces the upregulation of HSF1-dependent HSPB1, and when HSF1 is knocked down, HSPB1 expression decreases and cells become more sensitive to erastin (Sun et al. 2015). On the other hand, heat shock treatment increases HSF1 and HSPB1 expression and resistance to ferroptosis. Combined treatment of erastin and celastrol, a potential anticancer agent, causes death in non-small cell lung cancer cells by activating the ROS-mitochondrial fission-mitochondrial autophagy axis and stimulating HSF1 and HSPs expression. When HSF1 is knocked down, the tumor becomes more sensitive to the treatment (Liu et al. 2021c).

HSF1 and GPX4 are dose- and time-dependently downregulated by palmitic acid-induced ferroptosis, but HSF1 overexpression improves iron homeostasis and restores cellular activity by upregulating iron metabolism-related genes, including FTH1, TFRC, and SLC40A1 (Wang et al. 2021). HMOX1 can induce or prevent ferroptosis based on its effect on iron levels and antioxidant capacity. HMOX1 transcription is regulated by HSF1 (Inouye et al. 2018), implying that HSF1 is a mechanism to both resist and promote ferroptosis through the HSF1-HMOX1-Fe^{2+} pathway.

HSF1 also regulates ferroptosis through proteins other than HSPs. 4-HNE, a lipid metabolite formed from lipid peroxidation products, resists ferroptosis by activating HSF1 through MAPK14 and promoting HSF1-dependent transcription of PROM2 (Brown et al. 2021). Supplementation of L-threonine in *Caenorhabditis elegans* activates HSF1 and increases the transcription of ferritin to resist ferroptosis (Kim et al. 2022). Further investigation is needed to fully understand the role of HSF1 in the regulation of ferroptosis and to elucidate the molecular mechanisms underlying its function. This will require the use of advanced techniques to investigate the interactions between HSF1 and other key regulators of ferroptosis, as well as studies that examine the role of HSF1 in different cellular and environmental contexts.

5.5 Conclusions and Perspectives

HSPs play a crucial role in maintaining protein homeostasis and regulating ferroptosis. These heat shock proteins have two mechanisms for regulating protein homeostasis: (1) HSPs directly bind to misfolded proteins to refold them or mark them for degradation and (2) HSPs control the activity of stress sensors, such as HSF1 and ERN1, by directly binding to them. In the absence of stress, only a few HSPs bind to stress sensors to inhibit their activity and prevent activation. In the presence of stress, HSPs must be induced to be upregulated and have a higher affinity for misfolded proteins, resulting in their disengagement from the stress sensor and activation of the signaling pathway. The stabilization and correct function of proteins are extremely important for regulating ferroptosis. HSPs regulate ferroptosis in various contexts by stabilizing different protein substrates or

promoting protein degradation. The effect of HSPs on ferroptosis depends on the substrate and certain HSPs, such as HSPA5 and HSP90, have a bidirectional effect at different positions (Zhu et al. 2017; Wu et al. 2022c). The need for multiple HSPs to work together highlights the importance of further exploring the combined role of multiple HSPs in ferroptosis.

In addition, HSPs are important in tumor development and are widely used as potential clinical biomarkers for the diagnosis and prognosis of cancer patients. HSPs, such as HSP70 and HSP90, are targets in cancer therapy and their inhibitors are widely applied. For example, ruxolitinib, a JAK inhibitor, decreases HSP70 and HSP90 expression and inhibits breast tumor growth (Tavallai et al. 2016). A better understanding of the relationship between HSPs and ferroptosis could contribute to the development of more therapeutic tools for cancer and ferroptosis-related diseases. Despite extensive research, the precise role of HSPs in ferroptosis remains unclear. Some HSPs have been shown to have a dual role in regulating ferroptosis, while the interplay between HSPs, apoptosis, and autophagy in this process is not well understood. Additionally, the relationship between different subcellular localizations of HSPs and their impact on ferroptosis requires further investigation. The identification of specific biomarkers associated with HSP-dependent ferroptosis could facilitate its translation into practical applications (Liu et al. 2021b; Chen et al. 2021c).

Conflict of Interest The authors declare no conflicts of interest or financial interests.

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The Ongoing Search for a Biomarker of Ferroptosis

6

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Abstract

A regulated form of cell death characterized by iron-dependent lipid peroxidation, ferroptosis contributes to a wide variety of pathophysiological processes. There are high hopes for therapeutically modulating ferroptosis. However, the difficulty of detecting ferroptosis in vivo remains a major obstacle. Unlike other forms of regulated cell death, defining the presence and progression of ferroptosis is hampered by the lack of receptors, ligands, or an unambiguous executioner. Establishing a simple and reliable method to detect ongoing ferroptosis in the bio-samples of living organisms would greatly aid the clarification of many outstanding questions. In this chapter, we outline the current concepts of ferroptosis biomarkers and the most promising approaches for visualizing ferroptosis in vivo.

6.1 Definition and Regulation of Ferroptosis

Ferroptosis is a unique form of cell death in which iron-dependent accumulation of lipid peroxides and eventual plasma membrane rupture occur (Dixon et al. 2012). A prerequisite for ferroptosis to proceed is the presence of a sufficient pool of polyunsaturated fatty acids (PUFAs) in the plasma membrane (Doll et al. 2017; Yang et al. 2016). Upon ferroptotic stimuli, the PUFAs are peroxidized in iron-dependent processes (enzymatically by lipoxygenases (Yang et al. 2016) and nonenzymatically by the so-called Fenton reaction (Braughler et al. 1986; Pratt et al. 2011)), resulting in pore formation in biological membranes (Riegman et al. 2020; Pedrera et al.

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2021). Eukaryotic cells have evolved several potent protective mechanisms to counteract this detrimental accumulation of peroxides. Generally, it is assumed that a loss of these antioxidant protective mechanisms or disturbance of cellular redox homeostasis favors reactive oxygen species and lipid peroxide accumulation, which ultimately leads to cell death.

The first identified and arguably most important anti-ferroptotic mechanism is the glutathione (GSH)/glutathione peroxidase 4 (GPX4) system. GPX4 is an enzyme expressed in both the cytosol and mitochondria, where it directly catalyzes lipid peroxide reduction, oxidizing GSH as a cofactor (Mao et al. 2021). GPX4 not only represents an indispensable cornerstone for maintaining cellular redox balance under physiological conditions (Friedmann Angeli et al. 2014), but also plays a pathophysiological role in cancer cell resistance to oxidative stress (Yang et al. 2014). GPX4 function is dependent on the cellular GSH pool, which in turn relies on cystine import into the cell, as cystine is a critical substrate for GSH synthesis. The membrane-bound enzyme system Xc^- is the most relevant importer of cystine, the inhibition of which triggers ferroptosis in many cells (Dixon et al. 2012). Nevertheless, other sources of cystine supply have been described, for example, the catabolism of extracellular protein (Armenta et al. 2022).

A second major protective mechanism against ferroptosis is independent of GSH and GPX4. The central element in this mechanism is oxidoreductase ferroptosis suppressor protein 1 (FSP1, previously known as AIFM2), which reduces the membrane-bound radical scavenger ubiquinone (CoQ_{10}) by consuming NADPH (Doll et al. 2019; Bersuker et al. 2019). Recently, it was reported that the inhibitory effect of FSP1 on ferroptosis was mediated not only by CoQ_{10}, but also by vitamin K (Mishima et al. 2022), which is also a potent inhibitor of lipid peroxidation (Kolbrink et al. 2022). Another GPX4-independent protector against ferroptosis is GTP cyclohydrolase 1 (GCH1), which is involved in di- and tetrahydrobiopterin synthesis, limits PUFA peroxidation, and acts as a CoQ_{10} reducer (Kraft et al. 2020; Soula et al. 2020). Furthermore, the enzyme dihydroorotate dehydrogenase (DHODH) reduces CoQ_{10} in the mitochondria and thereby contributes to protection against ferroptotic stress of the mitochondrial membranes (Mao et al. 2021).

In conclusion, we have broad insight into the regulatory mechanisms and determinants of lipid peroxidation and the processes that determine cell sensitivity to ferroptosis. Nevertheless, detecting ongoing ferroptosis in patients is extremely difficult, which hinders the identification of the precise clinical scenarios in which ferroptosis modulation would be useful. This issue arises from a discrepancy between the methodology with which ferroptosis was initially discovered and defined and the methodology currently available in vivo. Ferroptosis was defined in vitro (Fig. 6.1) as a form of regulated cell death that can be induced by specific ferroptosis inducers (e.g., erastin, RSL3) and conversely can be prevented by specific small-molecule inhibitors of lipid peroxidation (ferrostatins or liproxstatins (Zilka et al. 2017)). Additionally, lipid peroxide levels can be quantified, e.g., using redox-sensitive dyes (Drummen et al. 2002; Aldini et al. 2001), to support a conclusion of ferroptotic cell death.

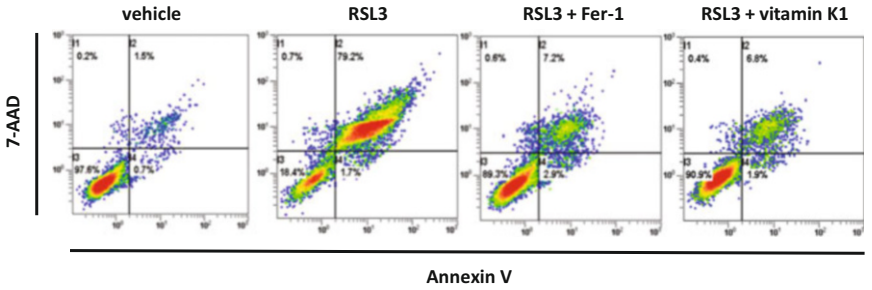

Fig. 6.1 The concept of ferroptosis in vitro. Ferroptosis can be induced in murine NIH3T3 cells through exposure to RSL3, a direct inhibitor of GPX4. The resulting cell death can be averted by specific inhibitors of lipid peroxidation such as ferrostatin-1 (Fer-1) or vitamin K1. Cells were stimulated at 37 °C for 24 h with 5 μM RSL3 alone or with 1 μM Fer-1 or 10 μM vitamin K1 as indicated. Cell death was quantified by fluorescence-activated cell sorting (FACS) using 7-amino-actinomycin D (7-AAD) and phosphatidylserine accessibility (annexin V staining) as markers. Note: The original data were published previously (Kolbrink et al. 2022)

However, there is no clearly definable limit for the lipid peroxidation levels leading to ferroptosis, which is largely due to the fact that the individual cell resistance depends strongly on the expressed cellular protective mechanisms. This issue is even more pronounced in the living organism and application of in vitro methods is challenging for three reasons: (1) the stimulus triggering ferroptosis cannot be as clearly defined as in cell culture. Often, in vivo stimuli that are thought to induce ferroptosis can simultaneously induce other forms of cell death (for example, ischemia-reperfusion injury [IRI] leads to necroptosis, ferroptosis, and pyroptosis). (2) Organisms do not comprise a homogeneous cell population; rather, they are composed of heterogeneous cells with varying behavior and expression of anti-ferroptotic systems. (3) While ferroptosis inhibitors are suitable for detecting the involvement of ferroptosis in animal models, they only enable retrospective conclusions. Therefore, this approach cannot aid the identification of ongoing ferroptosis.

Consequently, reliable biomarkers are required that can be measured in bio-samples (e.g., serum, biopsy tissue). In the following sections, we will discuss the available evidence on biomarkers currently used as markers of ferroptosis.

6.2 Acyl-CoA Synthetase Long-Chain Family Member 4 (ACSL4)

6.2.1 The Role of ACSL4 in Ferroptosis

The first candidate identified as a potential ferroptosis biomarker was the enzyme ACSL4. The ACSL family members catalyze coenzyme A (CoA) ligation to long-chain fatty acids, which are then metabolized through β-oxidation, glycerolipid, or cholesteryl ester synthesis pathways (Kim et al. 2001; Yan et al. 2015). As a unique

characteristic compared to the other ACSLs, ACSL4 has a marked preference for activating PUFAs such as arachidonic acid (AA) (Kim et al. 2001; Tang et al. 2018), which are required for ferroptosis. An oxidative lipidomics analysis in cells undergoing ferroptosis revealed that AA- and adrenic acid (AdA)-containing species in phosphatidylethanolamine (PE) were the preferred substrates for oxidation in ferroptosis (Kagan et al. 2017). Subsequent ACSL4-mediated AA and AdA activation is a necessary step for generating AA-PE and AdA-PE. *Acsl4* knockout reduced both AA-CoA and AdA-CoA, and AA- and AdA-containing PE species levels, thereby rendering cells insusceptible to ferroptosis (Doll et al. 2017; Kagan et al. 2017).

From these experiments published in 2017, we learned that ACSL4 expression is necessary for ferroptosis, but it took another five years until we understood, why ACSL4 alone is insufficient for ferroptosis: a further activation step of ACSL4 is crucial for the execution of ferroptosis. Upon ferroptosis induction, the protein kinase C βII (PKCβII) senses sublethal levels of lipid peroxides in a positive feedback mechanism, thereupon phosphorylating the inactive form of ACSL4 at Thr328. Subsequently, ACSL4 dimerizes and performs its function, providing the PUFA levels required for ferroptosis to proceed and allowing the cell to die by ferroptosis (Zhang et al. 2022). However, whether ACSL4 is an obligatory component of all contexts of ferroptotic cell death has not been conclusively answered. Several studies have demonstrated that *Acsl4* knockout prevented ferroptosis induced by indirect inhibition of GPX4 (by cystine deprivation through inhibition of the Xc^- system) (Muller et al. 2017; Yuan et al. 2016). Additionally, a recent study on the genetic regulation of ferroptosis postulated that ACSL4 is required for ferroptosis caused by direct inhibition of GPX4. However, that study assigned only a minor role to ACSL4 in ferroptosis induced by indirect inhibition of GPX4 (Magtanong et al. 2022), which partially contradicted previous evidence (Muller et al. 2017; Yuan et al. 2016). Notably, these deeper mechanistic observations are currently limited to in vitro settings. In contrast, ferroptosis in vivo presents a considerably more complex situation.

6.2.2 ACSL4 as a Biomarker of Pathophysiological Ferroptosis

The potential suitability of ACSL4 as a biomarker of ferroptosis emerged with the observation that ACSL4 expression is markedly upregulated in cancer cells upon ferroptosis induction in vitro (Yuan et al. 2016). To understand the further context: research on ferroptosis in vivo reported that the kidney was generally the first organ that is particularly susceptible to ferroptosis. This is illustrated by the fact that adult mice died of kidney failure after inducible *Gpx4* knockout (Friedmann Angeli et al. 2014), whereas complete (homozygous) knockout of *Gpx4* was embryonically lethal (Yant et al. 2003; Imai et al. 2003). In pathophysiological ferroptosis, renal IRI was identified as a disease model particularly strongly driven by ferroptosis (Linkermann et al. 2014).

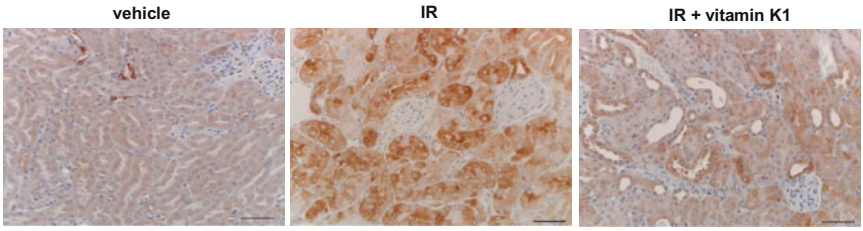

Fig. 6.2 ACSL4 expression in a mouse model of renal IRI. Male C57BL/6 J mice underwent 35 min bilateral renal pedicle clamping followed by 48 h reperfusion (IR). One group (IR + vitamin K1) received a single intraperitoneal injection of 25 mg/kg bodyweight of the ferroptosis inhibiting agent vitamin K1 15 min before the procedure. The kidney sections immunohistochemically stained for ACSL4 expression clearly demonstrate that IR greatly increased renal tubule ACSL4 expression compared to that in untreated animals (vehicle), which was prevented by the administration of vitamin K1 (scale bars = 50 μm). Note: The original data were published previously (Kolbrink et al. 2022)

The first report to assign ACSL4 a ferroptosis biomarker role in vivo was from our laboratory. Using a mouse model of kidney IRI, we demonstrated that the renal expression levels of ACSL4 correlated with tissue damage and impaired kidney function, were dependent on the ischemia time, and increased during the subsequent reperfusion period (Muller et al. 2017). Furthermore, the application of ferroptosis inhibitors (Fig. 6.2) reduced kidney damage and ACSL4 expression (Kolbrink et al. 2022).

In addition to its property as a biomarker in the renal IRI setting, ACSL4 also has direct pathophysiological importance in ferroptosis induction, as evidenced by the fact that the specific *Acsl4* knockout in renal tubules protected against IRI (Wang et al. 2022). Based on animal studies, similar pathophysiological significance was attributed to ACSL4 in IRI of various organs and tissues other than the kidney: carried by ACSL4, ferroptosis enhanced organ damage in IRI of the gut, heart, and brain (Li et al. 2019; Fan et al. 2021; Cui et al. 2021) and contributed to diabetic kidney disease (Wang et al. 2020), cardiac remodeling (Pei et al. 2021), and rhabdomyolysis in exertional heat stroke (He et al. 2022b). Moreover, ACSL4 expression upregulation was evident in necrotizing enterocolitis in mouse models, where it was suppressed by ferroptosis inhibition, and in humans (Dang et al. 2022).

These aforementioned ACSL4-dependent diseases all share the pathophysiologic characteristic that cell death processes lead to organ damage. This contrasts with a group of diseases in which it is not cell death but their excessive proliferation that presents a major problem for the organism as a whole, namely malignant neoplasms or cancer. ACSL4-dependent ferroptosis is also considered important in cancer. In this context, however, it is predominantly not considered harmful, but rather as a desirable event for eliminating malignant cells. Thus, as opposed to diseases in which the issue is excessive cell death, ferroptosis induction, and not prevention, is a prospective therapeutic approach in neoplastic diseases.

In mice, ACSL4 promoted immunogenic tumor ferroptosis and limited tumor growth (Liao et al. 2022). Furthermore, ionizing radiation treatment of tumor cells

and xenografts induced ACSL4 expression and enhanced ferroptosis (Lei et al. 2020). Therefore, ACSL4 appears to mediate ferroptosis in malignant tumors, potentially contributing to antitumor immunity. Supporting this assumption, increased ACSL4 expression in tumor tissue, which presumably implies greater tumor susceptibility to ferroptosis, was associated with significantly better prognosis in patients with breast cancer (Sha et al. 2021).

In conclusion, studies proposing ACSL4 as a ferroptosis biomarker predominantly compared ACSL4 protein expression in diseased tissue with that in healthy tissue and correlated the expression with the extent of tissue damage and lipid peroxidation. An increase in total protein expression therefore appears to correlate with increased ferroptosis occurrence. Based on this approach, ACSL4 primarily represents a semiquantitative ferroptosis marker, although the amount of ACSL4 required for ferroptosis in vivo has not been conclusively determined. A possible future refinement of this approach could be to specifically detect ACSL4 phosphorylated at Thr328, as this phosphorylation event appears to represent an important checkpoint in ACSL4-dependent ferroptosis (Zhang et al. 2022).

6.3 Transferrin Receptor 1 (TfR1)

6.3.1 The Role of TfR1 in Ferroptosis

As a key characteristic in the initial description of ferroptosis, it was clearly demonstrated that an exogenous supply of iron significantly sensitized cells to ferroptosis, whereas iron chelation completely inhibited ferroptosis (Dixon et al. 2012). As described above, the presence of intracellular iron is essential for ferroptosis, as the processes leading to lipid peroxidation are iron-dependent. However, iron ions cannot cross unhampered across cell membranes as the intracellular iron pool is tightly controlled for cell survival and function. Moreover, only a very minor portion of body iron is present in its free, ionized form; the largest portion is protein-bound, e.g., to the plasma transport protein transferrin, and cannot be easily released. Therefore, it is unsurprising that proteins responsible for iron import into cells also play a role in ferroptosis. One such protein is TfR1, which is expressed on the plasma membrane in response to intracellular iron demand. Upon binding Fe^{3+}-loaded transferrin, the TfR1-transferrin complex is internalized into endosomes, where Fe^{3+} is reduced to Fe^{2+} and subsequently transported to its cellular destinations (Aisen 2004).

Interestingly, decades before the first description of ferroptosis, it was clear that TfR1 is significantly involved in cellular redox metabolism. Although ferroptosis was not known by its present name then, it was observed that an oxidative form of cell death was dependent on TfR1-mediated cellular import of iron and that inhibiting TfR1 via specific antibodies prevented this cell death (Tampo et al. 2003; Kotamraju et al. 2002). Fittingly, oncogene-mediated upregulation of TfR1 sensitized cells to ferroptosis, whereas knocking down TfR1 exerted the opposite effect (Yang and Stockwell 2008; Gao et al. 2015). The physiological mechanisms

of TfR1 regulation also influence ferroptosis. Therefore, TfR1 is a target of the ubiquitin ligase E3 HUWE1/MULE, and ubiquitination of TfR1 followed by proteasomal degradation inhibited ferroptosis (Wu et al. 2022). Overall, it can be assumed that the intracellular iron pool, which is regulated by TfR1, is a key regulator of ferroptosis, which highlights TfR1 as a potential therapeutic target.

6.3.2 TfR1 as Biomarker of Ferroptosis

Brent Stockwell's group identified TfR1 as a protein of interest in a large screening searching for a specific detection biomarker of ongoing ferroptosis (Feng et al. 2020). For this purpose, they immunized mice with plasma membrane fragments of ferroptotic tumor cells, isolated antibody-producing plasma cell clones from their spleens, and examined the resulting monoclonal antibodies for their ability to detect cells undergoing ferroptosis in an immunofluorescence assay. The most promising of these antibodies was validated for its specificity for ferroptosis in several cell lines in vitro. Subsequently, immunoprecipitation and mass spectrometry identified TfR1 as its antigen. In a further step, the same team used machine learning to develop a ferroptosis identification technique using TfR1 immunostaining in an automated and low-bias manner. In a transformed cell line, immunostaining against TfR1 and F-actin, and nuclear staining, enabled the automated identification of ferroptotic cells with >90% accuracy (Jin et al. 2022). It must be noted that the specificity of TfR1 as a ferroptosis marker is apparent only with immunofluorescence; therefore, this methodology must be applied exactly as described. The total protein expression of TfR1 detected in western blots did not appear to change after the induction of ferroptosis in cell culture (Song et al. 2020; Ma et al. 2016).

Within ferroptosis-driven disease models, however, TfR1 expression behaves differently. Total TfR1 expression increased in rodent heart and liver IRI and correlated with lipid peroxidation levels and cell death (Tang et al. 2021; Wu et al. 2022). Furthermore, TfR1 expression was markedly enhanced over time in mice with traumatic brain injury, which was largely suppressed by ferroptosis inhibitors (Chen et al. 2021). In contrast, the role of TfR1 in cancer has not been conclusively elucidated. It has long been known that TfR1 is significantly upregulated in diverse cancers (Shen et al. 2018), which may explain the increased sensitivity of tumor cells to ferroptosis. Nevertheless, the extent to which TfR1 can be used to detect ferroptosis in vivo in this setting has not been determined.

Overall, TfR1 as a ferroptosis biomarker can currently be used to specifically detect ferroptosis in vitro via immunofluorescence. However, the limited in vivo evidence to date suggests that, similarly to ACSL4, TfR1 is a semiquantitative marker whose expression increases in ferroptosis-damaged tissue.

6.4 Lipid Peroxidation-Associated Protein Adducts

6.4.1 Accumulation of Lipid Peroxidation By-products during Ferroptosis

The concept of lipid peroxidation was established long before ferroptosis was described (Niki 2009). A result of harmful oxidative stress, lipid peroxidation is not a desirable process in the vast majority of cases, which spurred efforts to measure lipid peroxidation. As direct measurement of the manifold forms of lipid peroxides is extremely difficult, especially in living organisms, alternative means were sought to quantify the extent of lipid peroxidation. In biological systems, lipid peroxides are constantly degraded, and a large number of different aldehydes are formed during these degradation processes. Fortunately, these aldehydes are not difficult to detect. Therefore, the extent of lipid peroxidation can be inferred from the amount of these aldehydes. Well-established molecules for this purpose are malondialdehyde (MDA) and 4-hydroxynonenal (4-HNE) (Esterbauer et al. 1991; Spickett 2013). Aldehyde accumulation can also be detected during ferroptosis (which is regularly done in a wide variety of studies), but these molecules are not specific ferroptosis markers, as they are also constantly present under physiological conditions (Niki 2009). Yet, in higher, unphysiological concentrations, MDA and 4-HNE modify proteins in biological systems, and specific antibodies can be generated against the resulting adducts (Toyokuni et al. 1994; Yamada et al. 2001). Accordingly, better specificity for detecting ferroptosis could be assumed, and the 4-HNE modified proteins are particularly promising candidates.

6.4.2 4-HNE Protein Adducts as Biomarkers of Ferroptosis

As early as 1995, the Japanese group of Shinya Toyokuni generated five antibodies against the 4-HNE histidine adduct (Toyokuni et al. 1995). Using these antibodies, they studied an iron-mediated cell death (which would currently be referred to as ferroptosis) in kidney cells several years before ferroptosis was first described (Ozeki et al. 2005). Recently, they reexamined their antibodies and discovered that one of them recognizes ferroptotic cells in immunocytochemistry and immunohistochemistry with high sensitivity and specificity. Additionally, cells stained by this antibody were markedly increased in ferroptosis-driven disease models. In the same work, they further used this antibody to study ferroptosis in aging processes. In aging rats, they observed a massive increase in cells in various organs that could be stained by the antibody, leading to the assumption that they had detected ongoing multi-organ ferroptosis. In our opinion, however, the antibody staining did not correlate well with ferroptotic cell death in that model, as the corresponding animal would certainly not have survived such pronounced organ necrosis (Zheng et al. 2021). In this setting, therefore, we do not consider the antibody ferroptosis-specific and would rather assume that 4-HNE protein adducts were increased to antibody-detectable levels during senescence. Nevertheless, a second research group also

from Japan generated an antibody against ferroptotic cells that also recognizes 4-HNE adducts. However, this was only used in cell culture (Kobayashi et al. 2021). Therefore, the anti-HNE antibodies appear to be good in vitro markers of ferroptosis, but share the trait of limited evaluability in vivo with ACSL4 and TfR1.

6.5 Innovative Approaches

The underlying concept of the ferroptosis biomarkers discussed above is the detection of (increased) expression of specific proteins via antibodies. The details thereof differ, for example, ACSL4 and TfR1 are physiologically expressed proteins, whereas the 4-HNE adducts are random by-products of lipid peroxidation. Nonetheless, the basic idea of detecting differentially expressed markers is consistent. One possible approach to refine this methodology is to limit bias with machine learning (Jin et al. 2022), but to what extent this will become widespread is unknown.

In addition to these efforts, the genome has received much attention regarding ferroptosis. To date, hundreds of genes have described relevance in ferroptosis as drivers, inhibitors, and potential markers. Nevertheless, other than the few highlighted in this chapter, none of them are sufficiently specific to be suitable as a biomarker. The number of ferroptosis-related genes is constantly increasing and is nearly impossible to follow. Therefore, we refer to the "FerrDb" database, where a constantly updated compilation can be found (Zhou and Bao 2020).

There are creative and innovative new approaches to detect ferroptosis. Cellular changes in lipid composition (so-called lipidomics) can be visualized in tissue samples via matrix-assisted laser desorption/ionization imaging mass spectrometry, thereby detecting very specific changes in the lipidome during the course of tissue injury (Martín-Saiz et al. 2022). If it were possible to determine a specific lipid profile for ferroptosis, it could potentially be made visible in tissues. However, the complexity and high cost of the method are presently a major obstacle to routine use.

In addition, mass spectrometry can evaluate not only the lipidome, but also analyze the totality of all proteins in a cell, i.e., the proteome. Mass spectrometry can even detect specific posttranslational modifications of proteins. One posttranslational modification that can occur during ferroptosis is protein carbonylation, which is the addition of a reactive carbonyl group to a protein. This typically occurs through electrophilic unsaturated aldehydes formed during lipid peroxidation, for example MDA or 4-HNE. Mass spectrometry can be used to study exact carbonylation sites during ferroptosis, some of which appear to be ferroptosis-specific (Chen et al. 2018). Many more impressive subdisciplines have evolved from mass spectrometric analysis of the proteome, such as the study of the functional status of reactive cysteine residues on proteins, known as the cysteinome. As with the lipidome and protein carbonylations, specific changes can also be observed in the cysteinome during ferroptosis, although it is currently unclear whether these would have potential as biomarkers (Wang and Wang 2022).

The supreme discipline of all these -omics, however, is combining them into the so-called multi-omics. The overarching concept is that considering more -omics

leads to a more precise analysis. A recently published research letter provided a multi-omics approach to the prognostic and therapeutic relevance of ferroptosis in melanoma patients. This analysis included four molecular levels: mRNAs, microRNAs, DNA methylation sites, and a proteomic analysis of 49 proteins. Subsequently, a ferroptosis score was established that described the extent of ferroptosis not as a binary yes/no decision, but as a multifactorial numerical value. The ferroptosis score predicted outcomes and could potentially guide therapy (He et al. 2022a). This is undoubtedly a highly elegant and innovative technique, although the necessary resource investment is enormous and it is therefore probably not suitable for widespread practical use yet.

6.6 Conclusion

Currently, a small selection of biomarkers is available to detect ferroptosis in vitro or in vivo. To date, none of these markers has been established as superior and all have certain limitations. In particular, the unequivocal and definitive detection of ongoing ferroptosis in living organisms currently remains difficult, as the "common" biomarkers are only suitable as semiquantitative parameters so far. The release of the proteoglycan decorin (DCN) has recently been identified as a specific biomarker of ferroptosis, distinguishing it from other cell death modalities, both in vitro and in vivo (Liu et al. 2022). Additionally, there are several new and innovative ideas to either refine existing methodology or generate entirely novel approaches, but these are currently either not yet mature or are extremely complex and expensive. A feasible pragmatic approach would be to use a combination of the currently available "simple" biomarkers. In summary, the in vivo situation in particular requires substantial further research efforts to understand how, when, and where ferroptosis can be detected reliably.

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p53 and Ferroptosis

7

Shun Jiang and Yangchun Xie

Abstract

Ferroptosis is a form of oxidative cell death that relies on intracellular iron accumulation and lipid peroxidation. Recent studies have shown that the tumor suppressor p53 can regulate ferroptosis either positively or negatively, in addition to its well-known effects on apoptosis and other non-apoptotic forms of cell death, including necroptosis and autophagy. The mechanism by which p53 regulates ferroptosis can be transcriptional-dependent or independent. On one hand, p53 enhances ferroptosis by inhibiting SLC7A11 (solute carrier family 7 member 11) transcription, promoting the expression of SAT1 (spermidine/spermine N1-acetyltransferase 1) and GLS2 (glutaminase 2), or interacting with SLC25A28 (mitoferrin-2) in mitochondria. On the other hand, p53 suppresses ferroptosis by inhibiting DPP4 (dipeptidyl peptidase 4) activity or inducing CDKN1A/p21 (cyclin-dependent kinase inhibitor 1 A) and iPLA2β expression. This chapter provides an overview of the recent discoveries concerning the role of p53 in regulating ferroptosis.

7.1 Introduction

The tumor protein p53, also known as p53, plays a crucial role in the maintenance of multicellular organisms (Surget et al. 2013). Discovered in 1979, p53 has been described as "the guardian of the genome" due to its function in conserving stability and preventing genome mutation, thereby acting as a tumor suppressor (May and May 1999). Moreover, p53 has various mechanisms for its anticancer function and

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works through several pathways involved in cell death. External and internal stress signals stabilize and activate p53 (Kruiswijk et al. 2015). Activated p53 induces apoptosis by transactivating proapoptotic genes (e.g., BAX, BAD, BAK) or directly binding to antiapoptotic mitochondrial proteins (e.g., BCL-2, BCL-XL) (Huang and Strasser 2000; Galluzzi et al. 2008). p53 also plays a positive role in oxidative stress-induced necroptosis. During oxidative stress, p53 directly binds to cyclophilin D to open the mitochondrial permeability transition pore or transcriptionally activates necrosis-related factor, a long noncoding RNA (lncRNA), to elevate RIPK1/RIPK3 levels (Vaseva et al. 2012; Wang et al. 2016b).

Regarding autophagy, p53 can both promote and inhibit the autophagic process, depending on its subcellular localization. Nuclear p53 induces autophagy by transcriptionally upregulating AMPK (AMP-activated protein kinase) and DRAM (damage-regulated autophagy modulator), whereas cytoplasmic p53 inhibits autophagy by inducing Beclin-1 degradation (Maiuri et al. 2010; Feng et al. 2007; Criollo et al. 2009; Kang et al. 2011). Recently, emerging evidence has suggested that p53 is also involved in a novel non-apoptotic cell death process called ferroptosis, which will be discussed in this chapter.

7.1.1 Ferroptosis Basics

Ferroptosis is a newly discovered form of regulated cell death (RCD) that relies on intracellular iron accumulation and lipid peroxidation (Stockwell et al. 2017; Xie et al. 2016; Liu et al. 2022). First described in 2012, ferroptosis is characterized morphologically, biochemically, and genetically as distinct from other types of cell death, such as apoptosis, necroptosis, and autophagic cell death (Dixon et al. 2012). It is primarily caused by iron-mediated lipid oxidative damage, which can be inhibited by lipophilic antioxidants (e.g., ferrostatin-1, liproxstatin-1) and iron chelators (e.g., deferoxamine mesylate), but not by apoptosis-, necroptosis-, and autophagy-specific inhibitors (e.g., Z-VAD-FMK, BOC-D-FMK, wortmannin, and necrostatin-1) (Skouta et al. 2014; Friedmann Angeli et al. 2014). Two small molecule compounds, erastin and RSL3, were initially found to induce ferroptosis, respectively by indirect or direct inhibition of the membrane lipid repair enzyme GPX4 (glutathione peroxidase 4) (Dolma et al. 2003; Yang and Stockwell 2008; Yang et al. 2014; Dixon et al. 2012). Specifically, erastin inhibits the cystine/glutamate antiporter system Xc- on cell membranes, reducing cellular uptake of cysteine and blocking the synthesis of GPX4 substrates GSH (glutathione), which in turn triggers the accumulation of membrane lipid peroxides and ferroptosis (Dixon et al. 2012).

Recent studies have shown that phospholipids, the natural molecules that make up cell membranes, play a crucial role in driving ferroptosis in cells. Membrane phospholipids (PEs), which are rich in polyunsaturated fatty acids (PUFAs) such as arachidonic acid (AA), are the key substances involved (Kagan et al. 2017; Doll et al. 2017; Yang et al. 2016). The metabolism of AA involves pathways including lipoxygenases (LOXs). Under the action of LOXs and other oxidizing enzymes,

membrane PEs produce malondialdehyde and other oxidative end products, which react with proteins and nucleic acids to alter cell structure and function, thus producing toxicity (Gaschler and Stockwell 2017; Chen et al. 2021d). Core regulators such as System Xc-, GPX4, NRF2 (nuclear factor-E2-related factor 2), and ACSL4 (Acyl-coenzyme A synthetase long-chain 4) have been identified as involved in the control of ferroptosis (Dixon et al. 2012; Yang et al. 2014; Sun et al. 2016; Chen et al. 2017; Doll et al. 2017). However, the final effector in the execution of ferroptosis remains unclear. Currently, abnormal activation of ferroptosis pathways has been shown to be involved in a variety of pathophysiological processes, including cancer cell death, neurodegenerative lesions, ischemia-reperfusion injury, and antiviral immunity (Friedmann Angeli et al. 2014; Skouta et al. 2014; Matsushita et al. 2015; Do Van et al. 2016; Linkermann et al. 2014; Sun et al. 2015; Chen et al. 2021b; Tang et al. 2021).

7.2 p53 as a pro-Ferroptosis Regulator

7.2.1 Transcriptional Suppression of SLC7A11

The best-known mechanisms of p53 in tumor suppression involve initiating regulated cell death (RCD) and cell cycle arrest via transcriptional-dependent or -independent mechanisms. Posttranslational modifications of p53 can heavily impact its transcriptional and oncosuppressive functions. For example, $p53^{3KR}$, an acetylation-deficient p53 variant (with loss of K98 acetylation and also referred to as $p53^{K98R}$), fails to induce apoptosis and cell cycle arrest, but retains the capacity for tumor suppression as wild-type p53, indicating an unknown activity of p53 in tumor suppression (Li et al. 2012).

SLC7A11 (solute carrier family 7 member 11), a crucial component of the cystine/glutamate antiporter system Xc-, is identified as a transcriptional target of p53 through microarray screening (Jiang et al. 2015). In certain human cancer cells expressing wild-type p53, such as human breast cancer MCF7 cells and human osteosarcoma U2OS cells, as well as in mouse embryonic fibroblasts (MEFs), SLC7A11 is transcriptionally repressed upon induction of p53 expression, which is completely abrogated in the absence of p53. Notably, similar results are also observed in cell lines with induced p533KR expression. Genetic suppression of SLC7A11 and the subsequent inhibition of system Xc- impairs cystine uptake and glutathione (GSH) synthesis, leading to the accumulation of lipid reactive oxygen species (ROS) and ferroptosis (Dixon et al. 2012) (Fig. 7.1). Therefore, reduced SLC7A11 expression and cystine uptake levels are considered necessary conditions for ferroptosis in tet-on $p53^{3KR}$-inducible cells, which resolves a missing puzzle piece in p53-mediated tumor suppression (Jennis et al. 2016).

At present, there are various compounds that have been identified to induce ferroptosis partly through p53-mediated inhibition of SLC7A11 transcription in different cancer cells, such as pseudolaric acid B, bavachin, and flubendazole (Wang et al. 2018; Luo et al. 2021; Zhou et al. 2021). Furthermore, ALOX12 has

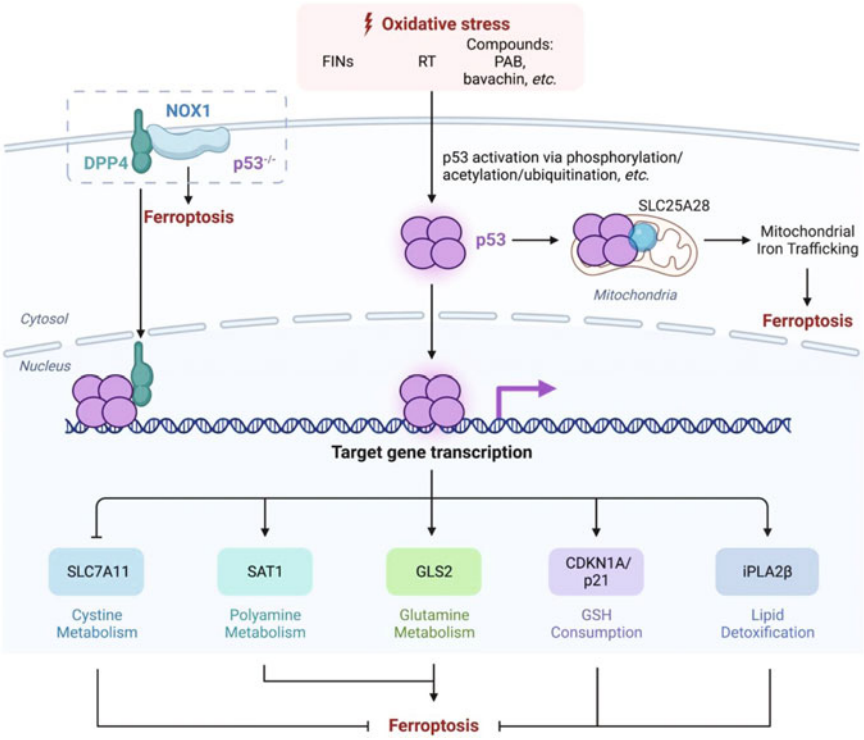

Fig. 7.1 The bidirectional regulatory role of p53 in the control of ferroptosis. On one hand, p53 enhances ferroptosis by inhibiting SLC7A11 transcription, promoting the expression of SAT1 and GLS2, or interacting with SLC25A28 in mitochondria. On the other hand, p53 suppresses ferroptosis by inhibiting DPP4 activity or inducing CDKN1A/p21 and iPLA2β expression

been identified as the required factor of p53-SLC7A11 axis-dependent ferroptosis in U2OS cells and human lung cancer H1299 Tet-on $p53^{3KR}$ cells (Chu et al. 2019). Genetic inactivation of ALOX12 is sufficient to abrogate p53-mediated tumor suppression (Chu et al. 2019). Mechanistically, SLC7A11 directly interacts with ALOX12 to modulate its activity and function. Additionally, in a recent study, STAT6 was found to inhibit ferroptosis and alleviate acute lung injury by competitively binding with CREB-binding protein, inhibiting P53 acetylation, and transcriptionally restoring SLC7A11 expression (Yang et al. 2022b). However, further investigation is required to determine how p53 might contribute to other components of ferroptosis, as not all p53-induced ferroptosis is via inhibition of SLC7A11.

7.2.2 Transcriptional Activation of SAT1

In addition to its role in cystine metabolism, p53 has been implicated in the regulation of polyamine metabolism, which involves organic compounds containing two

or more amino groups (Ou et al. 2016). Polyamines play important roles in regulating cellular growth, proliferation, differentiation, and programmed cell death(Moschou and Roubelakis-Angelakis 2014).

SAT1 (spermidine/spermine N1-acetyltransferase 1, arginine/arginine 1-acetyltransferase 1) is a rate-limiting enzyme that acetylates spermidine and spermine to form N1-acetylspermidine and N1-acetylspermine, respectively, in the presence of acetyl-coenzyme A. Repressed SAT1 expression and impaired spermidine/spermine depletion have been associated with various pathological processes, including tumorigenesis (Thomas and Thomas 2003). Recent studies have identified SAT1 as another transcriptional target of p53, as revealed by RNA sequencing in human melanoma A375 cells (Ou et al. 2016) (Fig. 7.1). Transcriptional induction of SAT1 by p53 contributes to ROS-mediated ferroptotic cell death, but not apoptosis, which is associated with the hyper-activation of ALOX15 (arachidonate 15-lipoxygenase), an iron-binding enzyme that oxidizes PUFAs. Furthermore, an ALOX15-specific inhibitor PD146176 impairs SAT1-induced ferroptotic cell death, establishing a p53-SAT1-ALOX15 axis in the ferroptosis pathway (Ou et al. 2016).

7.2.3 Transcriptional Activation of GLS2

Glutamine metabolism is an additional target in p53-mediated ferroptosis. Recent studies have shown that glutamine can induce ferroptosis during amino acid starvation by converting to glutamate with the help of mitochondrial glutaminases (GLS). Although two isoforms of GLS exist, only GLS2 is responsible for ferroptosis (Gao et al. 2015).

An African-specific single nucleotide polymorphism (SNP) in p53, the Ser47 variant (referred to as S47), has been found to attenuate p53 function in trans-activating target proteins, including GLS2, resulting in impaired ferroptotic cell death following treatment with erastin. Silencing GLS2 in wild-type MEFs showed a similar phenotype to the impaired ferroptotic cell death observed in S47 MEFs (Jennis et al. 2016) (Fig. 7.1). However, transformation with E1A and RAS restored p53 function in trans-activating GLS2 and oncosuppression, potentially overcoming resistance to cisplatin and erastin-induced cell death in humans with the S47 variant (Basu et al. 2016). Thus, a p53-mediated ferroptosis network has emerged, based on fine-tuning of p53 transcriptome.

7.2.4 Mitochondrial Translocation and Interaction with SLC25A28

There is evidence that demonstrates p53's involvement in regulating ferroptosis in mitochondria. Mitochondrial translocation of p53 contributes to the enhancement of ferroptosis by bromodomain-containing protein 7 (BRD7) in hepatic stellate cells (HSCs) (Zhang et al. 2020). BRD7 knockout significantly reduces serine392 phosphorylation and p53 expression in mitochondria, as opposed to in the cytoplasm and

nucleus, while knockin of BRD7 markedly increases them (Zhang et al. 2020). The S392A mutant impairs BRD7-mediated p53 mitochondrial translocation and ferroptosis in HSCs (Zhang et al. 2020). SLC25A28 (mitoferrin-2) is a crucial target that directly binds to p53 in the mitochondria, mediating mitochondrial iron trafficking in BRD7-enhanced ferroptosis, which is inhibited upon S392A mutant or p53 knockdown (Zhang et al. 2020) (Fig. 7.1). Furthermore, SLC25A28 knockdown completely abolishes mitochondrial iron accumulation, which impairs BRD7-enhanced ferroptosis in HSCs (Zhang et al. 2020). Thus, induction of ferroptosis to eliminate HSCs could effectively improve the pathological changes of liver fibrosis and serve as a potential anti-fibrotic therapeutic strategy.

7.3 p53 as an Anti-Ferroptosis Regulator

7.3.1 Transcriptional Activation of CDKN1A/p21

Recent evidence suggests that p53 plays a negative regulatory role in ferroptosis, which is fully dependent on the stabilization of wild-type p53 protein and transactivation of the canonical p53 target gene CDKN1A/p21 (cyclin-dependent kinase inhibitor 1A responsible for cell cycle arrest) (Tarangelo et al. 2018). Notably, in line with a prior study showing that p21 activation only occurs in cells expressing wild-type p53 but not those with mutant p53 (el-Deiry et al. 1993), the $p53^{3KR}$ mutant fails to constitutively express CDKN1A/p21, possibly explaining why opposite results were obtained with wild-type p53 versus the $p53^{3KR}$ mutant in the regulation of ferroptosis.

Pretreatment with nutlin-3 (a small-molecule MDM2 inhibitor) increases p53 expression levels and simultaneously suppresses erastin2-induced cell death (a specific system Xc- inhibitor) in $p53^{+/+}$ cells, but not in $p53^{-/-}$ cells. Likewise, nutlin-3 pretreatment delays the onset of cell death upon cystine deprivation, a process blocked by ferrostatin-1, indicating that the delayed ferroptosis requires the expression and stabilization of wild-type p53 (Tarangelo et al. 2018). Nutlin-3 upregulates CDKN1A mRNA and p21 protein in $p53^{+/+}$ but not $p53^{-/-}$ cells. In contrast, nutlin-3 pretreatment fails to protect against erastin2-induced ferroptosis in CDKN1A-deficient cells, suggesting a further requirement for p21 to suppress ferroptosis (Fig. 7.1). Mechanistically, the p53-p21 pathway delays the execution of ferroptosis by reducing the consumption of GSH (glutathione), rather than CDK4/6 inhibition or enhanced NRF2 (nuclear factor-E2-related factor 2) activity. However, precisely how p21 promotes GSH biosynthesis or conservation remains unclear.

7.3.2 Posttranslational Inhibition of DPP4 Activity

p53 is functionally inactivated or mutated in approximately 50% of human cancers, including colorectal cancer cells (CRC) (Fearon 2011). Recently, evidence has

shown that deletion and mutation of p53 in CRCs promote ferroptosis by enhancing the activity of DPP4 (dipeptidyl peptidase-4), demonstrating for the first time that p53 regulates ferroptosis in a transcription-independent pathway (Xie et al. 2017).

Ferroptosis is RAS mutation-oriented when discovered in 2012 (Dixon et al. 2012). Although ferroptosis was initially discovered to be RAS mutation-oriented (Dixon et al. 2012), alteration of RAS status within CRCs does not affect cell death induced by erastin. However, functional p53 deficiency sensitizes CRCs to ferroptotic cell death instead of non-CRCs such as U2OS and MCF7. In these non-CRCs, p53 acts as a pro-ferroptosis mediator through transcriptional suppression of SLC7A11, whereas in CRCs, a novel mechanism of p53 regulating ferroptosis was discovered through a protease inhibitor library screening. Remarkably, only DPP4 inhibitors block erastin-induced cell death in p53-deficient CRCs. DPP4, also known as CD26 (cluster of differentiation 26), is an enzyme expressed on the surface of most cell types and is associated with immune regulation, glucose metabolism, and oxidative stress (Reinhold and Brocke 2014; De et al. 2019; Chua et al. 2014). Mechanistically, p53 limits ferroptosis by promoting nuclear translocation of DPP4, which impairs the membrane DPP4 activity in lipid peroxidation (Fig. 7.1). Of note, there is almost no DPP4 expression in U2OS and MCF7 cells, suggesting that the p53-DPP4 network is not predominant in these cells.

7.3.3 Transcriptional Activation of iPLA2β

The calcium-independent phospholipase iPLA2β is another target of p53 during ROS-induced ferroptosis in cancer cells that express wild-type p53 (such as U2OS, MCF7, A375, and human lung cancer A549 cells) (Chen et al. 2021a). Upon p53 activation by either nutlin or doxorubicin treatment, the mRNA levels of iPLA2β are induced at the early stage of stress responses or under low levels of stress, but are diminished at the late stage of stress responses or under high levels of stress (Chen et al. 2021a). As a phospholipase involved in lipid detoxification, iPLA2β downregulates the levels of peroxidized membrane lipids upon ROS stress and effectively suppresses p53-promoted ferroptosis (Fig. 7.1). On the contrary, endogenous iPLA2β depletion sensitizes tumor cells to ROS-induced ferroptosis and enhances p53-mediated tumor suppression (Chen et al. 2021a). Therefore, repression of p53-driven ferroptosis by iPLA2β plays a critical role in the tumorigenesis of cancers that overexpress iPLA2β, and targeting iPLA2β to activate ferroptosis in human cancers is a promising therapeutic strategy.

7.4 Conclusion

Over the past few years, studies have revealed emerging functions of p53 in the ferroptosis network, highlighting its unconventional role in regulating RCD. It is evident that p53 becomes activated in response to a series of stressors, including DNA damage, oxidative, or metabolic stress. The critical events leading to the

activation of the p53 network involve posttranslational modifications and transcriptional regulation of numerous target genes. Acetylation, phosphorylation, and ubiquitination have been shown to be crucial for p53-mediated ferroptosis (Jiang et al. 2015; Wang et al. 2016a; Zhang et al. 2020; Wang et al. 2023). However, p53 regulation of its target genes, such as SLC7A11, SAT1, GLS2, and CDKN1A/p21, exhibits a bidirectional role in the control of ferroptosis in different contexts (Jiang et al. 2015; Wang et al. 2018; Ou et al. 2016; Jennis et al. 2016; Tarangelo et al. 2018). In addition, p53 or its target genes can be modulated by SNP, noncoding RNAs (including lncRNAs and microRNAs), SOCS1 (suppressor of cytokine signaling 1), SIRT3 (sirtuin 3), USP7 (ubiquitin-specific protease 7), or CUL9 (cullin9) to engage in ferroptosis (Basu et al. 2016; Mao et al. 2018; Saint-Germain et al. 2017; Wang et al. 2018; Lu et al. 2020; Wang et al. 2019a; Jin et al. 2021; Wang et al. 2019b; Chen et al. 2021c; Yang et al. 2022a). Due to the complexity of p53 activity, potential modifications and novel target genes still need to be identified. Furthermore, the subcellular localization of p53 plays a crucial role in the sensitivity of cells to ferroptosis. For example, nuclear p53 promotes ferroptosis in lung cancer cells but inhibits it in colorectal cancer cells (Mao et al. 2018; Xie et al. 2017). Additionally, p53 mitochondrial translocation promotes ferroptosis in HSCs (Zhang et al. 2020). Thus, further understanding of the molecular mechanisms by which p53 modulates ferroptosis may provide rich insights into the opposing effects in different cellular contexts.

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Ferroptosis in Cardiovascular Disease

8

Jason K. Higa, Nicholas K. Kawasaki, and Takashi Matsui

Abstract

Ferroptosis is a relatively newly discovered form of regulated cell death that is distinct from other forms of cell death. Ferroptosis has been observed in a variety of tissues and diseases, including the heart. The heart depends on iron as a cofactor in many enzymes and in catalyzing reactions supplying the constant energy needs of the heart. Erythrocytes also require iron for binding and transporting oxygen in heme. The metabolic demands of the heart and the constant perfusion of blood through the myocardium put it at risk of iron overload and consequent oxidative stress and lipid peroxidation—all hallmarks of ferroptosis. Disruptions to iron and redox homeostasis can induce ferroptosis in cardiomyocytes. Iron homeostasis is maintained by multiple iron transport and storage mechanisms, and redox balance is maintained by multiple pathways, including glutathione peroxidase (GPX4) and potential new cellular antioxidant mechanisms. Together, these antioxidant pathways are a cellular multipronged system in combating oxidative stress and lipid peroxidation. This chapter describes how various cardiac pathologies disrupt iron and redox homeostasis and facilitate ferroptosis. Later, we discuss current therapies for dealing with cardiovascular iron overload diseases and strategies for potential new anti-ferroptotic therapies.

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8.1 Introduction

Ferroptosis is a type of regulated cell death mediated by iron-dependent lipid peroxidation, which distinguishes it from other forms of cell death (Dixon et al. 2012). While accidental cell death caused by physical, chemical, or mechanical stimuli cannot be prevented, regulated cell death can be attenuated by altering the activity of endogenous signal transduction pathways (Galluzzi et al. 2015). Regulated cell death, such as apoptosis, is critical in the pathogenesis of many cardiac diseases, including acute myocardial infarction (MI) and heart failure (Konstantinidis et al. 2012). However, ferroptosis is distinct from apoptosis and other forms of cell death (Dixon et al. 2012). Two major pathophysiological features characterize ferroptosis: iron dependency and the accumulation of lipid peroxides (Hirschhorn and Stockwell 2019). These features are not shared with apoptosis nor other types of regulated cell death (Dixon et al. 2012). The pathophysiological role of ferroptosis in the heart has become a burgeoning field of study that has yielded many publications since the last edition of this book. In that time, ferroptosis has transitioned from a new discovery into an increasingly important factor in cell death in the heart (Fang et al. 2023; Yang et al. 2022b).

Damage to cardiac tissue can result in adverse left ventricular (LV) remodeling, which includes ventricular dilatation and fibrosis. Adverse LV remodeling is an important prognostic factor for heart failure (Bhatt et al. 2017). In cardiac ischemia, the magnitude of cardiomyocyte cell death is directly proportional to the future risk of LV remodeling and heart failure (Mckay et al. 1986). The death and loss of cardiomyocytes caused by an acute MI or cardiomyopathy can result in cardiac pump dysfunction and subsequent heart failure. The adult human heart has a limited ability to repair and compensate for losses in cardiomyocytes, as adults have limited cardiomyocyte proliferation rates after the neonatal period (Hirai et al. 2016). While studies have demonstrated cardiomyocyte proliferation and reprogramming of fibroblasts into cardiomyocytes under certain conditions (Abad et al. 2017; Senyo et al. 2013), there are currently no well-established therapeutic approaches or methods that restore cardiac function after losing functional cardiomyocytes in pathological settings. Therefore, preventing massive cardiomyocyte death due to cardiac injury remains a reasonable strategy for preventing and treating heart failure following cardiac insults or injuries such as an acute MI or cardiomyopathy.

Myocardial reperfusion therapies, such as primary percutaneous coronary intervention (PCI), reduce the initial infarct size in patients presenting with an acute MI by restoring blood flow to the infarcted area. However, the process of reperfusion induces further injury in the myocardium, known as reperfusion injury (Hausenloy and Yellon 2013). In patients with ST-segment elevation MI (STEMI), intramyocardial hemorrhage is a frequent complication that occurs after successful myocardial reperfusion and is an independent predictor of adverse LV remodeling (Carrick et al. 2016; Ganame et al. 2009). A report using cardiac magnetic resonance imaging (MRI) showed that all patients in the study who had adverse LV remodeling also displayed accumulated residual myocardial iron in the myocardium within the infarct zone (Bulluck et al. 2016). We demonstrated that in mouse models, iron

accumulates in the myocardium following ischemia-reperfusion (I/R) injury (Baba et al. 2018). In the report, we also showed that ferrostatin-1, a ferroptosis inhibitor (Dixon et al. 2012), inhibited Fe (III)-citrate-induced cell death in adult mouse cardiomyocytes (Baba et al. 2018). Reports using *in vivo* surgical models in mice demonstrated that ferroptosis plays an important role in the pathogenesis of I/R injury (Chen et al. 2021; Fang et al. 2019; Gao et al. 2015; Li et al. 2019a; Miyamoto et al. 2022). Further studies showed that inhibition of ferroptosis protects the heart against cardiomyopathy, like that seen in response to doxorubicin (DOX) treatment (Fang et al. 2019; Kitakata et al. 2021; Tadokoro et al. 2020).

These findings strongly suggest that ferroptosis, a form of iron-dependent regulated cell death, is a potential target for novel therapies targeting cardiac diseases, including acute MI and cardiomyopathy. We review the role of ferroptosis in cardiovascular disease in this chapter.

8.2 Iron in the Heart

8.2.1 The Physiological Role of Iron in the Heart

The heart is constantly beating and contracting to circulate and distribute blood throughout the body, thus it needs a constant supply of energy to fuel cardiomyocyte contractions. Therefore, cardiomyocytes are rich in mitochondria, which generate the adenosine triphosphate (ATP) needed to supply the constant energy demands of the heart. Blood is a fluid tissue that contains many iron-rich components in both its cellular and liquid fractions. Red blood cells (RBCs), or erythrocytes, are a major component of blood and are the primary carriers of oxygen to the tissues of the body. RBCs rely on intracellular stores of hemoglobin to bind oxygen for transport, and each hemoglobin molecule contains four molecules of heme. Iron is central to the structure of heme and essential for its ability to bind diatomic oxygen. Heme oxygenase-1 (HO-1) catalyzes the catabolism of heme into Fe^{2+} and biliverdin as part of the normal turnover of RBCs and recycling of iron in the body and has become increasingly important in studies of ferroptosis in the heart.

Iron is also critical for mitochondrial function and is needed in iron-sulfur clusters that are essential cofactors for proteins involved in several mitochondrial functions, including beta-oxidation, the citric acid cycle, and oxidative phosphorylation (Stehling and Lill 2013). In oxidative phosphorylation, the properties of iron-sulfur clusters allow the transfer of electrons through the mitochondrial complexes of the electron transport chain, which generates not only ATP but also reactive oxygen species (ROS) (Murphy 2009). Iron-sulfur clusters are also essential for heme synthesis.

8.2.2 The Pathological Role of Excess Iron in the Heart

Since the 1970s, researchers have reported correlations between excess cardiac iron deposition and cardiac dysfunction (Buja and Roberts 1971), and research attempting to link and implicate the causative effects of excess iron in cardiac disease continues to this day. In the 1990s, researchers hypothesized that ferritin and overall body iron stores and homeostasis contributed to cardiovascular disease (Salonen et al. 1992), but later studies and analyses failed to support the hypothesis that serum ferritin and body iron dysregulation are linked to cardiovascular disease (Knuiman et al. 2003; Sempos et al. 2000).

Despite the lack of support for the serum ferritin hypothesis, other studies have associated pathologies that cause excess iron deposition with cardiovascular dysfunction. Hemochromatosis is a condition characterized by iron overload in the body and can be caused by either hereditary factors that affect genes associated with iron homeostasis, or acquired through medical interventions such as frequent blood transfusions (Babitt and Lin 2011; Kohgo et al. 2008). Genetic hemochromatosis is associated with LV dysfunction (Carpenter et al. 2013), and chronic transfusions can cause cardiac iron overload as viewed through T2* MRI (De Montalembert et al. 2017).

Genetic disorders that affect hemoglobin synthesis and metabolism are also associated with cardiovascular disease (Taher et al. 2013). Hemoglobin relies on iron as an essential cofactor that allows heme to bind to oxygen, but hemoglobin is also a potential source of extracellular iron if hemolysis occurs. Beta-thalassemia is characterized by inefficient erythropoiesis, an inability to handle free iron, and is associated with an increased risk of heart failure (De Montalembert et al. 2017; Hahalis et al. 2005). In sickle-cell disease, a mutation in the gene encoding hemoglobin causes the protein to misfold and warp RBCs into an elongated shape, affecting their transport and durability in the cardiovascular system (Wood 2016). This atypical shape also makes sickle-cells more prone to hemolysis and releasing hemoglobin into the extracellular space (Wood 2016). Myocardial iron loading is also observed in sickle-cell disease (Wood et al. 2004), and this increased iron deposition is directly linked to myocardial dysfunction (Fujikura et al. 2018).

Research since the last edition of this book has revealed an increasing importance of iron and ferroptosis in acute and chronic cardiovascular diseases. Ischemia-reperfusion models have shown increased myocardial iron deposition and elevated ferroptotic markers. Various causes of cardiomyopathy, such as post-I/R LV remodeling, doxorubicin treatment, pressure overload-induced hypertrophy, and diabetes, have all shown increased ferroptosis (Fang et al. 2023; Yang et al. 2022b) (Fig. 8.1).

Thus, iron is an essential element for cardiovascular function in both cardiomyocyte metabolism and oxygen transport via hemoglobin, but these functions also mean that the heart is potentially exposed to both intracellular and extracellular sources of free iron. Therefore, the heart is vulnerable to derangements in iron homeostasis both inside and out, which can lead to ferroptosis and other iron-overload pathologies.

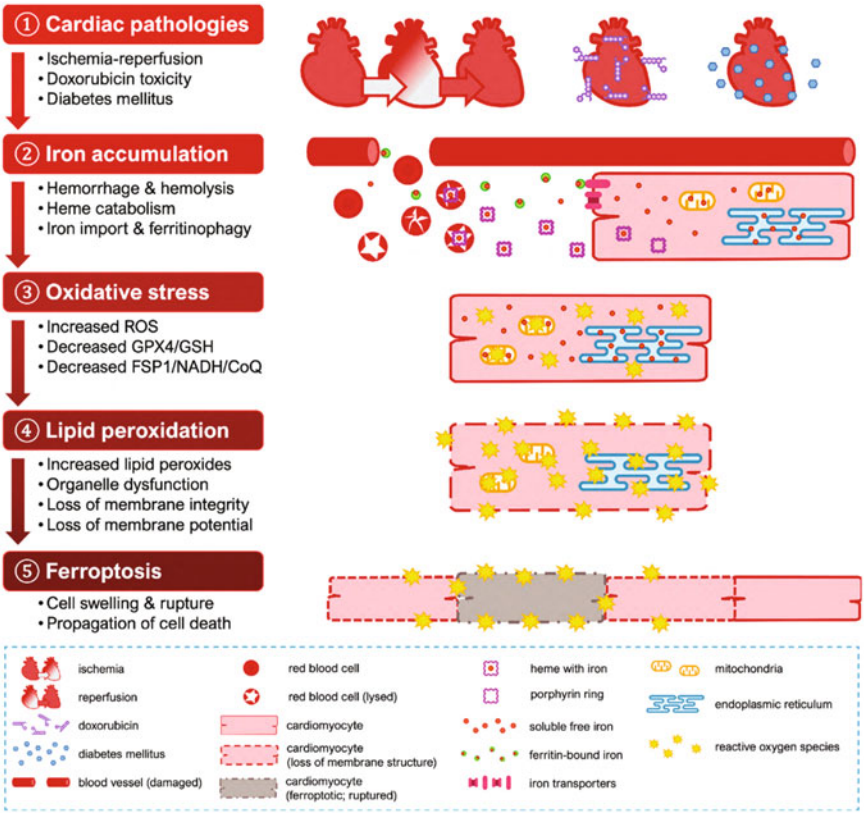

Fig. 8.1 Stages of cardiac ferroptosis. (1) Various cardiac pathologies such as ischemia-reperfusion (I/R) injury, doxorubicin-induced cardiomyopathy, and diabetes mellitus can cause myocardial damage. (2) Damage to the heart or its vasculature can result in the deposition, accumulation, and increased availability of redox-active iron inside cardiomyocytes and organelles. (3) Cardiac pathologies like I/R can also lower antioxidant defenses and cause oxidative stress due to increased reactive oxygen species (ROS) production. (4) Increased ROS and oxidative stress favors the formation of lipid peroxides, which can compromise membrane structure and result in a lack of membrane integrity and function. (5) Ferroptosis occurs as a result of the catastrophic accumulation of lipid peroxides, which leads to cell swelling and rupture, and may propagate along connected cardiomyocytes

8.2.3 The Effects of Iron Homeostasis on Cell Function

Although iron is a key component in triggering ferroptotic mechanisms, cardiomyocytes need iron as a cofactor for intracellular enzymes. Iron is especially vital for mitochondrial activity that supplies the energy needed for ion transport in cardiac electrochemical activity and muscle contraction (Griffiths 2012). Iron serves as a critical catalyst for several steps in aerobic respiration and generating ATP required for the constant energy demands of cardiomyocytes (Levi and Rovida 2009). Iron deficiency impairs mitochondrial aerobic respiration and results in an

eventual LV dysfunction and heart failure (Haddad et al. 2017). Since both iron deficiency and excess iron cause cardiac pathologies, a homeostatic balance must be struck between having too little or too much intracellular iron inside the heart. Therefore, cardiac muscle requires mechanisms that maintain iron homeostasis within a normal range.

Iron homeostasis is dynamic and can change by altering the contents of the labile iron pool—an intracellular reserve of soluble iron (Kakhlon and Cabantchik 2002) (Fig. 8.2). The iron in the labile iron pool can participate in redox reactions and can also be targeted by chelators. The labile property of the iron pool refers to how the cell can import, export, or sequester iron using proteins or organelles to adjust iron concentrations in the pool and regulate redox activity (Kakhlon and Cabantchik 2002). The primary mechanism of iron import into the cell is through the binding of transferrin-bound iron to transferrin receptors and consequent receptor-mediated endocytosis (Hentze et al. 2010; Kakhlon and Cabantchik 2002). Alternatively, ferroportin is the primary means of iron export from the cell and decreasing iron in the labile iron pool (Hentze et al. 2010). Another means of regulating the labile iron pool is through ferritin, which binds to soluble iron, thus sequestering it and allowing iron to be stored within the cell in a form that cannot easily participate in redox reactions (Arosio and Levi 2002). Conversely, ferritinophagy, a specific form of autophagy targeting ferritin, reverses this process and releases iron back into the labile iron pool (Hou et al. 2016; Santana-Codina and Mancias 2018). Pathways that mediate ferritinophagy have been identified in cardiac tissue, such as those involving Nuclear Receptor Coactivator 4 alpha and beta (NCOA4α & NCOAβ) activity (Gao et al. 2016; Ito et al. 2021). Disrupting lysosomal activity also increases intracellular iron levels, providing further evidence of the important effect of autophagy in intracellular iron homeostasis (Hou et al. 2016, Santana-Codina and Mancias 2018). Heme is also a source of potentially labile iron, and HO-1 has become increasingly important in studies of iron homeostasis and redox balance in the heart, as HO-1 activity catabolizes heme and releases its core Fe^{2+} into the labile iron pool (Fang et al. 2019) (Fig. 8.2).

8.3 Ferroptosis and Reactive Oxygen Species (ROS) Production

8.3.1 Iron Homeostasis and ROS Generation

Iron storage is not limited to ferritin in the cytosol, and excess iron can be stored in the mitochondria. Cardiac muscle needs a constant supply of ATP to keep up with the energy demands of a constantly beating heart. Thus, it follows that cardiomyocytes are also abundant in mitochondria, which require iron as cofactors in ATP synthesis (Levi and Rovida 2009). Cardiac mitochondria are also able to store excess iron within iron-sulfur clusters and mitochondrial ferritin (FtMt), a mitochondria-specific form of the iron storage protein (Drysdale et al. 2002). Mitochondria are a hotbed of ROS, which are generated by oxidative phosphorylation occurring within the mitochondria during aerobic ATP production (Levi and

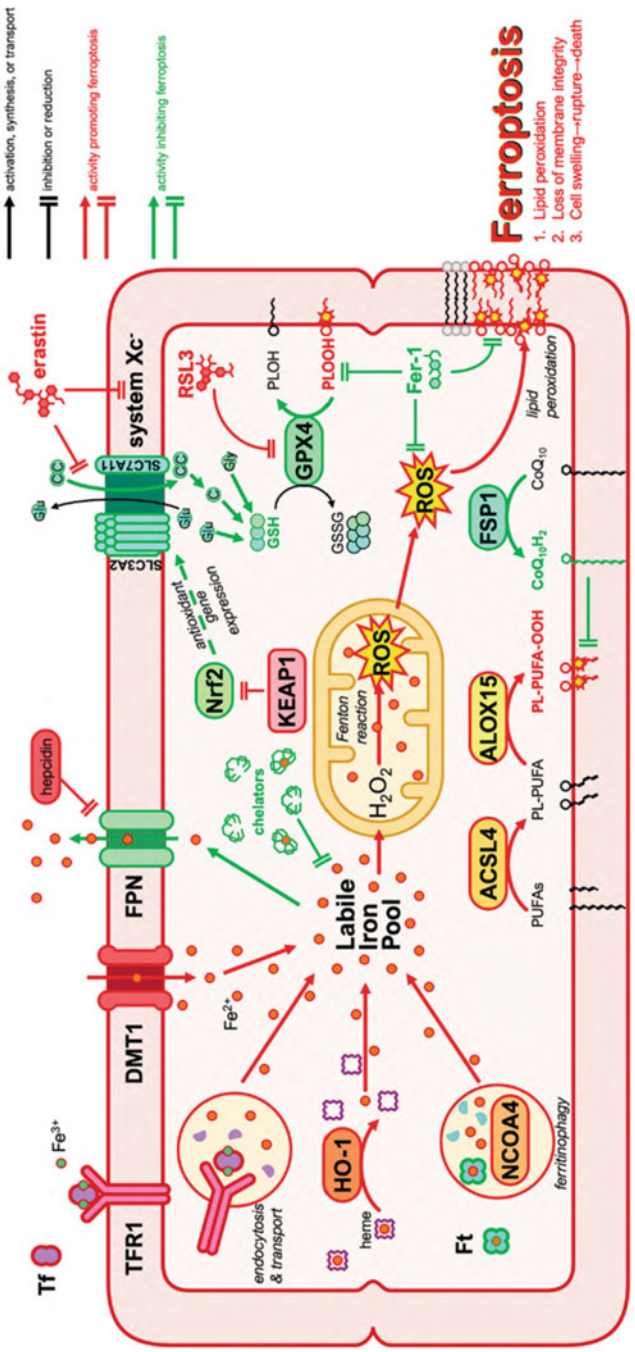

Fig. 8.2 Mechanisms of ferroptosis in cardiomyocytes. Red outlines indicate proteins or cell activity that promote ferroptosis. Green outlines indicate proteins or cell activity that promote antioxidant activity, inhibition of ferroptosis, or both. Pointed arrows indicate activation, synthesis, or transport. Blunt double-lined arrows indicate inhibition or reduction of its target. *ACSL4* acyl-CoA synthetase long chain family member 4, *ALOX15* arachidonate 15-lipoxygenase, *C* cysteine, *CC* cystine, CoQ_{10} coenzyme Q_{10}, $CoQ_{10}H_2$ coenzyme Q_{10} (reduced), *DMT1* Divalent Metal Transporter 1, *Glu* glutamate, *Fer-1* ferrostatin-1, *FPN* ferroportin, *FSP1* ferroptosis suppressor protein 1 (also AIFM2), *Ft* ferritin, *Gly* glycine, *GPX4* glutathione peroxidase 4, *GSH* glutathione (reduced), *GSSG* glutathione disulfide, *HO-1* heme oxygenase-1, *KEAP1* Kelch-like ECH-associated protein 1, *NCOA4* nuclear receptor coactivator 4, *Nrf2* nuclear factor erythroid 2-related factor 2, *PL* phospholipid, *PL-PUFA* phospholipid containing polyunsaturated fatty acid(s), *PL-PUFA-OOH* phospholipid hydroperoxide containing polyunsaturated fatty acid, *PLOH* phospholipid alcohol, *PLOOH* phospholipid hydroperoxide, *PUFA* polyunsaturated fatty acid, *ROS* reactive oxygen species, *SLC3A2* solute carrier family 3 member 2 (also 4F2HC), *SLC7A11* solute carrier family 7 member 11, *system Xc-* cystine/glutamate antiporter, *Tf* transferrin, *TFR1* transferrin receptor 1

Rovida 2009). The generated ROS can also interact with iron-sulfur clusters within the mitochondria and catalyze the Fenton reaction, in which iron can interact with ROS and H_2O_2 to generate even more ROS (Thomas et al. 2009).

Altered rates of iron import or export or increased release of iron from storage can cause iron to accumulate in the labile iron pool and trigger ferroptosis. Transferrin and its transferrin receptor (Tfr1) are the primary mode of iron import into the cell (Fig. 8.2). Increasing the rate of iron transport can predispose a cell to ferroptosis by causing it to accumulate iron. Lapatinib is a tyrosine kinase receptor inhibitor that increases transferrin transport and causes ferroptotic activity (Ma et al. 2016). Tfr1was also identified as a ferroptosis-specific marker as determined by a screen of monoclonal antibodies that identified 3F3-FMA, an antibody specific for ferroptosis and Tfr1. Immunofluorescence and confocal microscopy were used to verify the location of ferroptosis and the anti-Tfr1 antibody, which both colocalized at the plasma membrane of multiple cell lines treated with RSL3 (Feng et al. 2020), although this needs to be confirmed in cardiac cells. The specificity of Tfr1 as a marker for ferroptosis was validated by experiments showing that induction of apoptosis with staurosporine and induction of necrosis with H_2O_2 did not increase anti-Tfr1 staining (Feng et al. 2020). Divalent Metal Transporter 1 (DMT1) is a transmembrane protein that facilitates the uptake of ferrous iron (Fe^{2+}) into cells (Gunshin et al. 1997). A study that used an *in vitro* hypoxia-reoxygenation (H/R) model with neonatal cardiomyocytes demonstrated that overexpression of DMT1 promoted H/R-induced ferroptosis (Song et al. 2021). Accordingly, knockdown of DMT1 prevented intracellular accumulation of ferrous iron and significantly inhibited H/R-induced cell ferroptosis (Song et al. 2021). In a similar vein to increasing iron import via DMT1 or transferrin and Tfr1, decreasing the rate of iron export can also cause a cell to accumulate iron and predispose it to ferroptosis. Siramesine, a sigma-2 receptor ligand that was originally developed for treatment of depression, decreases ferroportin activity and induces ferroptosis by causing excess iron to accumulate without the ability to rid the cell of excess iron (Ma et al. 2016).

8.3.2 Glutathione Peroxidase 4 (GPX4)

Ferroptosis was initially discovered through small-molecule inhibitors that disrupted the production of glutathione and GPX4 (Dixon et al. 2012) (Fig. 8.2). GPX4 uses glutathione in the reduction of lipid peroxides and the prevention of oxidative damage and cell death that could result from the accumulation of lipid peroxide radicals (Friedmann Angeli and Conrad 2018). GPX4 is a selenoprotein that uses its selenocysteine to reduce hydroperoxides and lipid hydroperoxides in ferroptosis (Ingold et al. 2018; Seibt et al. 2019). Selenocysteine is integral to GPX4, as replacement of the selenium group with sulfur essentially replaces selenocysteine with cysteine, which induces ferroptosis (Ingold et al. 2018). The high catalytic activity of selenocysteine allows GPX4 to constantly target hydroperoxides and reduce oxidative stress in the cell (Conrad 2009). GPX4 works by preventing 15-lipoxygenases from oxidizing polyunsaturated phosphatidylethanolamines

(PEs), specifically on arachidonoyl and adrenoyl fatty acids (Stockwell et al. 2017; Wenzel et al. 2017). GPX4 specifically reduces hydroperoxy-PEs that are created by a combination of phosphatidylethanolamine-binding protein 1 (PEBP1) and 15-lipoxygenases (Wenzel et al. 2017). This process is ferroptosis-specific as inhibition of arachidonoyl and adrenoyl esterification prevents ferroptosis (Kagan et al. 2017). Moreover, loss of acyl-CoA synthetase long-chain family member 4 (ACSL4) and lysophosphatidylcholine acyltransferase 3 (LPCAT3)—both enzymes that esterify polyunsaturated fatty acids—also confers an anti-ferroptotic effect (Stockwell et al. 2017). In contrast, apoptosis oxidizes cardiolipins and not PEs, further distinguishing ferroptosis as using a different form of lipid peroxidation (Orrenius and Zhivotovsky 2005).

Ferroptosis-inducing compounds are currently classified into two categories: class 1 and class 2 ferroptosis inducers (Yang et al. 2014), although this will likely change in the future as new ferroptotic mechanisms have been discovered. Class 1 ferroptosis inducers, such as erastin, target the cystine/glutamate transporter (system Xc- or xCT). Cystine is an essential reactant in the synthesis of glutathione. Therefore, inhibition of system Xc- or the gene that encodes system Xc- will deplete the cell of cysteine and glutathione. Overexpression of solute carrier family 7 member 11 (SLC7A11), the gene that encodes system Xc-, inhibits ROS-induced ferroptosis (Jiang et al. 2015). Furthermore, SLC7A11 deletion combined with a high-iron diet triggers ferroptosis in murine macrophages (Jiang et al. 2015) and cardiomyocytes (Baba et al. 2018). Class 2 ferroptosis inducers, such as Ras-selective lethal 3 (RSL3), work downstream of class 1 inhibitors and directly target and inhibit GPX4 activity. Other small-molecule inducers of ferroptosis have been discovered, although their mechanisms are not as well-defined as class 1 and class 2 ferroptosis inducers (Yu et al. 2017).

GPX4 has powerful protective effects in cardiac tissue, and overexpression of GPX4 prevents ischemia/reperfusion-associated cardiac dysfunction (Dabkowski et al. 2008). GPX4 is also found in the mitochondria, but mitochondrial GPX4 may play a lesser role than cytosolic GPX4 in ferroptosis, as mitochondria-specific overexpression of GPX4 failed to prevent ferroptosis in cultured cardiomyocytes subjected to H/R (Miyamoto et al. 2022). In accordance with the protective role of GPX4 against ferroptosis, inhibition or loss of GPX4 activity results in decreased antioxidant defenses, accumulation of lipid peroxides, and consequent ferroptosis (Yang and Stockwell 2016). Haploinsufficient GPX4 mice show increased carbonyl stress and 4-hydroxynonenal (HNE)-adducts in cardiac tissue (Katunga et al. 2015). Those mice also displayed cardiac hypertrophy and cardiac fibrosis after being fed a high-fat, high-sucrose diet model (Katunga et al. 2015). Genetic GPX4 knockout models showed similar markers of ferroptosis and cardiomyopathies, which were reduced when GPX4 knockout mice were fed an antioxidant-rich diet supplemented with vitamin E (Imai et al. 2017). However, removal of this diet from the GPX4 knockout mice resulted in sudden cardiac death (Imai et al. 2017). Additional pathways further upstream of glutathione synthesis and GPX4 can also induce ferroptosis. These include the mevalonate pathway, which controls the synthesis of selenoproteins such as GPX4 (Yang and Stockwell 2016) and the sulfur-transfer

pathway, which are important in cysteine biosynthesis and consequent glutathione formation (Yu et al. 2017). Taken together, GPX4 plays an integral role in mediating ferroptosis in cardiomyocytes by attenuating oxidative stress and preventing ROS accumulation.

The role of mitochondria in ferroptosis is more evident than it has been before. One of the first hints of the involvement of mitochondria was a study showing that prenatal knockout of mitochondrial thioredoxin reductase in cardiomyocytes, a reducing selenoprotein similar to GPX4, led to congestive heart disease in murine models (Conrad 2009). The involvement of mitochondria in cardiac ferroptosis is also supported by a study showing that cardiac hypertrophy can be induced through iron overload of mitochondria in cardiomyocytes (Tang et al. 2019). The resulting cardiac dysfunction seems to be caused by an accumulation of lipid peroxides in mitochondrial membranes (Tang et al. 2019). Cells treated with an antioxidant that specifically targets the mitochondria were rescued from the damage caused by lipid peroxidation (Fang et al. 2019). Additionally, cells treated with RSL3, a GPX4 inhibitor, displayed abnormal mitochondrial morphology and reduced function (Jelinek et al. 2018). In the case of human hearts, Raf kinase inhibitor protein (RKIP) is upregulated in patients who suffer from heart failure. RKIP is a member of the phosphatidylethanolamine binding protein (PEBP) family, thus linking this clinical research data to lipid peroxidation and heart function (Wolf et al. 2018). Mitochondria do not produce GSH, therefore they need to transport it from the cytosol, but this mechanism is not completely clear. However, two putative carrier proteins, solute carrier family 25 member 10 (SLC25A10; also dicarboxylate carrier, DIC) and solute carrier family 25 member 11 (SLC25A11; also oxoglutarate carrier, OGC), have been identified as being protective against oxidative stress, although fused membrane vesicle assays with these carriers show that they do not directly transport GSH (Jang et al. 2021).

So far, we established that the inherent redox properties of iron generate ROS, cardiomyocytes continually generate ROS through mitochondrial activity, and the antioxidant glutathione has an important role in scavenging free radicals and preventing cell death caused by the accumulation of ROS generated from normal cardiac activity and pathological conditions (Van Der Pol et al. 2018). Therefore, it follows that the labile iron pool, mitochondria, and glutathione are all involved in ferroptosis, and that derangements in any of these components can result in a combination of excess iron accumulation, ROS generation, and lipid peroxidation, all of which are hallmarks of ferroptosis.

8.3.3 Ferroptosis Suppressor Protein 1 (FSP1)

Ferroptosis suppressor protein 1 (FSP1) was previously known as apoptosis-inducing factor mitochondria-associated 2 (AIFM2), but was renamed to FSP1 after its potent anti-ferroptotic effects were recently discovered (Bersuker et al. 2019; Doll et al. 2019) (Fig. 8.2). FSP1 localizes to the plasma membrane and catalyzes the reduction of ubiquinone (CoQ_{10}) to ubiquinol ($CoQ_{10}H_2$), the latter of

which is an antioxidant that can reduce nearby lipid peroxide radicals in the cell membrane and reduce them to phospholipid hydroperoxides, which in turn can be further reduced by GPX4 into phospholipid alcohols (Bersuker et al. 2019). FSP1 can also repair ferroptotic membrane damage by recruiting the endosomal sorting complexes required for transport III (ESCRT-III) (Dai et al. 2020). ESCRT-III anti-ferroptotic activity is specific to FSP1 and is independent of the FSP1/CoQ_{10} pathway, suggesting that the effects of FSP1 are carried through multiple anti-ferroptotic mechanisms.

FSP1 also compensates for losses in GPX4 activity, as overexpression of FSP1 confers resistance to RSL3-induced ferroptosis in GPX4 knockout cell culture models (Bersuker et al. 2019, Doll et al. 2019). Glutathione is not a substrate for FSP1, and glutathione levels are also unaffected by changes in FSP1 activity, further decoupling FSP1 from the GPX4 pathway (Bersuker et al. 2019). Therefore, FSP1 has complementary anti-ferroptotic effects that are independent of GPX4 and can compensate for losses in GPX4 activity (Bersuker et al. 2019; Doll et al. 2019).

8.3.4 Mitochondria and Dihydroorotate Dehydrogenase (DHODH)

The close relationship between mitochondria and ferroptosis is almost indisputable, as mitochondria are pivotal in iron homeostasis and redox reactions and display marked changes in their morphology and metabolism in response to ferroptotic conditions (Dixon et al. 2012; Doll et al. 2017; Gao et al. 2019). However, whether mitochondria are a key component of ferroptosis has come under question, and research has shifted toward investigating the extent to which mitochondrial are involved and whether they are necessary or play more of a supporting role in ferroptosis.

Studies investigating the localization of lipid peroxide accumulation and mechanisms of action of anti-ferroptotic agents have not converged on the mitochondria, making it less likely that the mitochondria are the primary initiator of ferroptotic mechanisms (Doll et al. 2019; Gaschler et al. 2018; Hirata et al. 2023; Riegman et al. 2020). Depletion of mitochondria via mitophagy did not rescue HT1080 cells from RSL3-induced ferroptosis, and inhibition of the electron transport chain and mitochondrial uncoupling failed to prevent RSL3-induced accumulation of lipid ROS in different noncardiac cell culture lines (Gao et al. 2019). Therefore, the mitochondria are not necessary for ferroptosis mediated through the GSH/GPX4 pathway (Gao et al. 2019). However, cells depleted of mitochondria showed increased resistance to ferroptosis induced by erastin or cysteine deprivation (Gao et al. 2019). Since erastin inhibits system Xc- and decreases import and intracellular levels of cysteine, it appears that mitochondria play an important role in ferroptosis caused by cysteine-poor conditions (Gao et al. 2019). This was confirmed by experiments using inhibitors of the tricarboxylic acid (TCA) and electron transport chain, which also suppressed ferroptosis (Gao et al. 2019). Therefore, mitochondrial contributions to ferroptosis may be dependent on the presence of cysteine and independent from GPX4 activity.

Despite the increasing evidence that mitochondria are not necessary for ferroptosis, it does not mean that mitochondria cannot initiate their own ferroptotic mechanisms. Dihydroorotate dehydrogenase (DHODH) is an enzyme involved with pyrimidine synthesis and is located in the mitochondrial inner membrane. Inhibition of DHODH sensitized cancer cells with low levels of GPX4 to ferroptosis, and deletion of DHODH caused lipid peroxidation and ferroptotic cell death (Mao et al. 2021). GPX4 knockdown increases expression of DHODH; however, DHODH knockout in cells that express high levels of GPX4 did not exhibit lipid peroxidation (Mao et al. 2021). Taken together, DHODH regulation of ferroptosis is not dependent on GPX4, giving further evidence that mitochondrial ferroptotic activity is independent from GPX4. Furthermore, the different subcellular locations of DHODH versus FSP1 suggest a possible alternate DHODH pathway of triggering ferroptosis, and that the normal functions of GPX4, FSP1, and a potential DHODH pathway may help compensate for oxidative stress when one of those pathways are compromised.

8.3.5 Lipid Peroxidation

Disruption of either GPX4 or the FSP1/CoQ_{10} axis eventually tips the scale in favor of ROS formation, which ultimately results in the accumulation of lipid-based ROS, especially in the form of lipid hydroperoxides (Yang and Stockwell 2016). Lipid-based ROS are more than a downstream target of increased ROS formation—they are a critical mechanism and a distinguishing and defining component of ferroptosis (Latunde-Dada 2017). Phosphatidylethanolamines were a primary suspect among possible lipid peroxides that could contribute to ferroptosis, as oxidized PEs have been observed in heart disease for decades, as ischemia disrupts the arrangement of PEs in the sarcolemma of cardiomyocytes (Post et al. 1995). Furthermore, culturing cardiomyocytes in the presence of N,N-dimethylethanolamine, which affects the configuration of PEs, limits cell damage caused by ischemia and metabolic inhibition (Post et al. 1995). Oxidized PEs have been implicated as a lipid-based ROS critical for ferroptosis signaling (D'herde and Krysko 2017). Additionally, by-products of lipid peroxidation, such as MDA (malondialdehyde) and 4-HNE, are also used as consistent markers for detecting ferroptosis (Feng et al. 2020).

It was previously thought that organelles could be the primary sites of ferroptosis, but the plasma membrane has resurfaced in importance regarding the accumulation of lipid-based ROS in subcellular compartments (Bersuker et al. 2019; Doll et al. 2019; Hirata et al. 2023; Riegman et al. 2020). Interest in the plasma membrane was boosted by the discovery of the FSP1/CoQ_{10} axis, which acts at the plasma membrane instead of organelles (Bersuker et al. 2019; Doll et al. 2019; Hirata et al. 2023). Ferroptosis has shown the ability to spread between cells in a wave-like pattern (Linkermann et al. 2014a; Riegman et al. 2020) which led to investigations into the potential involvement of the plasma membrane in the propagation of ferroptotic cell death. One study revealed that iron and lipid peroxides are essential for propagation of ferroptotic cell death, and that ferroptotic lipid peroxidation compromises the

structure of the plasma membrane (Riegman et al. 2020). The altered structure of the ferroptotic plasma membrane leads to pore formation, which contributes to osmotic swelling and rounding of cells undergoing ferroptosis (Hirata et al. 2023, Riegman et al. 2020). The increased cell volume caused by osmotic swelling results in increased membrane tension and activation of mechanosensitive cation channels (Hirata et al. 2023). Cation channels appear to play a role in ferroptosis, as increased Ca^{2+} influx has been observed before ferroptotic cell rupture. Additionally, treatment with ruthenium red, a broad cation channel blocker, results in decreased LDH release from cells treated with RSL3 and other inducers of ferroptosis (Hirata et al. 2023). Piezo1 is a mechanosensitive nonselective cation channel that opens in response to membrane distortion and appears to facilitate ferroptosis as evidenced by Piezo knockout and knockdown cell lines being significantly more resistant to RSL3-induced ferroptosis (Hirata et al. 2023). Loss of cation concentration gradients and equilibration of cytosolic cations with extracellular fluid are also observed in ferroptosis (Hirata et al. 2023), which would render cardiomyocytes unable to conduct electrical impulses. Cellular swelling, compromised membrane integrity, and the collapse of concentration gradients caused by ferroptosis eventually result in cell rupture and death (Hirata et al. 2023; Riegman et al. 2020).

Lipid-based ROS can also propagate to the membranes of other intracellular compartments (Torii et al. 2016). Mitochondria were an initial target of studies investigating the effects of ferroptosis on organelles because of how excess iron accumulation in mitochondria causes lipid peroxidation of mitochondrial membranes (Fang et al. 2019). Lysosomes were studied due to their vital role in autophagy and release of iron from ferritin stores (Gao et al. 2016). The endoplasmic reticulum (ER) was also studied due to it being a central site of lipid synthesis and containing the majority of the total lipid bilayers in a cell (Gaschler et al. 2018). An imaging study in MCF7 breast cancer cells used an iridium-based transition metal complex that accumulates in the ER and showed increased ER viscosity in response to ferroptosis (Hao et al. 2021). Another study of the subcellular localization of ferrostatin-1 revealed that ferrostatin-1 suppressed lipid peroxidation localized at the ER (Gaschler et al. 2018).

Polyunsaturated fatty acids (PUFA) have been studied in the context of lipid peroxidation and oxidative stress due to their roles as essential membrane components and free radical scavengers (Hulbert et al. 2014). However, the radical-scavenging properties of PUFAs also make them a target for oxidation (D'herde and Krysko 2017). This has led to studies investigating enzymes involved in lipid metabolism and biosynthesis of PUFAs. Studies of ACSL4, an enzyme involved in PUFA metabolism, revealed that knocking out ACSL4 increased cell resistance to ferroptosis in breast cancer cell lines, and inhibition of ACSL4 in intestinal I/R mouse models also inhibited ferroptosis (Doll et al. 2017; Li et al. 2019b) (Fig. 8.2). PUFAs are substrates for ALOX15, a lipoxygenase that catalyzes the formation of lipid peroxide radicals and signaling molecules. Increased ALOX15 activity is associated with increased lipid peroxidation and sensitivity to ferroptosis (Ma et al. 2022). A study in cancer cells demonstrated that enrichment of PUFAs predisposes cells to ferroptotic death by GPX4 inhibition (Beatty et al. 2021).

Another study showed that membrane complexes consisting of PEBP1 and 15-lipoxygenases triggered the formation of PUFA-based ROS in disease models of asthma, kidney failure, and brain trauma (Wenzel et al. 2017).

Although it might seem like PUFAs are complicit in ferroptosis, they are also vital components of cellular membranes, and PUFA deficiency results in adverse outcomes in multiple diseases (Calon et al. 2005; Pachikian et al. 2011). Higher PUFA intake also reduces the risk of some cardiovascular diseases, including nonfatal MI and sudden death due to coronary artery disease (Bucher et al. 2002). Therefore, the role of PUFAs in normal cardiac function and cardiac ferroptosis might be similar to that of iron: both iron and lipid-based ROS are necessary for ferroptosis, but a deficiency in either iron or PUFA results in other adverse cardiac outcomes (Bucher et al. 2002; Von Haehling et al. 2015). One hypothesis behind the relationship between ferroptosis, PUFA, and lipid-based ROS states that ferroptosis might be a consequence of cells incorporating PUFAs into membranes during the evolution of the cell (Stockwell et al. 2017).

In contrast to PUFAs, monounsaturated fatty acids (MUFAs) appear to have an anti-ferroptotic effect. MUFAs were able to suppress erastin-induced ferroptosis by cotreatment with either oleic acid or palmitoleic acid in cell culture models (Magtanong et al. 2019), although this result was not obtained from a cardiac cell line. The mechanism behind how MUFAs suppress ferroptosis was shown to be independent of chelation, changes in GPX4 activity, or direct free radical scavenging in cell-free assays (Magtanong et al. 2019). Therefore, MUFAs may act in parallel or downstream of GSH/GPX4 activity. Nevertheless, MUFAs block membrane lipid ROS accumulation as detected by fluorescent markers, and this activity seems to be preferentially targeted to the plasma membrane (Magtanong et al. 2019). Treatment with exogenous MUFAs also decreases PUFA incorporation into phospholipids, further offsetting the potential sensitizing effects of PUFAs toward ferroptosis (Magtanong et al. 2019). The anti-ferroptotic effects of MUFAs are dependent on the presence of acyl-coenzyme A synthetase long-chain family member 3 (ACSL3), as exogenous oleic acid treatment was rendered less effective in preventing ferroptosis in ACSL3 loss-of-function cell culture models as compared to wild-type cell culture lines (Magtanong et al. 2019).

Other lipids and enzymes involved in lipid metabolism have been found to affect ferroptosis. Alpha-eleostearic acid was found to enhance cell death in a GSH/GPX4-dependent manner when cotreated with inhibitors of glutathione synthesis (Beatty et al. 2021). The ferroptotic effects of alpha-eleostearic acid are dependent on ACSL1, another acyl-CoA synthetase long-chain family member that forms peroxides from alpha-eleostearic acid (Beatty et al. 2021).

8.4 Pathological Features of Ferroptosis in the Heart

Ferroptosis has emerged as an increasingly important factor in I/R injury of the heart. A study estimated that ~40% of cell death can be attributed to ferroptosis in cardiac I/R models (Miyamoto et al. 2022). This estimate is supported by a study in rat I/R

models that used pharmacological agents that inhibit apoptosis, necroptosis, and ferroptosis. The study concluded that apoptosis and ferroptosis are the primary types of regulated cell death in cardiac I/R injury (Luo et al. 2022). However, ferroptosis can still occur in the heart even if apoptosis is suppressed (Fang et al. 2019). Several cardiac pathologies can lead to excess iron deposition and accumulation, increased ROS production, and pathological alterations in membrane lipids, which are all vital elements of ferroptosis (Fig. 8.1).

8.4.1 Ferroptosis in Ischemia-Reperfusion (I/R) Injury

An increasing number of studies have observed ferroptosis during cardiac I/R injury. I/R injury results from temporary deprivation of blood, oxygen, and nutrients to the myocardium during ischemia, and metabolic changes in response to restoration of blood flow to the infarcted area during reperfusion (Murphy and Steenbergen 2008). I/R injury frequently occurs as a consequence of coronary artery disease and MIs and subsequent intervention (Asanuma et al. 1997). ROS production is characteristic of I/R injury and increases in the myocardium during the ischemic phase (Cadenas 2018; Zweier et al. 1987). Although reperfusion restores oxygen and nutrients to infarcted tissue, reperfusion causes yet another increase in ROS production (Zweier et al. 1987). The one-two combination of ROS production during both phases of I/R leads to an acute and marked overproduction of ROS (Zweier et al. 1987). I/R also causes mitochondria to produce more ROS, which results from altered mitochondrial metabolism during hypoxia and ischemia, and the sudden influx of oxygen upon reperfusion (Zweier et al. 1987).

I/R injury shares multiple features in common with ferroptosis, including release of labile free iron, increased ROS production, lipid peroxidation, and ER stress. I/R causes an increase in free hemoglobin and heme concentrations during open heart surgery (Das et al. 1992). The release of heme also leads to the release of free iron and increased ROS production from the labile iron pool (Das et al. 1992). HO-1 catalyzes the release of Fe^{2+} from heme, and an increasing number of studies are linking HO-1 to ferroptosis, making it a significant factor in I/R-induced ferroptosis. HO-1 is upregulated in response to H/R in cultured cardiomyocytes and in I/R injury in rat hearts leading to iron overload in the endoplasmic reticulum (Miyamoto et al. 2022). GPX4 is also affected by I/R, and I/R injury in mouse hearts decreases GPX4 expression throughout the myocardium (Miyamoto et al. 2022). Therefore, I/R injury not only generates ROS, but it increases redox-active iron and decreases endogenous cellular defenses against oxidative stress.

I/R-induced ROS production can lead to the formation of lipid peroxides (Fig. 8.1). The role of lipid peroxides in I/R injury is supported by clinical evidence showing that serum lipid peroxide concentrations are higher in patients with coronary heart disease compared to peers without heart disease (Kesavulu et al. 2001). Lipid peroxidation may occur during the ischemic phase of I/R injury, as suggested by a study showing that lipid peroxides and ferroptotic markers increased during the hypoxic phase in H/R treatment of H9c2 cells (Ma et al. 2022). A variety of lipid

peroxide types have been identified as contributing to ferroptosis. Surgical I/R injury models in rats have shown increased oxidized phospholipid (OxPL) and mitochondrial oxidized adrenoyl-phosphatidylethanolamine levels that result in ferroptotic cardiomyocyte death (Jang et al. 2021; Yeang et al. 2019). I/R also affects levels of enzymes involved in lipid metabolism, such as ACSL4, which increases during I/R and increases sensitivity to ferroptosis in *in vitro* H/R and *in vivo* I/R intestinal models (Li et al. 2019b), although this has yet to be directly demonstrated in the heart. Ischemic and hypoxic cardiomyocyte damage is also associated with increased arachidonic acid 15-lipoxygenase-1 (ALOX15) expression (Ma et al. 2022). ALOX15 catalyzes the peroxidation of PUFAs and overexpression of ALOX15 increases cell death due to hypoxia (Ma et al. 2022). PUFAs have been celebrated for their antioxidant properties and seeming cardiovascular benefits (Oppedisano et al. 2020), but if combined with increased ALOX15 activity, PUFAs become increasingly oxidized and increase susceptibility to ferroptosis (Ma et al. 2022). ALOX15 knockout mice fed with a PUFA-enriched diet showed increased resistance to I/R injury and better cardiac function recovery than wild-type mice fed with the same PUFA-rich diet (Ma et al. 2022).

I/R also induces pathological changes in organelle function. I/R causes derangements in mitochondrial function and compromises mitochondrial membrane integrity, which can lead to ferroptosis (Jang et al. 2021; Meerson et al. 1982; Miyamoto et al. 2022; Paradies et al. 1999). When H9c2 cells are treated with inhibitors of electron transport chain complexes I and III or ATP synthase, the addition of RSL3 causes a marked increase in cell death (Jang et al. 2021). The mitochondria do not produce GSH, therefore they must source and transport it from elsewhere. Treatment with RSL3 or direct inhibition of the mitochondrial carrier proteins SLC25A10 (also DIC) and SLC25A11 (also OGC) results in decreased cellular and mitochondrial GSH levels and increased ROS production and ferroptosis (Jang et al. 2021). Treatment with RSL3 also inactivates mitochondrial GPX4, depletes mitochondrial GSH reserves, and increases mitochondrial ROS in H9c2 cells, which lead to ferroptosis and loss of membrane integrity and membrane potential (Jang et al. 2021).

ER stress is also a characteristic of I/R injury (Zhang et al. 2009). In the ER, oxygen accepts electrons from disulfide bonds formed during protein folding; however, disulfide bond formation also generates ROS (Chakravarthi et al. 2006; Malhotra et al. 2008; Zeeshan et al. 2016). Reduced glutathione (GSH)/oxidized glutathione (GSSG) also assists with oxidation during disulfide bond formation and with redox balance to prevent excess ROS formation in the ER (Chakravarthi et al. 2006; Malhotra et al. 2008). However, hypoxia experienced during ischemia inhibits disulfide bond formation, causing misfolding of proteins and ER stress (Rozpedek et al. 2016). ER stress further disrupts the redox balance within the ER and induces the formation of more added ROS (Zeeshan et al. 2016). The overproduction of ROS leads to ER lipid peroxidation and ferroptosis (Gaschler et al. 2018). HO-1 also contributes to ER lipid peroxidation, but does so by causing the accumulation of free iron in the ER in response to H/R instead of affecting protein folding (Miyamoto et al. 2022).

Ferroptosis-inducing drugs are known to cause ER stress, which further underscores the importance of the ER in ferroptosis (Lee et al. 2018). Ferroptosis-inducing drugs also cause activation of the PERK [protein kinase RNA (PKR)-like ER kinase]/eIF2α (eukaryotic initiation factor 2α)/ATF4 (activating transcription factor 4)/CHOP [C/EBP (CCAAT-enhancer-binding protein) homologous protein] signaling pathway (Lee et al. 2018). The PERK/eIF2α/ATF4/CHOP pathway is also notable in that it is involved in both ferroptosis and apoptosis (Lee et al. 2018). Although ferroptosis and apoptosis have distinct features and characteristics, e.g., iron-dependency in ferroptosis and caspase activation in apoptosis, common pathways like PERK/eIF2α/ATF4/CHOP could indicate possible cross-talk between ferroptosis and apoptosis, despite apoptosis not being necessary for ferroptosis (Fang et al. 2019; Lee et al. 2018).

Activating transcription factor 3 (ATF3) is a downstream target of ATF4 activity (Jiang et al. 2004) and has also been identified as a regulator of ferroptosis (Liu et al. 2022). ATF3 is a transient molecule that reaches its highest expression levels during early reperfusion and has shown protective effects against I/R injury (Liu et al. 2021). ATF3 overexpression inhibits ferroptosis caused by I/R injury and inhibits the effects of erastin and RSL3-induced ferroptosis in cell culture models. Conversely, ATF3 knockout decreases GPX4 protein expression and exacerbates the extent of I/R injury in murine models (Liu et al. 2022). ATF3 also binds to the promoter of Fanconi anemia complementation group D2 (FANCD2) and upregulates its expression. FANCD2 improved cell viability and inhibited ferroptosis in an H/R cell culture model using AC16 cells, thereby forming an anti-ferroptotic axis with ATF3 (Liu et al. 2022).

Ferroptosis may also involve ubiquitin pathways. Increased expression of ubiquitin-specific peptidase 22 (USP22), a deubiquitinase, was found to inhibit ferroptosis and limit myocardial I/R injury in rat models (Ma et al. 2020). The anti-ferroptotic mechanism of USP22 appears to be through a pathway involving sirtuin 1 (SIRT1), p53, and SLC7A11, the latter of which provides a bridge between USP22 and antioxidant activity (Ma et al. 2020). Ubiquitin-specific protease 7 (USP7) removes ubiquitin from p53, and a study that used H9c2 cells, rat models of I/R injury, and bioinformatic analysis revealed a pathway involving USP7, p53, and TfR1. The novel USP7/p53/TfR1 pathway connects ubiquitination with I/R injury, iron metabolism, and ferroptosis (Tang et al. 2021).

Several studies have also investigated the effects of cardiac ferroptosis on other tissues and cells. Inflammation has a well-established role in I/R injury and consequent ventricular remodeling, and studies are increasingly finding connections linking inflammation to cardiac ferroptosis. Cells subjected to I/R injury and ferroptosis release pro-inflammatory molecules such as damage-associated molecular patterns (DAMPs), which is thought to be due to compromised membrane integrity caused by ferroptotic membrane lipid peroxidation (Linkermann et al. 2014b). By-products of lipid peroxidation such as 4-HNE have also shown pro-inflammatory effects via Toll-like receptor 4 (TLR4). Cardiac I/R injury also activates Toll-like receptor adaptor molecule 1 (TICAM1) and TLR4 signaling in endothelial cells, which release pro-inflammatory molecules such as type

1 interferons (IFN) that attract neutrophils. Murine models of heart transplantation showed increased TLR4 signaling, neutrophil recruitment, and inflammation initiated by ferroptotic cell death, further linking cardiac damage with inflammation and ferroptosis (Li et al. 2019a). ELAVL1 (embryonic lethal-abnormal vision like protein 1) is an RNA-binding protein that regulates gene expression and has been linked to cardiac inflammation and ferroptosis. Increased ELAVL1 expression has also been observed in diabetic human hearts and surgical I/R mouse models. Conversely, ELAVL1 knockdown of ELAVL1 inhibits cardiac pyroptosis and ferroptosis. ELAVL1 also stabilizes and increases the expression of Beclin-1, which regulates autophagy associated with myocardial I/R injury (Chen et al. 2021).

8.4.2 Ferroptosis in Cardiomyopathy

8.4.2.1 Iron Overload and LV Remodeling

After an MI, the heart is left to deal with the aftermath of the myocardial damage and resulting impaired cardiac function. An MI can permanently damage coronary vessels in an infarcted region, which can cause intramyocardial hemorrhage upon reperfusion to the infarcted area (Das et al. 1992). Blood flow to damaged vessels could lead to hemorrhaging of blood components, including red blood cells, out of injured blood vessels and into the myocardium of the infarcted area (Betgem et al. 2015). This extravasation of red blood cells can lead to a subsequent release of hemoglobin, heme, and free iron. MRI T2 and T2* imaging provided further evidence of this phenomenon, as those imaging techniques can measure and quantify iron in cardiac tissue after MIs (Wood and Ghugre 2008). Studies that employed T2 and T2* imaging to study iron deposition in the myocardium also provided further evidence against the serum ferritin hypothesis and showed more localized and focal post-MI iron deposition in the myocardium rather than in the entire bloodstream (Kolnagou et al. 2006, Wood and Ghugre 2008). Increased iron deposition was observed in infarcted myocardium as detected by histological staining, T2*-weighted imaging and mapping, and quantitative susceptibility mapping (QSM) detection of magnetic susceptibility in a swine I/R model (Moon et al. 2020). QSM also detected elevated magnetic susceptibility in patients who experienced reperfusion after a hemorrhagic myocardial infarction, although the result should be considered preliminary since it is based on a small sample size (Moon et al. 2020). However, these studies are valuable, as detection of myocardial hemorrhage and iron deposition after I/R injury in human hearts is a very important first step toward targeting and managing ferroptosis in patients experiencing adverse cardiac events.

Intramyocardial hemorrhage is also associated with adverse LV remodeling, further cardiac events, and sudden death (Betgem et al. 2015). LV remodeling refers to histological and physiological changes in the myocardium that typically occur after a MI (Gajarsa and Kloner 2011; Sutton and Sharpe 2000). The changes that occur during LV remodeling attempt to adapt and compensate for losses in cardiac function from myocardial damage sustained during and after the MI (Sutton and Sharpe 2000). However, this compensation is not perfect, and the heart never reaches

the same level of efficiency it had before the MI (Sutton and Sharpe 2000). As a result, LV remodeling eventually leads to the future development of heart failure (Gajarsa and Kloner 2011; Konstam et al. 2011; Sutton and Sharpe 2000). One common link between LV remodeling and ferroptosis is the importance of ROS and redox balance. Treatment with dimethylthiourea, an antioxidant, prevented LV remodeling and subsequent heart failure in mice (Kinugawa et al. 2000). Another group obtained similar results in a genetic model by overexpressing glutathione peroxidase, which also prevented LV remodeling and failure (Shiomi et al. 2004).

Iron overload is strongly linked with cardiomyopathy and provides a foundation for ferroptosis through cardiac iron deposition (Gujja et al. 2010). As mentioned earlier in this chapter, patients who suffer from blood disorders such as thalassemia receive periodic transfusions, which can result in iron overload in the serum (Taher et al. 2013; Wood et al. 2004). However, this iron overload can also lead to cardiomyopathy caused by the accumulation of iron in cardiomyocytes (Berdoukas et al. 2015). A study of L-type Ca^{2+} channels provided a mechanism for this accumulation and showed that L-type Ca^{2+} channels are the dominant mechanism of iron entry into cardiomyocytes during iron-overload cardiomyopathy (Oudit et al. 2003).

However, iron overload cardiomyopathy is not solely caused by transfusions and can be idiopathic (Gujja et al. 2010). Furthermore, iron overload cardiomyopathy is often asymptomatic in its early stages (Gujja et al. 2010). Apelin and its APJ receptor have been implicated as a pathway that links several features of iron overload and cardiomyopathy. Apelin and APJ receptor are important regulators of embryonic cardiomyocyte development (Scott et al. 2007). However, apelin is also a marker of cardiovascular disease, and increased apelin concentrations are found in blood and cardiac tissue of patients with comorbid cardiomyopathy and heart failure (Berry et al. 2004; Foldes et al. 2003; Miettinen et al. 2007). Another study showed that apelin increases sideroflexin1 (SFXN-1)-mediated iron overload, ferritinophagy, and cardiac hypertrophy. If iron-overload resulting from apelin signaling can also induce ferroptosis, apelin and APJ receptor could be an important ferroptotic mechanism and target for preventing ferroptosis in the heart (Tang et al. 2019).

Ferritinophagy has been the subject of multiple studies due to its ability to affect iron concentrations in the labile iron pool. NCOA4 has emerged as a vital regulatory component of ferritinophagy in the heart. A study subjected NCOA4-deficient mice to transverse aortic constriction (TAC) pressure overload and observed attenuated cardiac remodeling and decreased formation of MDA and 4-HNE lipid peroxide by-products in NCOA4-deficient mice as compared to counterparts with intact NCOA4 genes (Ito et al. 2021). NCOA4-deficient hearts also showed altered iron metabolism, as hearts with normally functioning NCOA4 showed greater levels of ferrous iron (Fe^{2+}) in response to TAC pressure overload, whereas NCOA4-deficient hearts showed greater levels of ferric iron (Fe^{3+}) and lower levels of ferrous iron to ferritin heavy chain 1 (FTH1) (Ito et al. 2021).

BTB and CNC homology 1 (BACH1) is another transcription factor that binds to heme and regulates iron and heme metabolism by downregulating ferritin proteins FTH1 and ferritin light chain 1 (FTL1) and HO-1 (Nishizawa et al. 2020). BACH1

also affects the synthesis of glutathione by downregulating several proteins in the GSH/GPX4 antioxidant pathway such as glutamate-cysteine ligase modifier subunit (Gclm), glutamate-cysteine ligase catalytic subunit (Gclc), and solute carrier family 7 member 11 (SLC40A11). Generally, most of the genes repressed by BACH1 have some anti-ferroptotic activity (Nishizawa et al. 2020). These anti-ferroptotic genes were upregulated in mouse embryonic fibroblasts (MEFs) treated with erastin, suggesting that these genes may form a coordinated compensatory antioxidant mechanism in response to ferroptotic activity. BACH1 has also been shown to promote ferroptosis by blunting the antioxidant response and increasing mitochondrial labile iron. The pro-ferroptotic activity of BACH1 is further supported by MI I/R models using BACH1 knockout mice, which show smaller infarct sizes, better cardiac recovery, and better survival than wild-type mice subjected to the same I/R procedure. (Nishizawa et al. 2020)

Cardiomyopathy can result in oxidative stress due to increased ROS production (Berdoukas et al. 2015). In addition to redox stress caused by ROS, iron-catalyzed ROS production also damages mtDNA and decreases mitochondrial respiration in cardiomyocytes, which further exacerbates the severity of cardiomyopathy (Gao et al. 2010). However, it must be noted that although oxidative stress is linked and often seen in cardiomyopathy, there are mechanisms unrelated to oxidative stress that can still cause cardiomyopathy (Gammella et al. 2015).

8.4.3 Diabetic Cardiomyopathy

In diabetic cardiomyopathy, the heart experiences additional oxidative stress due to diabetic metabolic derangements (Cai and Kang 2001). Lipid peroxidation also occurs in diabetic cardiomyopathy (Garcia-Bunuel 1984), and one study implicates ferroptosis as being essential for diabetic cardiomyopathy (Wang et al. 2022a). Pathways involving AMP-activated protein kinase (AMPK) have been investigated as a possible mechanism linking diabetic cardiomyopathy with ferroptosis. AMPK increases glucose uptake and lipid oxidation in nutrient-deficient conditions (Lin and Hardie 2018). Increased AMPK activity in nutrient-poor conditions was associated with decreased sensitivity to ferroptosis murine models of renal I/R injury in mice (Lee et al. 2020). Alterations in AMPK activity also change the biosynthesis of PUFAs, as AMPK deletion increases the production of PEs (Lee et al. 2020). The effects of AMPK on ferroptosis also occur outside of the mitochondria, further suggesting that mitochondria-independent ferroptotic mechanisms may exist, although this has yet to be confirmed in cardiac tissue (Lee et al. 2020). AMPK activity also increases Nrf2 (Nuclear factor erythroid 2-related factor 2) activation via an AMPK/Akt/GSK3β/Nrf2 pathway, thus linking AMPK to cellular antioxidant responses and defenses against oxidative stress (Sun et al. 2020; Wang et al. 2022a).

8.4.4 Chemical-Induced cardiomyopathy

Cardiomyopathy can also be caused by cytotoxic molecules. Doxorubicin (DOX) is a chemotherapeutic agent that belongs to the anthracycline class of anti-neoplastic drugs (Thorn et al. 2011). DOX exerts its cytotoxic effects by binding to DNA and inducing cell death (Thorn et al. 2011). However, the cytotoxicity of DOX leads to its well-documented cardiotoxicity along with resultant cardiomyopathy (Chatterjee et al. 2010). Doxorubicin triggers iron-mediated ROS production, mitochondrial dysfunction, and dysregulation of sarcoplasmic reticulum Ca^{2+} flux, all of which contribute to its cardiotoxic effects (Thorn et al. 2011). However, given the effectiveness of doxorubicin in chemotherapy, its use will probably continue despite its cardiotoxic effects, and recent studies are focusing on other DOX delivery methods that result in less toxicity (Tacar et al. 2013).

The first study that revealed a strong link between doxorubicin and ferroptosis investigated the development of doxorubicin-induced cardiomyopathy in mice that were defective in apoptotic or necroptotic mechanisms (Fang et al. 2019). The necroptosis-defective mice showed cardiac ferroptotic cell death in response to doxorubicin treatment (Fang et al. 2019). Ferrostatin-1 also successfully improved the survival of control and receptor interacting protein kinase 3 (Ripk3) knockout mice after treatment with doxorubicin (Fang et al. 2019). Dexrazoxane (DXZ), an iron chelator, was also used in that study to alleviate the effects and symptoms of doxorubicin treatment (Fang et al. 2019). This landmark study was particularly impactful in cardiac ferroptosis research, as it integrated cell death, labile iron, redox balance, and multiple mechanisms of ferroptosis specifically in the context of cardiac tissue, instead of cancer or other organs. Most importantly, the study showed that ferroptosis and doxorubicin-induced cardiomyopathy did not involve necroptosis, and that ferroptosis was not dependent on apoptosis (Fang et al. 2019). This was supported by a study that used zVAD-FMK (zVAD), an apoptosis inhibitor, and showed that zVAD-FMK was unable to reduce mitochondrial lipid peroxidation caused by doxorubicin (Tadokoro et al. 2020).

Further studies confirmed that ferroptosis is the major form of regulated cell death in cardiomyocytes after doxorubicin treatment (Tadokoro et al. 2020). A study implicated mitochondria as being a central part of doxorubicin-induced ferroptosis in the heart and demonstrated increased iron accumulation and lipid peroxide formation in the mitochondria, but not in the cytosol nor other organelles (Tadokoro et al. 2020). Doxorubicin is also a chelating agent for iron, and the same study used chelators specific for ferrous and ferric ions to determine whether one species of iron ion plays a larger role in ferroptosis. The authors concluded that doxorubicin-ferric iron complexes and free ferrous iron are the major reactants in Fenton reactions caused by doxorubicin treatment (Tadokoro et al. 2020).

Studies further investigating the effects of DXZ in remediating the cardiotoxic effects of DOX revealed that high mobility group box 1 (HMGB1) may be a common link between the effects of DOX and DXZ. DOX induces expression of HMGB1, and overexpression of HMGB1 increased cell susceptibility to ferroptosis (Zhang et al. 2021). Conversely, co-treatment of DOX with either DXZ or Fer-1

resulted in decreased HGMB1 activity compared to DOX alone, as well as increased cell viability and decreased production of lipid peroxidation by-products (Zhang et al. 2021).

Other pathways have been associated with DOX-induced cardiomyopathy. MITOL/MARCH5 is an E3 ubiquitin ligase localized to the outer mitochondrial membrane, and decreased MITOL expression results in decreased GPX4 and induces ferroptosis in rat ventricular cardiomyocytes (Kitakata et al. 2021). MITOL knockdown increased cultured cardiomyocyte susceptibility to DOX-induced ferroptosis, and in turn, DOX decreases MITOL in a dose-dependent manner (Kitakata et al. 2021). MITOL also has crosstalk with other pathways involved in ferroptosis, as siRNA knockdown of MITOL in rat ventricular cardiomyocytes increased protein expression in the eIF2α/ATF4 pathway (Kitakata et al. 2021).

DOX also increases cardiomyocyte expression of methyltransferase-like 14 (METTL14), an m6A modification enzyme that targets KCNQ1OT1, a noncoding RNA that acts as a miRNA sponge (Zhuang et al. 2021). Increased expression of METTL14 and KCNQ1OT1 led to increased expression of Tfr1, iron accumulation, and ferroptosis (Zhuang et al. 2021). Silencing of METTL14 and knockdown of KCNQ1OT1 restored levels of several miRNA species, decreased intracellular iron in DOX-treated cells, and suppressed ferroptosis (Zhuang et al. 2021). This study could be the first glimpse of the possible involvement of miRNAs in cardiac ferroptosis.

Although the majority of chemical-induced ferroptosis studies have concentrated on DOX as of this writing, a few groups have investigated the ferroptotic effects of other molecules involved in cardiac diseases. One study used lipopolysaccharides (LPSs), a common component of Gram-negative bacteria, to investigate the possible role of ferroptosis in sepsis-induced cardiomyopathy. In vitro cell culture treatment with LPS and intraperitoneal injections of LPS into mice increased the expression of islet cell autoantigen 69 (ICA69), a molecule with potential roles in inflammation and immune function (Kong et al. 2022). This correlates with the detection of increased ICA69 in peripheral blood mononuclear cells (PBMCs) collected from patients with sepsis that were hospitalized in intensive care units, and ICA69 levels were correlated with the severity of sepsis (Kong et al. 2022). Compared with their wild-type counterparts, ICA69 knockout mice treated with LPS showed decreased levels of pro-inflammatory cytokines, increased GPX4 expression, and decreased markers of ferroptosis such as MDA and PTGS2 (Kong et al. 2022). Thus, ICA69 could be a potential in vivo biomarker of ferroptosis that could be measured in a blood sample.

8.5 Potential Therapies Targeting Ferroptosis in Cardiovascular Disease

8.5.1 Regulation of Iron in the Labile Iron Pool

The labile iron pool is a dynamic source of intracellular iron that catalyzes ROS formation and potentially triggers consequent ferroptosis. One potential strategy that can limit the extent of ferroptotic cell death is to decrease the amount of iron in the labile iron pool by further increasing the activity of mechanisms that remove or sequester iron from the labile iron pool and prevent it from participating in redox reactions and ROS production.

8.5.2 Iron Storage and Transport

The labile iron pool can be decreased by keeping iron stored within ferritin. BACH1 increases ferroptosis by suppressing ferritin and ferroportin gene expression, and BACH1 knockout mice showed better resistance in response to increased labile iron pool levels (Nishizawa et al. 2020). HPPE is a substituted benzimidazole small molecule inhibitor of BACH1 that has neuroprotective effects in mouse models of Parkinson's disease, but could also potentially be used to inhibit ferroptosis in the heart as ferroptosis is also part of the pathophysiology of Parkinson's disease (Ahuja et al. 2021; Wang et al. 2022d).

Heme is another potential source of iron for the labile iron pool (Fig. 8.2). When heme is catabolized by HO-1, the porphyrin ring structure releases its central iron atom (Ryter and Tyrrell 2000). Zinc protophorphyrin IX (ZPP-IX), an HO-1 antagonist, has been used to protect against doxorubicin-induced ferroptosis and cell death in animal models (Fang et al. 2019). Therefore, HO-1 inhibitors could prevent the release of free iron from heme into the labile iron pool.

Inhibition of ferritinophagy is another potential strategy to decrease labile iron by preventing the release of iron from ferritin. This strategy was successful in studies that used lysosomal and autophagic inhibitors to decrease ROS production and ferroptosis (Gao et al. 2016; Torii et al. 2016). NCOA4 plays an important role in ferritinophagy in the heart, and decreased expression of NCOA4 was correlated to reduced levels of iron overload, reduced lipid oxidation, and attenuation of cardiac remodeling in response to TAC pressure overload. One group has developed a compound that inhibits NCOA4, ferritinophagy, and ferroptosis (Fang et al. 2021). The compound reduced cerebral ischemic injury caused by transient cerebral artery occlusion, therefore NCOA4 inhibitors could be another class of anti-ferroptotic drugs that target ferritinophagy (Ito et al. 2021).

Ferroportin can also decrease iron in the labile iron pool by exporting iron from the cell (Gardenghi et al. 2007; Lakhal-Littleton et al. 2015) (Fig. 8.2). Overexpression of ferroportin decreases ROS production and inhibits cell death under ferroptotic conditions (Ma et al. 2016). Therefore, increasing ferroportin activity may be a way to inhibit ferroptosis by reducing available iron in the labile

iron pool. A study using cardiac-specific ferroportin knockout mice demonstrated that deletion of ferroportin increases accumulation of iron in cardiomyocytes and cardiac ferroportin is necessary for preserving cardiac function (Lakhal-Littleton et al. 2015). Additionally, hepcidin is an endogenous inhibitor of ferroportin that causes internalization and degradation of ferroportin (Hentze et al. 2010). Although the mechanisms of the hepcidin-ferroportin axis on iron homeostasis in the heart are complex (Lakhal-Littleton et al. 2015), controlling ferroportin could be an effective strategy for inhibiting ferroptosis in cardiac disease. Dimethyl fumarate (DMF) is a Nrf2 activator and hepcidin inhibitor that also upregulates ferroportin and ferritin expression (Belcher et al. 2017), therefore DMF and other hepcidin inhibitors may be a new potential class of anti-ferroptotic molecules that work by increasing ferroportin activity.

Inhibition of iron import by inhibiting transferrin and transferrin receptors could be another strategy to inhibit ferroptosis by limiting iron transport and uptake into cells, thus preventing more iron from being added into the labile iron pool. A study employing this strategy showed that transferrin knockdown reduced cell death, ROS, and ferroptosis in several breast cancer cell lines (Ma et al. 2016). P22077 is a USP7 inhibitor that decreases TfR1 protein expression via a USP7/p53/TfR1 pathway. P22077 treatment was able to decrease Tfr1 expression, increase GPX activity, decrease lipid peroxide formation, and inhibit ferroptosis in H9c2 cells subjected to H/R (Tang et al. 2021). siRNA knockdown of Tfr1 achieved similar anti-ferroptotic effects (Tang et al. 2021). miRNA pathways also affect the expression of Tfr1 in cardiomyocytes, and miR-7a-5p levels were inversely correlated with Tfr1 expression and sensitivity to ferroptosis (Zhuang et al. 2021). One group discovered a compound derived from a marine natural product that inhibits Tfr1 and caused iron deprivation in breast cancer cells (Wu et al. 2022), and could be also used to prevent ferroptosis. Therefore, small molecule, siRNA, and miRNA-based therapeutics that downregulate or inhibit Tfr1 could be explored for possible anti-ferroptotic therapies.

8.5.3 Iron Chelation

The solubility of iron allows it to be transported in the fluids of the body, but it also means that iron can also be chelated (Kakhlon and Cabantchik 2002). Chelation traps and sequesters iron, thus effectively removing it from the labile iron pool and preventing it from participating in redox reactions and ROS generation (Gammella et al. 2016). The use of chelators in the prevention of ferroptosis is an active area of research as many chelating compounds are approved for medical use in humans (Britton et al. 2002; Mobarra et al. 2016) (Fig. 8.2). Chelation therapy is already used to treat iron-overload diseases and prevent iron-overload-induced cardiomyopathy (Gujja et al. 2010).

Deferoxamine (DFO) is a chelator with decades of use in the treatment of iron overload in patients with hematological disorders (Pennell et al. 2006; Propper et al. 1977). Multiple studies show that DFO reduces cytosolic ROS production and

inhibits ferroptotic cell death (Kwon et al. 2015; Ooko et al. 2015). DFO also shows cardioprotective effects by inducing ischemic preconditioning-like effects and altering ROS production in I/R models (Dendorfer et al. 2005). DFO also protects against derangements in cardiac electrophysiology in a gerbil model of iron-overload cardiomyopathy (Obejero-Paz et al. 2003) and is successfully used in preventing iron-overload cardiomyopathy caused by hereditary hemochromatosis (Tauchenova et al. 2016). The relatively long history of DFO use in treating hematological disorders makes it an attractive potential treatment in the prevention of ferroptosis during acute myocardial events, although this has not been tested in clinical trials as of this writing.

Dexrazoxane (DXZ) is another chelator with notable clinical use in protecting the heart against the cardiotoxic effects of doxorubicin (Swain et al. 1997). DXZ prevents ferroptosis by maintaining mitochondrial ATP levels and inhibiting phospholipid oxidation (Koleini et al. 2019; Nagai et al. 2017). DXZ displays cardioprotective effects in I/R models by inhibiting ROS production, apoptosis, and promoting angiogenesis (Neckar et al. 2012). DXZ also shows cardioprotective effects in animal models of doxorubicin-induced cardiomyopathy by protecting myocardial mitochondria against the effects of doxorubicin (Fang et al. 2019; Hasinoff et al. 2003; Lebrecht et al. 2007). DXZ may also confer additional protective effects by inhibiting HMGB1, a protein that has been implicated in ferroptosis and doxorubicin-induced cardiomyopathy (Zhang et al. 2021).

A selected few iron chelators also can target organelles, such as chelators that cross into the mitochondria. Mitochondria-targeted chelators could potentially decrease the ability of excess iron to trigger ROS overproduction and mitochondrial dysfunction. 2,2'-bipyridyl (2BP) is a membrane-permeable metal chelator that can enter mitochondria and chelate mitochondrial iron (Dixon et al. 2012). Several studies have used 2BP to target and affect mitochondrial iron, with 2BP consistently showcasing an ability to inhibit ferroptosis (Dixon et al. 2012; Gao et al. 2015). In in vivo animal models of I/R injury, treatment with 2BP preserved cardiac function against I/R injury, whereas the DFO-treated group showed no significant protection (Chang et al. 2016). Further studies are required to see if the mitochondrial targeting of 2BP is related to the ferroptotic mechanisms involving the mitochondria that are described earlier in this chapter.

The endoplasmic reticulum is also a potential target for chelation, as FerroFarRed (FFR), a chelator that targets the ER, was able to improve cell survival in response to H/R (Miyamoto et al. 2022). The anti-ferroptotic effects shown by FFR and other organelle-specific chelators could lead to studies that investigate which organelles are linked to specific ferroptotic mechanisms, or a measurement of how much organelles contribute to ferroptosis.

8.5.4 ROS Inhibition

Research since the previous edition of this chapter has revealed multiple antioxidants and antioxidant systems involved in redox homeostasis and ferroptosis in the heart.

The discovery of new pathways has yielded many new possible strategies in controlling ROS and ferroptosis in the heart.

8.5.5 Glutathione (GSH) and Glutathione Peroxidase 4 (Gpx4) Activity

ROS generation is not limited to mitochondrial activity and ferroptosis. Other cardiac pathological conditions such as acute MI and I/R injury can also result in ROS overproduction (Cadenas 2018; Chouchani et al. 2016; Lim et al. 2009). Therefore, protective mechanisms are needed to maintain cardiac function and cardiomyocyte viability during these conditions. GPX4 is critical in preventing excess free radical generation and resultant cardiomyocyte death (Wortmann et al. 2013). GPX enzymes use GSH to donate electrons to reduce its substrates, therefore an adequate supply of glutathione is needed to maintain the antioxidant activity of GPX (Bellinger et al. 2009). Glutathione S-transferases are another important enzyme that conjugate GSH to substrates to remove them from the body (Ishikawa et al. 1986). Cardiac glutathione S-transferases catalyze the conjugation of GSH to 4-HNE, a lipid peroxide, and facilitate its removal from the heart (Ishikawa et al. 1986). GPX4 also modulates the levels of H_2O_2 in the matrix of myocardial mitochondria in rats (Antunes et al. 2002). Given all the conditions that could lead to oxidative stress, dysregulation of GSH production would result in the disruption of its protective antioxidant properties and cause oxidative stress and potential adverse cardiac outcomes (Antunes et al. 2002; Ishikawa and Sies 1984; Lim et al. 2009).

Mitochondria are a major site of ROS production that could contribute towards ferroptosis (Wu et al. 2018), and treatments targeting the mitochondria could blunt the contribution of mitochondria to ferroptotic cell death. Mitochondria-specific overexpression of GPX4 attenuates I/R injury and decreases lipid peroxidation (Dabkowski et al. 2008). XJB-5-131, a mitochondria-targeted ROS and electron scavenger, showed increased protection and recovery of cardiac function after I/R injury as compared to controls (Jang et al. 2021). Therefore, antioxidant small molecules that target the mitochondria like XJB-5-131 could be used to prevent ferroptosis.

Gene therapy targeting components of the GSH/GPX4 pathway could also be a new way of preventing ferroptosis. Adenoviral vectors were used to overexpress USP22 in the myocardium of rats subjected to myocardial I/R injury, and the increased USP22 increased GSH consumption through the SIRT1/p53/SLC7A11 axis and inhibited ferroptosis (Ma et al. 2020). Gene therapy that targets pathways that affect and enhance the expression and activity of GPX4 could also be explored. ATF3 has anti-ferroptotic effects and increasing its activity could restore GPX4 levels, as ATF3 reexpression using adenoviral vectors was able to restore GPX4 levels and maintain cardiac function in an ATF3 knockout mouse model (Liu et al. 2022).

8.5.6 Nrf2-Mediated Antioxidant Activity

Nrf2 is a well-studied transcription factor that regulates the expression of many genes related to cellular antioxidant defenses, including GPX4, GSH reductase, and SLC7A11 (Fig. 8.2). In its inactive state, Nrf2 is kept in check and inhibited by Kelch-like ECH-associated protein 1 (Keap1), which holds Nrf2 in the cytoplasm and targets it for ubiquitination and degradation. Oxidative stress releases Nrf2 from Keap1, which allows Nrf2 to translocate to the nucleus and bind to antioxidant response elements (AREs) located in the promoters of GPX4 and other genes that reduce oxidative stress and restore redox homeostasis.

Numerous studies have investigated the role of Nrf2 in the context of different cardiovascular diseases. Nrf2 protects cardiac cells against oxidative damage and cell death during ischemia-reperfusion injury (Howden 2013). In turn, both ischemic preconditioning and myocardial ischemic-reperfusion injury increase Nrf2 protein expression (Xu et al. 2014). Small molecules that upregulate Nrf2 and its downstream targets also protect against maladaptive cardiac remodeling and fibrosis (Xing et al. 2012). Decreases in Nrf2 activity have also been linked to increased sensitivity to ferroptosis. Nrf2 knockout models show that Nrf2 is necessary to protect against cardiac remodeling from pressure overload (Wang et al. 2015). Another study showed that overexpression of protein arginine methyltransferase 4 (PRMT4) inhibited Nrf2 activity and aggravated doxorubicin-induced cardiomyopathy (Wang et al. 2022b). Nrf2 also exhibits anti-inflammatory effects through its suppression of NF-κB activity, and treatment with the triterpenoid bardoxolone methyl (also called CDDO-Me) increased Nrf2 activity and improved post-MI cardiac recovery in rat models of chronic heart failure (Tian et al. 2019).

Several studies in the past few years have revealed a strong relationship between Nrf2, GPX4, and ferroptosis. Upregulation and overexpression of Nrf2 increase cellular resistance to ferroptosis in various cancer cell models (Fan et al. 2017; Shin et al. 2018). Nrf2 also binds to AREs in the promoters of several genes involved in GSH synthesis and GPX4 activity, such as SLC7A11, GSS, GCLC/GCLM, and GPX4 itself (Dodson et al. 2019). Lipid peroxides and by-products such as 4-HNE also activate the Keap1/Nrf2 pathway, thus activating cellular defenses against oxidative stress and ferroptosis (Cai et al. 2018; Gao et al. 2020). The well-established antioxidant activity of Nrf2 and its direct regulation of several proteins and enzymes in the GSH/GPX4 axis could lead to therapeutic options that combat the oxidative stresses of ferroptosis in cardiac disease.

Although there is increasing evidence that Nrf2 activity affects iron homeostasis by regulating genes that modulate the levels of free iron in the labile iron pool, the overall mechanism of how Nrf2 increases, decreases, or modulates free iron levels is not as clear. Nrf2 regulates genes involved in both heme synthesis and heme catabolism, which can remove or add iron to the labile iron pool. Nrf2 regulates heme synthesis by binding to AREs and increasing expression of ferrochelatase (FECH) (Kerins and Ooi 2018), which inserts ferrous ions into the protoporphyrin ring structure of heme, thus consuming free iron and removing it from the labile iron pool in the process. On the other hand, Nrf2 regulates heme catabolism by binding to

the promoter of HO-1 (Yang et al. 2022a), which catabolizes heme into biliverdin and releases free iron into the cytosol (Mu et al. 2021).

Despite its antioxidant activity, Nrf2 itself does not have a completely cardioprotective role. Granted, many studies show that increased Nrf2 activity protects against ferroptosis (Cui et al. 2021; La Rosa et al. 2021; Shin et al. 2018), but there are studies that suggest the opposite, as Nrf2 has been implicated in the progression and worsening of cardiac disease in certain contexts (Qin et al. 2016; Zang et al. 2020). In the setting of autophagy insufficiency, Nrf2 contributes to maladaptive cardiac remodeling (Qin et al. 2016). In a transgenic mouse model of mutant protein aggregation and reductive stress, disruption of Nrf2 signaling prevented reductive stress and preserved cardiac function (Kannan et al. 2013). Therefore, although Nrf2 protects against oxidative stress, the role of Nrf2 in cardiac disease is not a straightforward protective one and may change in the context of other cellular stresses. The Nrf2 target HO-1 further muddles the relationship between Nrf2 and ferroptosis, as HO-1 accelerates free iron release and erastin-induced ferroptosis (Kwon et al. 2015). More studies are needed to investigate the overall effect of Nrf2 on cellular iron stores, especially given the seemingly paradoxical role of HO-1.

Despite the lack of a clear pathway linking Nrf2 to the prevention or promotion of ferroptosis, there is a growing number of studies that successfully use methods of increasing Nrf2 activity to prevent ferroptosis and limit the extent of cardiac disease and injury. Investigating the effects of molecules that increase Nrf2 activity could yield new anti-ferroptotic drugs.

Natural products are a widely known source of antioxidants and have been investigated as potential anti-ferroptotic agents. Triterpenoid saponins have demonstrated increased Nrf2 activity and protection against doxorubicin-induced cardiomyopathy (Luo et al. 2021). Sulforaphane, a phytochemical found in cruciferous vegetables, activates an AMPK/Nrf2 pathway and protects against ferroptosis in murine high-fat diet models of diabetes (Wang et al. 2022a).

Two sedative agents have surprisingly shown potential anti-ferroptotic activity. A study using post-conditioning with etomidate (R-1-(1-ethylphenyl) imidazole-5-ethylester), a carboxylated imidazole sedative used in anesthesia, showed that etomidate increased Nrf2 levels and decreased ROS production in a rat model of I/R injury (Lv et al. 2021). Dexmedetomidine, an anxiolytic and sedative widely used in surgery, also showed increased Nrf2 activity and decreased sensitivity to ferroptosis in H9c2 cells and rat models of I/R injury (Wang et al. 2022c). The increased Nrf2 activity by dexmedetomidine also increased AMPK/GSK-3β, which lends more evidence for a AMPK pathway in ferroptosis (Lee et al. 2020; Wang et al. 2022c).

Although there are many studies investigating Nrf2 and ferroptosis, only a relatively small subset investigates this relationship in the heart. Despite this, the majority suggest a possible cardioprotective role of Nrf2 against ferroptosis. However, given the complicated relationship between Nrf2 and heart disease in general, Nrf2 and cardiac ferroptosis should be investigated in a variety of cardiac pathologies and models to determine whether Nrf2 has an overall cardioprotective

effect or Nrf2 acts in a context-specific manner influenced by other stresses in the heart.

8.5.7 GPX4-Independent Antioxidants

The revelation of FSP1 and its anti-ferroptotic mechanism has opened a new avenue for a potential whole new class of anti-ferroptotic drugs. Screening of existing compound libraries has already yielded a compound that inhibits FSP1 activity (Doll et al. 2019). Therefore, a similar screen of molecules that mimic the antioxidant properties of FSP1 or upregulate its expression could lead to the discovery of new anti-ferroptotic drugs. FSP1-like molecules or compounds that boost the expression or activity of FSP1 could be used to prevent ferroptosis in conditions with low GSH/GPX4 activity.

Several other compounds have shown GPX4-independent antioxidant and anti-ferroptotic activity. Tetrahydrobiopterin (BH4) is a metabolite of GTP and has shown antioxidant effects in cancer cells undergoing ferroptosis due to GPX4 inhibition (Soula et al. 2020). A decrease in BH4 alone did not increase lipid peroxidation levels, but when suppressed alongside GPX4, an increase in lipid ROS was observed. Treatment with BH2, the reduced form of BH4, was also able to restore cellular function in response to decreased BH4 and GPX4 activity. Since BH4 is derived from GTP, the anti-ferroptotic properties of BH4 make it a form of ferroptotic resistances that is not reliant on cysteine concentrations and can be the target of potential anti-ferroptotic therapeutics that do not rely on adequate levels of GPX4, GSH, or cysteine.

MitoTEMPO, a mitochondria-targeted antioxidant, also prevented DOX-induced ferroptosis and cardiomyopathy (Fang et al. 2019). DHODH is located at the mitochondrial inner membrane, and its activity is coupled to the reduction of CoQ to $CoQH_2$. DHODH inhibition increased the ratio of oxidized CoQ to $CoQH_2$ and increased susceptibility to ferroptosis, whereas supplementation of mito$CoQH_2$ (a mitochondria-targeted analog of $CoQH_2$) provided some protection against RSL3-mediated ferroptosis (Mao et al. 2021). Taken together, mitochondria-targeted antioxidants or enzymes could prevent ferroptosis in conditions where mitochondria are significant contributors to oxidative stress.

Vitamins and natural products have also shown anti-ferroptotic activity, such as baicalein, a natural product and antioxidant that also shows inhibition of lipid peroxidation and ferroptosis (Shintoku et al. 2017). Lipid-soluble antioxidant vitamins and vitamin precursors have also shown anti-ferroptotic activity, including alpha-tocopherol (vitamin E) and beta carotene (Yang and Stockwell 2016). When researchers deprived GPX4 knockout mice of dietary vitamin E, the reduction in antioxidant intake induced endothelial cell detachment and the formation of thrombi in multiple organs (Wortmann et al. 2013). However, the reintroduction of the antioxidant vitamin back into the diet of the mice rescued them from cardiovascular catastrophe. Vitamin E also prevents ferroptosis in GPX4 knockout mice, albeit in a hepatocellular context (Carlson et al. 2016). Trolox, a water-soluble antioxidant

analog of vitamin E, also inhibits lipid peroxidation and prevents ferroptotic cell death in vitro (Shintoku et al. 2017).

Vitamin E is a major source of antioxidants in the human diet. Therefore, numerous studies have investigated potential benefits of vitamin E in cardiovascular disease prevention (Lonn et al. 2005; Stephens et al. 1996). Unfortunately, these studies have failed to yield consistent data that vitamin E improves cardiovascular health or prevents cardiovascular disease, with a randomized clinical study of over 500 patients in each group suggesting that vitamin E supplementation may actually increase the risk of heart failure (Lonn et al. 2005). However, that large-scale study and most cardiovascular vitamin E studies use alpha-tocopherol by itself and other studies suggest that other tocopherols besides alpha-tocopherol or a blend of tocopherols may have more potent antioxidant and cardioprotective effects (Devaraj and Jialal 2005; Ohrvall et al. 1996). The possible involvement of other tocopherols could lead to the development of diets or nutritional programs that leverage antioxidant vitamin research to prevent ferroptosis and cardiovascular disease.

8.5.8 Ferrostatin-1 and Other Small Molecule Inhibitors of Ferroptosis

Ferrostatin-1 is arguably the most researched anti-ferroptotic agent to date with its anti-ferroptotic effects demonstrated in a variety of cell lines, tissue types, and disease models (Gaschler et al. 2018; Skouta et al. 2014; Zilka et al. 2017). Ferrostatin-1 protects the heart against I/R injury and cardiomyopathy (Fang et al. 2019; Li et al. 2019a). The anti-ferroptotic mechanism of ferrostatin-1 is largely due to its structure and inherent antioxidant properties and ability to scavenge free radicals (Zilka et al. 2017). Ferrostatin-1 can neutralize oxidized lipids such as oxidized phosphatidylcholines (Stamenkovic et al. 2021). Ferrostatin-1 also rescues cardiomyocytes from DOX-induced cardiomyopathy in MITOL knockdown and knockout models ferroptosis (Fang et al. 2019; Kitakata et al. 2021). Ferrostatin-1 can accumulate in lysosomes, mitochondria, and the endoplasmic reticulum, but predominantly does so in the ER (Gaschler et al. 2018). Ferrostatin-1 also prevents ferroptotic cell death caused by the accumulation of iron in the labile iron pool, such as that caused by siramesine, a lysosome destabilizing detergent (Ma et al. 2016). However, to date, the use of ferrostatin-1 is preclinical and no human studies of ferrostatin-1 toxicology and safety with the goal of potential therapeutic use have been performed.

More anti-ferroptotic agents have been discovered since ferrostatin-1. Liproxstatin-1 was discovered in a small-molecule screen for ferroptosis inhibitors (Friedmann Angeli et al. 2014) and also works as a free radical scavenger that prevents PUFA oxidation (Zilka et al. 2017). Further studies have discovered more ferroptosis inhibitors, such as tetrahydronaphthyridinols (THNs), a class of compounds that improve cell viability and decrease ROS production more effectively than both ferrostatin-1 and liproxstatin-1 (Zilka et al. 2017). The diverse chemical structures of currently known ferroptosis inhibitors and their general free

radical-scavenging properties suggest that other antioxidants could have ferroptosis-inhibiting properties. If current anti-ferroptotic drugs like ferrostatin-1 and liproxstatin-1 do not pass clinical trials, a screen of small molecule antioxidants could yield more candidate compounds that could be added to the ferroptosis inhibitor pharmacopeia in the future.

Although ferroptosis in the heart does not depend on apoptosis, apoptosis still has a significant role in cardiac cell death in response to I/R injury. Combinations of anti-ferroptotic and anti-apoptotic drugs may yield even more protection against cardiac cell death in response to I/R (Tadokoro et al. 2020). Targeting pathways shared by both ferroptosis and apoptosis, such as the PERK/eIF2α/ATF4/CHOP pathway (Lee et al. 2018), may also yield therapies that could prevent I/R injury even better than therapies that only target ferroptosis or apoptosis in the heart.

8.5.9 Lipid Metabolism and Membrane Integrity

Enzymes that regulate lipid metabolism could also be potential targets for inhibiting ferroptosis. Lipoxygenases, such as ALOX15, are essential enzymes that metabolize arachidonic acid and are key in the synthesis of PUFA-derived compounds such as leukotrienes (De Caterina and Zampolli 2004). However, ALOX15 and PUFAs are also complicit in the lipid peroxidation that is characteristic of ferroptosis (Ma et al. 2022; Shintoku et al. 2017; Zilka et al. 2017). Antioxidant compounds like ferrostatin-1 and liproxstatin-1 prevent lipid peroxidation, but neither of them are substrates nor direct inhibitors of ALOX15 (Zilka et al. 2017). Several small molecule inhibitors of ALOX15 have been identified, such as PD146176, baicalein, and daidzein (Ma et al. 2022; Shintoku et al. 2017; Zilka et al. 2017). Treatment of cultured cardiomyocytes with ALOX15 inhibitors results in decreased lipid peroxidation and suppression of ferroptosis (Shintoku et al. 2017; Zilka et al. 2017), although this effect may be dependent on the presence of PUFAs (Ma et al. 2022). siRNA-mediated knockdown and mouse knockout models of ALOX15 also decrease lipid peroxidation and prevent ferroptotic cell death in in vivo and in vitro models of cardiac ferroptosis, therefore decreasing ALOX15 activity through gene therapy or upstream regulators could also inhibit ferroptosis (Ma et al. 2022; Shintoku et al. 2017).

ACSL4 catalyzes the metabolism of lipids, making it an attractive target for preventing lipid peroxidation and ferroptotic. Rosiglitazone, an anti-diabetic PPAR modulating drug, inhibits ACSL4 and showed anti-ferroptotic effects in intestinal H/R and I/R models by restoring GPX4 function, reducing lipid oxidation, and limiting the extent of I/R injury in mouse intestines (Li et al. 2019b). Similar anti-ferroptotic effects were also seen by siRNA knockdown of ACSL4 (Li et al. 2019b). 5-aminoimidazole-4-carboxamide ribonucleotide (AICAR) is an endogenous metabolite that stimulates AMPK activity that inhibits PUFA synthesis, thereby indirectly decreasing ACSL4 activity. AICAR decreases erastin-induced ferroptosis in cell culture models and decreases sensitivity to ferroptosis in renal I/R injury models (Lee et al. 2020). Other ACSL family members, such as ACSL1 and ACSL3, have

also shown pro-ferroptotic and anti-ferroptotic effects in noncardiac cell lines when incubated in conditions enriched with their lipid substrates (Beatty et al. 2021; Magtanong et al. 2019). Taken together, modulators of ACSL family proteins could be used as a new class of anti-ferroptotic drugs. Furthermore, given that the ferroptotic activity of these enzymes is affected by levels of their lipid substrates, supplementation of lipids that inhibit ferroptosis such as MUFAs or limited consumption of easily oxidized lipids such as PUFAs could also be an avenue of preventing ferroptosis through dietary means.

Oxidized phosphatidylcholine-containing phospholipids (OxPLs) are produced in response to I/R injury and cause cardiomyocyte cell death. E06, a murine natural antibody that targets OxPLs but not unoxidized phospholipids, was able to reduce infarct size in surgical I/R models in mice when overexpressed as a single-chain antibody fragment (Stamenkovic et al. 2021; Yeang et al. 2019). The development of antibodies, biologics, or other molecules that neutralize or reduce the production of OxPLs could yield a new class of anti-ferroptotic drugs.

Lipid peroxides also cause the formation of nanoscale pores in the plasma membrane of cells undergoing ferroptosis, which leads to osmotic swelling, rupture, and propagation of ferroptotic cell death (Riegman et al. 2020). A study of the formation of nanopores in ferroptosis used polyethylene glycols PEG1450 and PEG3350 to serve as osmoprotectants and was able to inhibit cell rupture caused by ferroptosis (Riegman et al. 2020). Activation of mechanosensitive cation channels and loss of cation concentrations gradients are also caused by changes in membrane composition during ferroptosis (Hirata et al. 2023). Treatment with 2-aminoethoxydiphenyl borate (2-APB), a broad inhibitor of TRP channels, was able to maintain Na^+/K^+ concentration gradients and suppress RSL3-induced ferroptosis (Hirata et al. 2023). Further studies of ion channels are needed to determine if there are some that are specific to ferroptosis. Screening of existing ion channel modulators and blockers could yield a new class of anti-ferroptotic drugs.

8.5.10 Inhibition of Inflammation

In light of all the studies linking inflammatory stimuli and ferroptosis, targeting inflammatory pathways and administering anti-inflammatory treatments could attenuate both cardiac inflammation and ferroptosis.

ELAVL1 is associated with inflammation, pyroptosis, and autophagy, and siRNA knockdown of ELAVL1 suppressed I/R-induced ferroptosis associated with Beclin-1-mediated autophagy (Chen et al. 2021). MS-444 is a small molecule inhibitor of ELAVL1 (also known as human antigen R or HuR) and although it has been mostly investigated in cancer cell lines and not in the heart, it could have potential anti-ferroptotic effects (Chaudhary et al. 2023).

Activation of inflammatory innate immune responses could also be targeted, as the release of DAMPs, TLR4 activation, and neutrophil recruitment are all characteristics of I/R injury. Depletion of neutrophils through treatment with Ly6g

antibodies was able to reduce the infarcted area in murine hearts subjected to I/R injury (Li et al. 2019a).

Molecules that promote both inflammation and ferroptosis, such as ICA69, could serve as targets of anti-ferroptotic interventions. Increased ICA69 expression was observed in murine models of sepsis-induced cardiomyopathy, and knockout murine models of ICA69 exhibited decreased pro-inflammatory and ferroptotic markers, therefore therapies that target and decrease ICA69 could lead to decreases in both inflammation and ferroptosis in patients with sepsis (Kong et al. 2022).

In summary, studies published since the previous edition of this book have opened multiple new avenues and potential mechanisms for inhibiting ferroptosis in cardiac tissues. These general strategies include: decreasing the availability of free redox-active iron, restoring antioxidant defenses and redox homeostasis, modulating membrane lipid metabolism, and inhibiting inflammation. Despite the wide variety of mechanisms and potential treatments, all of these pathways converge on ROS and lipid peroxide production as central key steps in the process of ferroptosis.

8.6 Conclusions

The constant activity of the heart requires a steady supply of energy to meet its metabolic demands. Iron and redox reactions are vital components of enzymes and metabolic processes that generate ATP to supply the heart with energy, and an imbalance in the homeostasis of either iron or redox reactions can trigger ferroptosis in the myocardium.

Cardiac pathologies such as I/R and ventricular cardiomyopathy share common elements with ferroptosis, such as excess iron accumulation in cardiomyocytes, oxidative stress, mitochondrial dysfunction, ER stress, and lipid peroxidation.

However, ferroptosis is not unique to the heart and is part of the pathophysiology of many diseases, including cancer, kidney disease, liver disease, and neurodegenerative diseases. Each year, researchers are discovering and confirming new ferroptotic mechanisms in a variety of pathologies, and current cardiac ferroptosis research has confirmed many of those mechanisms in the heart.

As the list of ferroptotic pathways and genes grows, the library of molecules that regulate or inhibit ferroptosis has also expanded. However, information of the safety and toxicity of these molecules in humans is scant, if not nonexistent. In the meantime, several drugs that chelate iron or boost antioxidant defenses are relatively safe and approved for medical use in many countries. Existing compound libraries can also be screened for pro- and anti-ferroptotic molecules, which is a strategy that several groups mentioned in this chapter have already implemented. Each newly discovered gene or pathway involved in ferroptosis is an opportunity to discover another potential compound or class of drugs that could be used to inhibit ferroptosis in the heart.

Ferroptosis has drawn increased attention over the past decade, and this has led to a rapid growth in research investigating ferroptosis in the heart. The expanding

interest and activity in this field makes for a positive and optimistic outlook for a variety of ferroptosis-targeted therapies in the near future.

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Understanding Ferroptosis from a Free Radical Perspective

9

Junichi Fujii

Abstract

Membrane disruption caused by iron-dependent phospholipid peroxidation is a distinctive feature of ferroptosis. Lipid peroxides that are produced either by iron-containing oxygenase enzymes or by oxygen radicals generated via free iron-mediated nonenzymatic reactions are likely involved in cell death. While actively proliferating cells are sensitive to ferroptosis, quiescent cells are somewhat resistant. Proliferating cells, as typified by tumorigenic cells, are prone to producing oxygen radical species because of their stimulated oxygen consumption due to active metabolism. Upon ferroptotic stimuli, ferritinophagy increases the levels of free iron by degrading ferritin and some iron-sulfur clusters associated with enzymes of the tricarboxylic acid cycle may also become a source for free iron in mitochondria. Substances with radical-scavenging ability can terminate radical chain reactions, thereby preventing ferroptosis-related diseases. The focus of this chapter is on the generation of radical species that initiate lipid peroxidation reactions and the importance of their removal to prevent cell death.

9.1 Introduction

Cells die through several pathways under various physiological or pathological conditions. While machinery involved in final process of many types of regulated cell death generally consists of unique proteins, peroxidized phospholipids are exceptionally responsible for ferroptosis via membrane destruction (Tang et al. 2019; Stockwell 2022). Cells with a cysteine (Cys) deficiency caused by inhibition

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of the cystine transporter xCT, also referred to as SLC7A11, or by the deprivation of extracellular cystine typically undergo ferroptosis (Dixon et al. 2012). Because glutathione peroxidase 4 (GPX4) plays a central role in the reductive detoxification of phospholipid hyroperoxides (PL-OOH), a defect in glutathione (GSH) synthesis due to an insufficient Cys supply leads to the malfunction of GPX4, which results in the accumulation of PL-OOH and eventual cell rupture (Seibt et al. 2019; Ursini and Maiorino 2020).

Polyunsaturated fatty acids (PUFA) are sensitive targets of hydroxyl radicals, which can be produced by Fenton chemistry in the presence of free iron and peroxides. The resulting alkoxyl radicals initiate chain reactions of lipid peroxidation (Niki 2009). Since free iron along with peroxides is the initiator for the lipid peroxidation, the removal of free iron effectively prevents cell death (Latunde-Dada 2017; Masaldan et al. 2019). Investigations concerning the pathways for ferroptosis execution have identified several genes that largely contribute to the processes of cell death as well as protection against it (Tarangelo et al. 2018; Kang et al. 2019; Gan 2021). Because most studies exploring anti-ferroptotic genes have been carried out under conditions in which xCT or GPX4 are inhibited, emerging genes largely function to compensate GPX4 function by suppressing lipid peroxidation. Intriguingly, however, a group of genes has been classified into phospholipid synthesis or its repairment, but show no direct association with lipid peroxidation (Rodencal and Dixon 2022). These results suggest that ferroptosis is tightly associated with the active metabolic state of cells and occurs due to declined capacity of detoxification of lipid peroxides. In this chapter, after a brief overview of the ferroptotic pathway, the sources of iron and radicals that may initiate the vicious cycle and cause eventual cell death are discussed.

9.2 Lipid Peroxidation Reaction in Ferroptosis

Since the first report on the definition of ferroptosis (Dixon et al. 2012), our knowledge of the properties of the cell death pathway that includes primary roles of lipid peroxidation and defense systems has accumulated abundantly (Stockwell 2022). The dysfunction of GPX4 alone causes ferroptosis in most cases, which implies that lipid peroxidation products that occur during normal metabolic processes are sufficient to reach lethal levels if they are not efficiently eliminated.

9.2.1 Radical Chain Reactions Responsible for Lipid Peroxidation

Many compounds, such as xCT inhibitors and GPX4 inhibitors, as well as pathogenic conditions, such as inflammation and ischemia-reperfusion injury, have been reported to induce ferroptosis, and there seems to be no dispute that lipid peroxides are the direct executor of this process (Stockwell 2022). A malfunction of GPX4 by a GSH insufficiency or the downregulation of GPX4 gene expression generally results in increased PL-OOH levels. Vitamin E (tocopherol: Toc) supplementation can

rescue cell death caused by xCT inhibition (Dixon et al. 2012) and a lethal phenotype of GPX4 gene ablation (Seibt et al. 2019). Since Toc is effective in scavenging lipid radicals, these observations support the notion that the radical chain reaction has a major role in PL-OOH formation and the subsequent execution of ferroptosis. Among organs, the kidney appears to be the most susceptible organ to ferroptosis under a GPX4 deficiency (Friedmann Angeli et al. 2014).

Alkyl radicals (L•) may be produced by the reaction of PUFA with other radical species that are likely originated from superoxide (Niki 2009). The reaction of L• with an oxygen molecule results in the production of alkylperoxyl radicals (LOO•), which, upon reaction with PUFA, is converted into LOOH with the simultaneous regeneration of L•. Thus, the radical chain reaction continues to produce LOOH as long as L•, PUFA, and oxygen molecule are present. The presence of free iron further stimulates the accumulation of LOOH through the generation of radical species.

Toc functionally compensates for a GPX4 deficiency, whereas the mechanisms responsible for the inhibition of ferroptosis by Toc and GPX4 are somewhat different. GPX4 is the enzyme that reduces PL-OOH to the alcohol form (PL-OH) using electrons from GSH (Ursini and Maiorino 2020). Meanwhile, Toc reduces L• to L and becomes a tocopherol radical (Toc•), which possesses low electron-abstracting potential and hence is much less cytotoxic. Accordingly, Toc-mediated radical scaventing terminates the chain reaction and suppresses the further production of PL-OOH. Coenzyme Q (CoQ) is synthesized in mitochondria, and a part of CoQ is transferred to the plasma membrane via STARD7 (Deshwal et al. 2023) and reduces lipid radicals, which include Toc•. The resulting radical form of CoQ can be reduced back by the ferroptosis suppressor protein 1 (FSP1) in a NAD(P)H-dependent manner (Bersuker et al. 2019; Doll et al. 2019). Vitamin K also suppresses lipid peroxidation by donating an electron and is converted into a vitamin K radical. FSP1 again reductively recycles the vitamin K radical and consequently suppresses ferroptosis (Mishima et al. 2022). Thus, FSP1 acts as an anti-ferroptosis enzyme that functions independently from GPX4. Tetrahydrobiopterin produced by GTP cyclohydrolase-1 (GCH1) indirectly maintains the CoQ redox state, and, consequently, GCH1 also suppresses ferroptosis (Kraft et al. 2020).

Ascorbate is present in the mM range in cell organelles and is a representative antioxidant that reduces Toc• back to Toc (Buettner 1993). In fact, ascorbate is a strong reducing agent against multiple radical species because of its ability to donate an electron. However, the reaction of ascorbate with free iron may generate radical species and exacerbate the oxidative state (Badu-Boateng and Naftalin 2019). This potentially harmful reaction based on redox coupling between ascorbate and iron appears to be a reason for why ascorbate does not emerge as an effective anti-ferroptotic agent. Consistent with this notion, the coadministration of ascorbate and an iron chelator may be able to prevent ferroptosis-related diseases by eliminating radical species more effectively. On the other hand, such cytotoxic action can be applicable for the treatment of malignant tumors. For example, a pharmacological dose of ascorbate can exert tumoricidal effects by means of generating radical species (Chen et al. 2007).

9.2.2 Roles of Free Iron in Ferroptosis

Iron may originate from either an intracellular or an extracellular source and is involved in radical formation via Fenton-type chemistry (Toyokuni et al. 2017). The iron fraction that can be chelated by high-affinity metal chelators is regarded as the labile iron pool (LIP) and accounts for 0.1–3% of the total cellular iron. However, the LIP alone is not sufficient for inducing ferroptosis. Ferritin is a ferric iron storage protein that prevents harmful free iron-mediated reactions from occurring. The degradation of ferritin is stimulated by means of autophagy, more specifically ferritinophagy, under conditions where xCT or GPX4 are inhibited (Chen et al. 2021) (Fig. 9.1). Toc is present in lysosomal membranes at concentrations that are approximately one-order in magnitude higher than in other subcellular compartments (Wang and Quinn 1999). Because lysosomes are rich in free iron among cell organelles, an enriched Toc content together with GPX4 associated with the membrane (Wu et al. 2019) could protect lysosomal membranes from peroxidation. It is therefore conceivable that ferritinophagy under a Toc deficiency or aberrant GPX4 function could result in elevated levels of PL-OOH, thus making the lysosomal and autolysosomal membrane vulnerable. Rupture of the vesicular structure may release iron into the cytosol, which augments the lipid peroxidation of the plasma membrane, eventually causing cell death. On the other hand, lipoxygenase (LOX) is activated and appears to be involved in lipid peroxidation for ferroptosis (Yang et al. 2016; Benatzy et al. 2022). Under conditions in which xCT is inhibited,

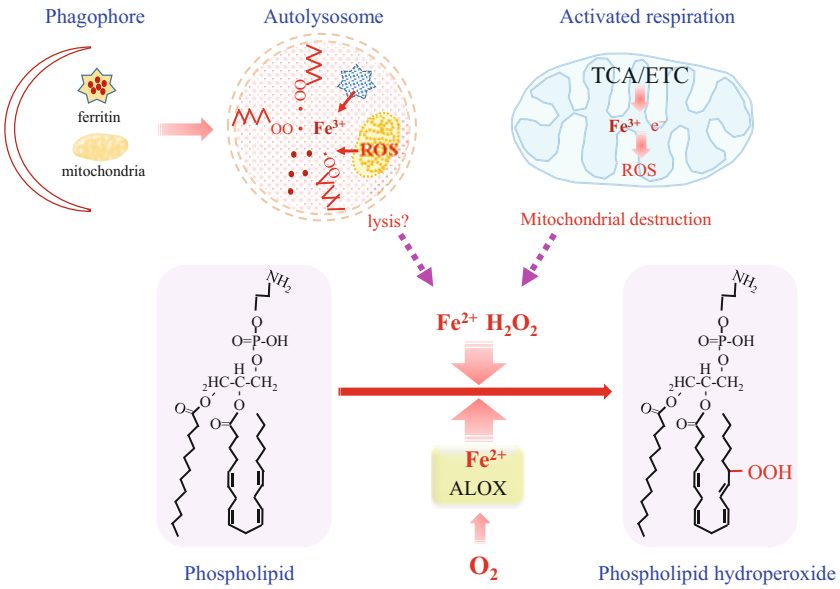

Fig. 9.1 Lipid peroxidation in ferroptosis. Phospholipids with PUFA are prone to be peroxidized by either hydrogen peroxide in the presence of free iron or by the enzymatic activity of ALOX. Free iron and ROS may also be provided from ferritinophagy and respiratory chain in mitochondria

phosphorylase kinase G2 (PHKG2) appears to increase the levels of cellular iron, which leads to the activation of lipoxygenase enzymes. We further discuss the sources and roles of iron in phospholipid peroxidation during ferroptosis in the following session.

9.2.3 Cys-GSH-GPX4 Axis as a Primary Defense System against Ferroptosis

Cys auxotrophy is quite different between in vitro and in vivo situations because of difference in the redox environment of cells. Cells are generally cultivated under atmospheric oxygen conditions, i.e., 21% O_2, which is approximately 4-times higher than those at in vivo conditions. In this oxygen status, Cys is largely converted into cystine in the culture medium. Cys/cystine are either taken up by cells or synthesized via the transsulfuration pathway in conjunction with Met metabolism in vivo. Despite the highly oxidative extracellular state, mouse plasma still contains significant amounts of Cys vs cystine at 18 μM vs 33 μM (Sato et al. 2005). Therefore, cells in most tissues, excluding the brain and immune cells, do not express xCT but utilize extracellular Cys or synthesize it through the transsulfuration pathway (Conrad and Sato 2012). Neutral amino acid transporters ASCT1 and ASCT2 are responsible for the uptake of Cys in ordinary cells, whereas neurons employ neutral amino acid transporter excitatory amino acid carrier type 1 (EAAC1) (Conrad and Sato 2012). These cells, however, possess the ability to express xCT upon an oxidative insult most likely via the activation of Nrf-2, which is a master regulator for redox-reactive genes (Yamamoto et al. 2018). Cystine incorporated into cells via xCT may be reduced to Cys by means of thioredoxin 1 and the thioredoxin-related protein 14 kDa (TRP14) (Pader et al. 2014). A significant amount of Cys is then secreted from cells, where it may act to maintain redox balance in the cellular microenvironment (Banjac et al. 2008). Accordingly, cultivated cells commonly depend on the expression of xCT to meet the demand of Cys for redox homeostasis.

Ferroptosis is typically induced in cultivated cells by a deficiency of intracellular Cys, which can occur by the inhibition of xCT or cultivation in cystine-free media. The dysfunction of GPX4 or Cys deprivation leads to the accumulation of PL-OOH, which results in membrane destruction and ferroptosis (Stockwell 2022). Since Cys is an essential building block for GSH, most studies have concluded that a Cys deficiency causes ferroptosis through a defect in GSH synthesis. However, there is a dissociation in the viability of cells caused by a Cys deficiency and that caused by GSH depletion (Banjac et al. 2008). In fact, Cys can be utilized to synthesize many cellular components that include taurine, coenzyme A, cysteamine, and iron-sulfur [Fe-S] clusters other than a building block for proteins and GSH. Cys is also the endogenous source of cellular mediators including hydrogen sulfide (H_2S) and persulfides (Filipovic et al. 2018). Persulfides, as represented by glutathione hydropersulfides (GSSH), terminate radical chain reactions by eliminating radical species efficiently and suppressing ferroptosis (Barayeu et al. 2023; Wu et al. 2022).

The widespread use of Cys may explain why the cessation of the Cys supply rather than the inhibition of GSH synthesis robustly induces ferroptosis.

As a nonessential amino acid, Cys can be produced through the transsulfuration pathway coupled with methionine (Met) metabolism; however, the contribution of the pathway to the supply of Cys appears to be limited to some organs, such as the liver (McBean 2012). In the transsulfuration pathway, cystathionine β-synthase (CBS) produces cystathionine from homocysteine, which is supplied from the metabolism of Met, and serine, after which, cystathionine γ-lyase (CSE) cleaves cystathionine to Cys and 2-oxo butyrate (Fujii et al. 2020). The ability to synthesize Cys tends to decline under cell culture conditions due to the downregulation of enzymes that are involved in the transsulfuration pathway for unknown reasons. Accordingly, most lines of cells, whether normal cells or cancer cells, depend on extracellular cystine for survival.

The malignant phenotype of cancer cells tends to be associated with the expression of xCT even in an in vivo situation (Ishimoto et al. 2011), which may be explained by the augmented requirement of Cys for active proliferation. For these reasons, ferroptosis is considered to be a promising target for anticancer therapy (Jiang et al. 2021; Lee and Roh 2022). On the other hand, quiescent cells are less sensitive to ferroptosis, as discussed below, which may be one of the reasons why cancer stem cells tend to avoid drug-induced cell death. Substantial differences in the redox state between ex vivo and in vivo conditions can be an obstacle to the application of results obtained from in vitro studies to cancer therapy. Genetic modification can convert CBS to cyst(e)inase, which hydrolytically depletes plasma Cys and the cystine pool in mice and nonhuman primates (Cramer et al. 2017). The application of cyst(e)inase to pancreatic ductal adenocarcinoma was found to be effective by inducing ferroptosis (Badgley et al. 2020), although at this time, this approach has not reached the stage of clinical trials for cancer patients (Lee and Roh 2022). Because Cys is a semi-essential amino acid, the depletion of plasma cyst(e)ine may influence the redox conditions of most cells that depend on extracellular cyst(e)ine.

9.2.4 Regulators of Phospholipid Peroxidation

Both enzymatic and radical-mediated nonenzymatic reactions are considered to be responsible for lipid peroxidation (Stoyanovsky et al. 2019). Exploration of factors that regulate ferroptosis has identified several genes that are robustly involved in ferroptosis. Arachidonate lipoxygenase (ALOX), an enzyme that requires nonheme iron for activity (Benatzy et al. 2022), has been shown to be responsible for LOOH formation in some cases of ferroptosis (Yang et al. 2016). Among the LOX family of enzymes, the aberrant activation of ALOX5, ALOX12, and ALOX15 reportedly results in the development of ferroptosis (Kagan et al. 2017; Chu et al. 2019). Catalytic iron in ALOX coordinates amino acids of the enzyme, but may uncontrollably catalyze peroxidation reactions of PUFA. An elevated iron supply, gene expression, and membrane localization have all been reported to activate ALOX

sufficiently to cause cell death (Yang et al. 2016; Ou et al. 2016; Shintoku et al. 2017). Cyclooxygenase (COX) is also an iron-containing PUFA oxygenase, but its direct involvement in ferroptosis has not been confirmed. This is probably because iron, which constitutes the catalytic center of COX, is in the form of heme that does not easily release iron due to its rigid structure. This difference in iron status appears to distinguish the contribution of the two PUFA oxygenases to ferroptosis.

Acyl-coenzyme A synthetase long-chain family member 4 (ACSL4) was also reported to dictate ferroptosis under conditions where xCT or GPX4 are inhibited (Yuan et al. 2016; Doll et al. 2017; Kagan et al. 2017). ACSL4 has no direct role in lipid peroxidation reactions, but it preferentially catalyzes the conjugation of arachidonate with CoA and stimulates its incorporation into phosphatidic acid, followed by phospholipids and triacylglycerol. Upon ferroptotic stimuli, PKCβII activates ACSL4 by phosphorylation, which further accelerates the incorporation of PUFA into phospholipids (Zhang et al. 2022). Since lipid peroxides recruit PKCβII to membranes and stimulate its activity, these reactions are considered to form a feedforward signal amplification cascade. Thus, the coordinate actions of ACSL4 with ALOX or nonenzymatic lipid peroxidation reactions result in the accumulation of PL-OOH in cell membranes. Contrary to this, calcium-independent phospholipase A2 encoded by PLA2G6 was found to suppress ferroptosis (Beharier et al. 2020; Chen et al. 2021a; Sun et al. 2021). This anti-ferroptotic action appears to be accomplished by the excision of LOOH from PL-OOH. Because the expression of high levels of PLA2G6 is associated with drug-resistance of some tumors, this anti-ferroptotic action may be responsible for the malignancy of melanoma and other tumors (Duffy et al. 2018).

9.3 Ferroptosis Occurs Preferentially in Metabolically Active Cells

An understanding of the sources of radical electrons and iron is of prime importance for the purpose of the prevention and treatment of ferroptosis-associated diseases. It is well recognized that proliferating cells rather than quiescent cells are prone to developing ferroptosis, which is a quite different hallmark from apoptotic cell death. We discuss how such a cell state determines cell fate here.

9.3.1 Association of Ferroptosis with Active Metabolic State

Cells in most tissues of healthy adults, excluding some cells such as those in bone marrow and reproductive organs, generally do not proliferate. Unlike cancer cells that continue to proliferate, cell-to-cell contact is a primary cause for the arrested proliferation of normal cells, which is defined as contact inhibition. E-cadhelin, which is the adhesion molecule constituting the adherent junction between adjacent cells, is involved in the suppression of ferroptosis by activating the tumor suppressor Merlin/NF2 and the Hippo signaling pathway (Wu et al. 2019). Resistance to contact

inhibition is one of the hallmarks of malignant cells, which may explain the high susceptibility to ferroptosis of mesenchymal cancer cells that exhibit a high metastatic potential (Viswanathan et al. 2017; Hangauer et al. 2017).

Cellular proliferation processes are divided into four phases, G_1, S, G_2, and M, which are promoted by the unique coupling of the cyclin/cyclin-dependent kinase (Cdk) and proceed in a cyclic manner (Havens et al. 2006). Generally speaking, high metabolic activity consumes more oxygen and, hence, is considered to produce more ROS compared to quiescent cells (Meacham et al. 2022). Metabolic reactions, including energy production in mitochondria, are uniquely regulated at each phase of the cell cycle, resulting in varying degrees of ROS release. Although the production of excessive levels of ROS may suppress mitotic spindle formation through the inhibition of Aurora kinase and result in mitotic arrest (Wang et al. 2017), moderate levels of ROS rather stimulate cell proliferation by modulating mitotic signaling (Finkel 2011). The cellular localization of GSH as well as the levels of antioxidant enzymes also changes synchronously with the cell cycle. The accumulation of proteins with oxidized Cys residues is found in the M phase (Patterson et al. 2019). However, this may not simply indicate a high production of ROS at the M phase because protein contents are balanced between their synthesis and degradation. In fact, it is known that lysosomes, which function alongside proteasomes to digest intracellular proteins, are scarce during the M phase (Nowosad and Besson 2022). As a result, genome integrity can be protected against lysosomal nucleases while the nuclear envelope is broken down (Odle et al. 2020). Inhibition of the serine/threonine kinase mechanistic target of rapamycin 1 (mTOR1), which is a critical regulator of autophagy, accomplishes this genome guard at the M phase. Thus, the accumulation of oxidized proteins at the M phase may not necessarily indicate the production of ROS at this stage. At present, it is still vague as to how much ROS are produced at each phase of the cell cycle and the nature of the mechanism that regulates them.

Cultivation in cystine-free media typically induces ferroptosis in cells with a defect in the transsulfuration pathway, but surprisingly, a double deficiency of cystine and Met rather suppresses cell death (Lee et al. 2017). As a matter of fact, upon Met restriction, cell cycle arrest occurs at the end of the G1 phase and the G2 phase, which is called the Hoffman effect (Kaiser 2020). While several changes occur in metabolism, Met-deficient cultivation leads to a decline in the content of S-adenosylmethionine (SAM), a Met metabolite acting as the methyl group donor (Fig. 9.2). Among many methylated compounds, cytosine with methylation at the 5 position must be inherited to newly synthesized daughter DNA during the S phase. DNA methyltransferase DNMT1 maintains the cytosine methylation at the CpG island during DNA replication (Chen and Zhang 2020). While excessive methylation of tumor-suppressor genes may be a cause for tumor development, overall defects in DNA methylation restrict the cell cycle progression. For example, disabling DNA methylation by means of DNMT1 gene ablation causes hemimethylation in the CpG island, ultimately leading to cell cycle arrest (Chen et al. 2007b). When cells cultivated under a double deficiency of Cys and Met are supplemented with SAM, cell cycle resumes and LOOH levels rise, ultimately

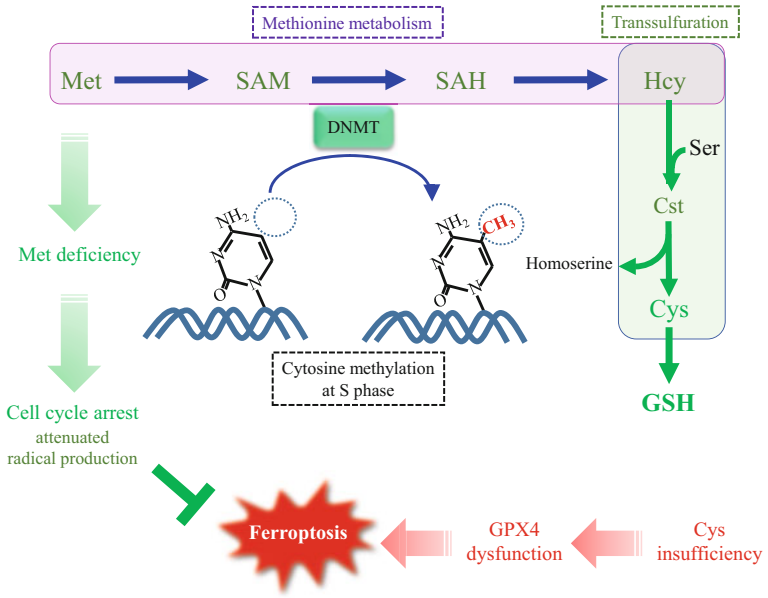

Fig. 9.2 Regulatory system of ferroptosis in association with methionine metabolism coupled with the transsulfuration pathway. Cys can be produced through a transsulfuration reaction coupled with Met metabolism. SAM is a methyl group donor for cytosine methylation during the replication of DNA at the S phase of the cell cycle. Cells with a SAM deficiency are unable to transmit methylation status to daughter DNA and undergo cell cycle arrest. GSH depletion caused by a Cys deficiency leads to the development of ferroptosis. While a Cys deficiency alone does not affect the mitotic ability of cells, a double deficiency of Cys and Met leads to cell quiescence and survival due to defective SAM production. DNMT, DNA methyltransferase; SAH, S-adenosylhomocysteine; Hcy, homocysteine, Cst, cystathionine, Ser, serine

leading to ferroptosis (Homma et al. 2022a). These collective observations suggest that ROS derived from active metabolism after the S phase initiates ferroptosis if the LOOH-scavenging system does not function properly.

9.3.2 Mitochondria as a Source for Radicals

The origin of the oxygen radical that triggers lipid peroxidation reactions appears to vary depending on the ferroptotic stimulus. The destruction of mitochondrial morphology, represented by fragmentation, cristae disappearance, and volume reduction, is a hallmark of ferroptotic cells (Dixon et al. 2012; Wang et al. 2020; Oh et al. 2022). Given that the mitochondrial electron transport chain (ETC) is considered to be the major source for the electron leak (Wong et al. 2017), it is conceivable that leaked electrons along with oxygen molecules form oxygen radicals that are responsible for the lipid peroxidation and consequent mitochondrial destruction. The inhibition of ETC complexes decreases mitochondrial membrane potential, which

is elevated upon ferroptotic stimuli, and suppresses cell death (Gao et al. 2019). Then, the use of specific inhibitors that suppress the leakage of electrons from each ETC complex without otherwise altering energy metabolism demonstrated that electrons leaked from ETC III but not from ETC I mainly contribute to the ferroptosis caused by cultivation under cystine-deprived conditions (Homma et al. 2021). Contrary to these results, however, the inhibition of ETC I in melanoma cells reportedly triggers the production of ROS and results in ferroptosis (Basit et al. 2017). These observations imply that radical sources for ferroptosis may differ depending on type of cell and the ferroptotic stimuli.

9.3.3 Sources of Free Iron in Mitochondrial

While free iron is released upon ferritinophagy, the source of the iron for lipid peroxidation within mitochondria remains ambiguous. In normal proliferating cells, iron is transferred from the cytosol to mitochondria after ferritinophagy to meet the demand for heme and [Fe-S] cluster formation. Mitochondria carry a unique ferritin, which stores iron and has a high structural similarity to cytosolic ferritin (Levi et al. 2021). The excessive degradation of mitochondrial ferritin by proteases may release free iron, which would result in ferroptosis through accleration in lipid peroxidation. Consistent with this possibility, the overexpression of mitochondrial ferritin alleviates erastin-induced ferroptosis (Wang et al. 2016) and cerebral damage caused by ischemia/reperfusion (Wang et al. 2021). Information on mitochondrial ferritin with respect to ferroptosis is still limited, and further investigations are needed to determine the significance of this mitochondrial iron-storage protein.

Cellular iron auxotrophy is controlled by the iron regulatory protein 1 (IRP1) which contains [4Fe-4S] clusters with a high susceptibility to cellular iron status. Under an iron insufficiency, the release of one iron atom from the [4Fe-4S] cluster causes cytosolic IRP1 to bind to the iron-responsive element in the mRNA that encodes for iron-metabolizing proteins, which results in regulation of the translation and the stability of the mRNA (Rouault 2006). Meanwhile, mitochondrial IRP2 acts as aconitase, which catalyzes the conjugation of oxaloacetate and the acetyl group of acetyl-CoA in the tricarboxylic acid (TCA) cycle. While iron in the form of heme and [Fe-S] clusters associated with proteins are generally not directly involved in radical formation, reactive oxygen and nitrogen oxide species may cause the release of iron from [Fe-S] cluster in proteins (Gardner et al. 1994; Castro et al. 2019). The reaction of ROS with aconitase releases ferrous iron and hydrogen peroxide, which results in the remodeling of the carbon metabolism by the TCA cycle, as typically observed in M1-polarized macrophages (O'Neill and Artyomov 2019). Succinate dehydrogenase, another enzyme in the TCA cycle, contains three [Fe-S] clusters, [2Fe-2S], [3Fe-4S], and [4Fe-4S], and is sensitive to inactivation by superoxide as well as itaconate, a metabolite produced via the broken TCA cycle in M1 macrophages. While TCA cycle remodeling causes phenotypic changes in the cells, little interest has developed regarding the released iron so far. This is because macrophages are known to be resistant to ferroptosis. Macrophages have unique iron

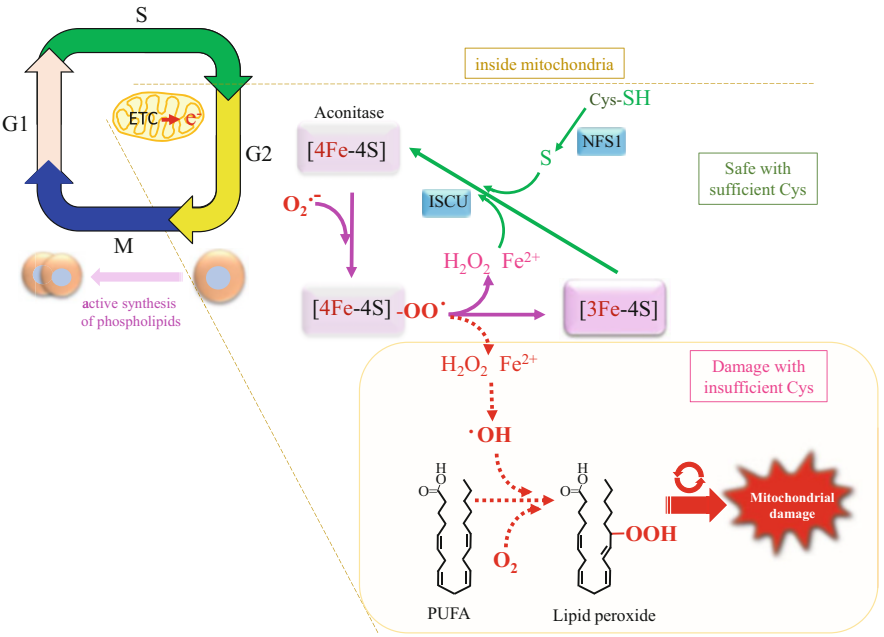

Fig. 9.3 A hypothetical mechanism for cell proliferation-associated lipid peroxidation in mitochondria. Superoxide is produced in proliferating cells due to active respiration

exclusion systems, the lysosomal iron pump protein natural resistance-associated macrophage protein 1 (Nramp1), and the iron export protein ferroportin, which may decrease the toxicity of iron (Haschka et al. 2021). Given that mitochondrial destruction is one of hallmarks of ferroptosis in ordinary cells and aconitase is present in abundant levels, it can be speculated that the released iron from [Fe-S] clusters and radicals that have leaked from ETC may coordinately be involved in lipid peroxidation in the mitochondrial membrane (Fujii et al. 2022). Based on this assumption, we propose a hypothetical model in which ROS originated from ETC attacks on the [4Fe-4S] cluster in aconitase 2, releases ferrous iron together with hydrogen peroxide, and collectively causes lipid peroxidation under a Cys deficiency (Fig. 9.3). Consistent with this notion, the [Fe-S] cluster assembly protein ISCU regulates iron status in mitochondria and suppresses the ferroptosis that is induced by dihydroartemisinin (Du et al. 2019). Cys desulfurase NFS1 which transfers sulfur from Cys for assembling [Fe-S] also suppresses ferroptosis in lung tumor cells (Alvarez et al. 2017). Modulating the assembly of [Fe-S] clusters by the cysteine desulfurase complex is activated by frataxin, which also results in the suppression of ferroptosis (Du et al. 2020). Other [Fe-S] cluster proteins may also be involved in this scenario. For example, proteins with CDGSH iron sulfur domains (CISDs) are proteins with [2Fe–2S] clusters and may be responsible for the [2Fe–2S] cluster relay between mitochondria and other cellular components (Mittler et al. 2019). While CISD1 and CISD3 stimulate ferroptosis, CIDS2 appears to confer resistance to ferroptosis (Tang et al. 2019). Since the functions of these proteins that

are associated with ferroptosis are vague at this moment, this issue needs further clarification.

Because [4Fe-4S] clusters in aconitase are prone to oxidative damage, ferrous iron along with hydrogen peroxide is released. The [4Fe-4S] cluster can be reconstructed by means of Cys desulfurase (NFS1) and the [Fe-S] cluster assembly protein (ISCU) in the presence of sufficient Cys. However, this process is dysfunctional when sufficient amounts of Cys are not available. As a result, released iron together with hydrogen peroxide may stimulate lipid peroxidation, leading to the destruction of the mitochondrial membrane structure.

9.4 Future Mission of Ferroptosis Research

Rapid advances in ferroptosis research would provide clues for the treatment of ferroptosis-related diseases by modulating the pathways. For the purpose of understanding and the early diagnosis of such diseases, the development of a simple and reliable method for the in situ identification of ferroptotic cells is awaited. On the other hand, the induction of ferroptosis may be a promising approach for the treatment of malignant tumors that are resistant to apoptosis (Viswanathan et al. 2017; Hangauer et al. 2017).

9.4.1 Approach to Detect Ferroptotic Cells In Situ

Apoptosis is typically executed by caspases that are activated through death receptor- or apoptosome-involved pathways and is responsible for a variety of physiological and pathological functions in the body (Nagata 2018). The detection of cleaved caspases by the antibodies and cleaved DNA by means of the TUNEL method are quite useful for in situ detection. Studies using cell culture systems have greatly contributed to the clarification of machineries responsible for ferroptosis; however, several problems associated with applying these findings to the pathological diagnosis of ferroptosis-related diseases have emerged. The identification of ferroptotic cells in situ would be of prime importance for the diagnosis of such diseases, but there appears to be no simple methods available for accomplishing this so far.

For the purpose of detecting ferroptotic cells in situ, it would be advantageous to establish specific antibodies for this purpose. 4-Hydroxy-4-nonenal (HNE) and related aldehydes are produced in lipid peroxidation reactions and, hence, can be regarded as a marker for ferroptotic cells (Toyokuni 2016; Saito 2021). These aldehydes are common products of the lipid peroxidation reactions under oxidative stress, but may not be formed at early process of ferroptosis. We attempted to establish monoclonal antibodies raised against ferroptotic cells, which were prepared by cultivation in the absence of cystine. One antibody, designated as FerAb, appears to recognize cells upon several ferroptotic stimuli, although the antigenic structure remains unidentified (Kobayashi et al. 2021). FerAb also recognizes HNE-modified proteins, but the cellular localization of the possible antigenic molecule appears to

differ from that of anti-HNE. FerAb could distinguish ferroptotic cells without fixation by means of a fluorescence activated cell sorter (FACS) (Homma et al. 2022b). The nature of this antibody would be beneficial because artifacts that may be produced during fixation can be avoided. Thus, FerAb appears to be one of the first generation antibodies that rather specifically detects ferroptotic cells. While the characterization of FerAb is still ongoing, the development of specific antibodies and the identification of their antigenic structures could facilitate the diagnosis of ferroptotic cells in situ.

9.4.2 Prevention and Treatment of Ferroptosis-related Diseases

Ferroptosis underlies the pathogenesis of many diseases, such as neurodegeneration and ischemic organ injuries, and, therefore, needs to be prevented for the sake of a healthy subject. Gene products, such as ACSL4, are involved in the execution of ferroptosis, but also have roles in normal physiology. Hence, their inhibition can suppress the ferroptosis-associated pathological state, but may also affect the physiological functions of ordinary cells. It would be more advantageous to scavenge radicals, which initiate or continue the chain reaction associated with lipid peroxidation, by means of natural or synthetic radical-scavenging compounds. For example, nitric oxide is inhaled for the treatment of certain respiratory diseases. Therapeutic effects of nitric oxide on some pulmonary injuries may be partly attributed to chain-terminating reactions, in addition to improving blood circulation.

Cancer cells that are less sensitive to apoptosis-inducing drugs have been reported to be susceptible to ferroptosis (Viswanathan et al. 2017; Hangauer et al. 2017). While Cys deprivation is effective in inducing ferroptosis in vitro, it may not be an ideal approach in vivo because Cys plays requisite roles in the redox homeostasis of all types of cells. Quiescent cancer cells, as represented by cancer stem cells, tend to be resistant to the treatment and may cause recurrence (Liang and Kaufmann 2023). For the purpose of the treatment of such cancer cells that are quiescent due to a Met insufficiency (Kaiser 2020), the administration of pharmacological doses of Met/SAM along with ferroptosis-inducing drugs may increase the efficacy of the cancer treatment by stimulating cell death.

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The NRF2-anti-ferroptosis Axis in Health and Disease

10

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Abstract

Despite a common endpoint, how a cell dies is dictated by the initiating stressor and downstream effectors that govern the pro-death response. One recently identified mode of cell death is ferroptosis, an iron- and lipid peroxidation-driven death cascade that is morphologically distinct from apoptotic, necrotic, and autophagic cell death. While the name "ferroptosis" was just recently coined in 2012, evidence supporting the existence of this pathway dates back several decades. Importantly, though ferroptosis was initially identified as a means to kill apoptosis-resistant cancer cells, its relevance in other pathological contexts continues to emerge. One critical deterrent to ferroptotic cell death is the transcription factor nuclear factor erythroid 2-related factor 2 (NRF2), which controls the expression of several key anti-ferroptotic mediators. Although the ability of NRF2 to prevent ferroptotic death was initially thought to derive primarily from its mediation of the antioxidant response, recent evidence has revealed NRF2 regulation of iron homeostasis and lipid peroxide reduction also plays a pivotal role. Here, we will provide a brief overview of the ferroptosis and NRF2 pathways, including the NRF2-mediated transcriptional programs that have been implicated in regulating the ferroptosis cascade. Additionally, we will discuss the evolving pathological relevance of ferroptosis, including how

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modulation of NRF2 has been shown to affect ferroptotic death, and what the future holds for this critical axis of cell survival.

10.1 Introduction

The balance between life and death in the cell is governed by an intricate network of signaling pathways that intertwine to activate or suppress a given cellular response depending on the initiating signal. In the case of programmed cell death, the upstream stressor plays a large part in determining the downstream death cascade that is initiated. Accordingly, an increasing number of cell death pathways have been identified, evolving from the initially recognized 3 morphotypes (i.e., apoptosis, autophagic cell death, and necrosis) to 12 distinct modes of cell death (Galluzzi et al. 2018). This infers that while the outcome may be the same, the steps and effectors needed to reach the death endpoint can differ greatly depending on the context and stress involved. Perhaps one of the more intriguing death cascades to recently emerge is ferroptosis, which requires both an excess of iron and lipid peroxides to take place. While ferroptosis was initially characterized as a novel mode of cell death in apoptosis-resistant cancer cells, its pathological relevance continues to emerge across other disease contexts as well, including neurodegeneration, diabetes, autoimmune disorders, ischemic injury, cardiovascular disease, and chronic liver disease (Jiang et al. 2021; Stockwell 2022; Anandhan et al. 2020; Tang et al. 2021). Additionally, an increasing number of pro- and anti-ferroptotic effectors continue to be discovered, further enhancing our understanding of the complexity of this death cascade.

Importantly, much like the other identified modes of programmed cell death, initiation of ferroptosis is mediated by a system of checks and balances that ensure it occurs only when certain conditions are met. At its core, these conditions can be broken down into two primary components: 1) increased lipid peroxidation, which can occur both enzymatically and nonenzymatically, and 2) an accumulation of labile, redox-active ferrous iron, which catalyzes the formation of harmful radicals via the Fenton reaction. Despite the seemingly simple combination of events needed for ferroptosis to occur, the possible causative factors that can lead to alterations in lipid peroxidation and the labile iron pool are quite complex. Thus, determining the pathological mechanisms that drive these key hallmarks of ferroptotic death, both in a cancer context and beyond, continues to represent an area of active interest. Critically, the expression of most of the anti-ferroptotic mitigators identified to date are mediated by the transcription factor, nuclear factor erythroid 2-related factor 2 (NRF2). As such, it is not surprising that NRF2 has continued to emerge as a primary target for activating or inhibiting ferroptosis depending on the pathological setting. In this chapter, we will briefly introduce the ferroptosis and NRF2 cascades, including a detailed overview of the NRF2 transcriptional targets that mediate ferroptosis. Finally, we will highlight evidence in the literature indicating that NRF2 plays an important role in determining ferroptotic fate across a variety of

pathological contexts and conclude with a discussion on what the future holds for the NRF2-ferroptosis field.

10.2 A Brief History of Ferroptosis

Similar to the inception of other areas of scientific research, the field of ferroptosis arose from efforts to solve an unsolved problem, i.e., overcoming acquired apoptosis-resistance in aggressive cancer subtypes. In fact, one of the basic tenets of the recently expanded hallmarks of cancer originally laid out by Hanahan and Weinberg is resisting cell death (Hanahan 2022), which has represented a significant obstacle to the generation of successful cancer therapeutics for decades. It was in this vein that Stockwell's group set out to screen for compounds capable of killing apoptosis-resistant cancer cells; in this case, those harboring oncogenic RAS mutations that facilitate their continued survival. It was in the early days of these screening attempts that erastin (Eradicator of RAS and ST-expressing cells) was discovered, which caused cell death in the absence of any indicators of apoptosis (Dolma et al. 2003). From there, Stockwell's group began to identify the fundamental aspects of this newly identified mode of cell death, showing in 2007 that erastin-induced cell death was oxidative by nature and could be reversed by the administration of lipophilic antioxidants, indicating the importance of membrane lipid peroxidation in driving this cascade. This same study also identified the first targets of erastin, voltage-dependent anion channel 2 and 3 (VDAC2/3), which at the time made sense based on the notion that mitochondrial dysfunction could produce the reactive species needed to promote membrane peroxidation (Yagoda et al. 2007). Later, in 2008, Stockwell's group discovered two homologous compounds that induced non-apoptotic death in a manner similar to erastin, which they named RSL3/5. They also demonstrated that iron chelation using deferoxamine could reverse both erastin and RSL3-induced cell death (Yang and Stockwell 2008), which was one of the earliest indications that iron accumulation, along with the already established increase in lipid peroxidation, was also a critical driver of this type of death. It is important to note that while the initial discoveries regarding erastin and RSL3 were being made, Marcus Conrad's group independently showed in 2008 that genetic ablation of glutathione peroxidase 4 (*GPX4*) in vitro, a critical enzyme that reduces lipid peroxides, also resulted in non-apoptotic cell death, which would also later prove to be an important early finding in the field (Seiler et al. 2008).

All of these discoveries led to one of the seminal moments in the field, the 2012 coining of the term "ferroptosis" for this newly identified mode of non-apoptotic cell death. The name arose following the definitive demonstration by Dixon and Stockwell that iron-dependent lipid peroxidation was promoting erastin-induced cancer cell death, a phenomenon also observed in glutamate-treated ex vivo brain slices, in a manner that was morphologically and molecularly distinct from apoptosis, autophagic cell death, and necrosis (Dixon et al. 2012). Furthermore, erastin, much like an excess of glutamate, was shown to inhibit system xc-, a cystine-glutamate antiporter, resulting in glutathione (GSH) depletion, increased oxidative

stress, and decreased function of critical antioxidant enzymes that use GSH as a cofactor. One such enzyme is GPX4, whose ferroptosis relevance was solidified in a series of 2014 studies, where it was identified as the target of RSL3, and Conrad's group showed that genetic ablation of *Gpx4* led to increased lipid peroxidation and ferroptosis-driven acute kidney failure in mice (Yang et al. 2014; Friedmann Angeli et al. 2014). Over the next few years, several enzymatic sources of lipid peroxidation were also shown to be associated with ferroptosis induction, including lipoxygenases 5, 12, and 15 (ALOX5/12/15), prostaglandin endoperoxide synthase 2 (PTGS2/COX-2), as well as the lipid metabolizing enzymes acyl-CoA synthetase long chain family member 4 (ACSL4) and lysophosphatidylcholine acyltransferase 3 (LPCAT3) (Yang et al. 2014; Dixon et al. 2015; Yang et al. 2016; Yuan et al. 2016). In 2016, it was also shown that nuclear receptor coactivator 4 (NCOA4)-dependent degradation of ferritin, a process termed ferritinophagy, could result in an increase of labile iron, thus promoting ferroptosis (Gao et al. 2016; Hou et al. 2016). Importantly, this was one of the first mechanistic indications of where the iron that promotes ferroptosis may originate, as up to this point its importance, but not source, was known. Identification of iron and lipid peroxides as drivers of ferroptotic death led to the pursuit of anti-ferroptotic effectors that might function in different intracellular compartments, particularly considering the abundance of phospholipids in the plasma and organellar membranes. This search led to the co-discovery of ferroptosis suppressor protein 1 (FSP1; previously named AIFM2) in 2019, and dihydroorotate dehydrogenase (DHODH) in 2021, which suppress lipid peroxidation in the plasma and mitochondrial membranes, respectively (Doll et al. 2019; Bersuker et al. 2019; Mao et al. 2021).

The studies included above represent just some of the key findings that served as the foundation for the ongoing development and emergence of the ferroptosis field. As the relevance of this pathway continues to expand, so too will the identification of novel effectors of this critical death cascade. A timeline of key discoveries made to date is provided in Fig. 10.1 (top portion).

10.3 The NRF2 Pathway and Its Relevance to Ferroptosis

Mentioned briefly above, the NRF2 signaling pathway and ferroptosis are intimately linked, as many of the proteins responsible for maintaining iron homeostasis and preventing lipid peroxidation are encoded by NRF2 target genes. NRF2 itself is primarily regulated at the protein level by the Cullin 3-Ring box 1-Kelch-like ECH-associated protein 1 (CUL3-RBX1-KEAP1) E3 ubiquitin ligase complex, which under basal, non-stressed conditions targets NRF2 for proteasomal degradation. However, in the presence of oxidative/electrophilic stress, autophagy dysfunction, or somatic mutations in *NFE2L2*/NRF2 or the genes that encode its degradation machinery, NRF2 can accumulate in the nucleus and activate the transcription of antioxidant response element (ARE)-containing genes (Fig. 10.2). While many of the NRF2 target genes involved in glutathione metabolism, phase I, II, and III xenobiotic detoxification, and iron storage/homeostasis (Fig. 10.2) are relevant in

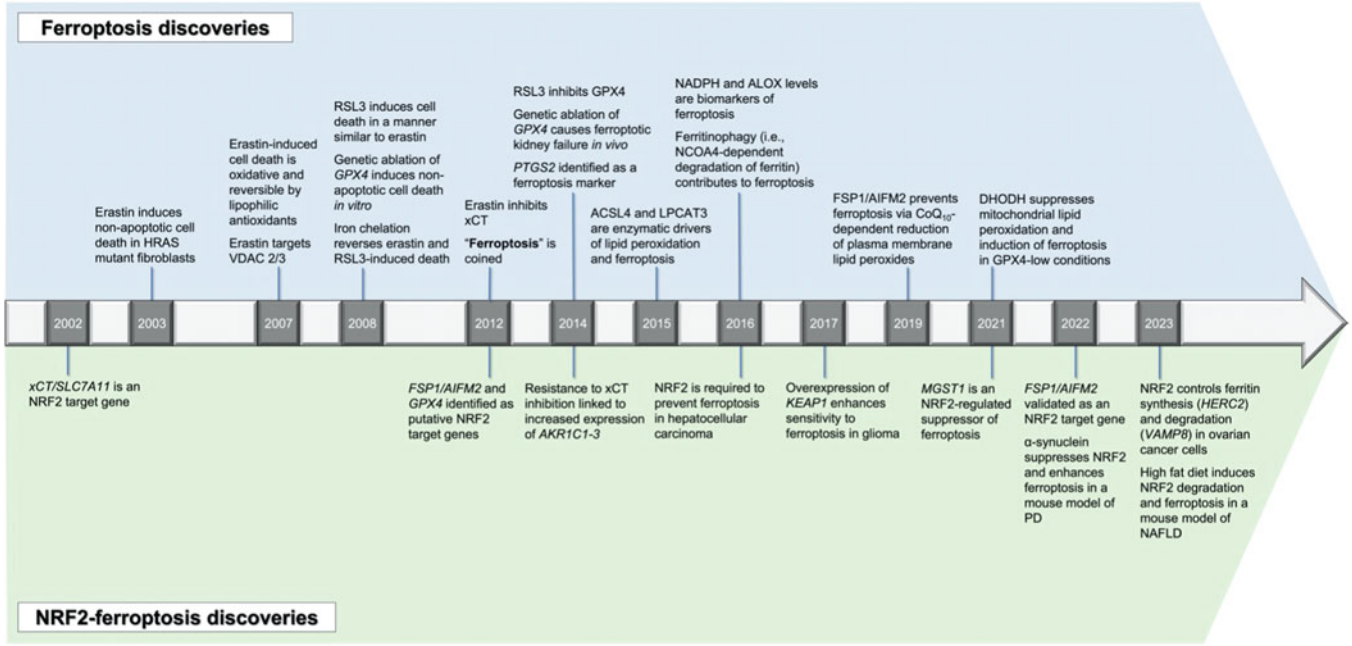

Fig. 10.1 Timeline of key ferroptosis and NRF2-related ferroptosis discoveries. The top half of the timeline (blue) contains the primary findings that have defined the ferroptosis field to date. The bottom half (green) indicates ferroptosis discoveries that are specifically related to NRF2

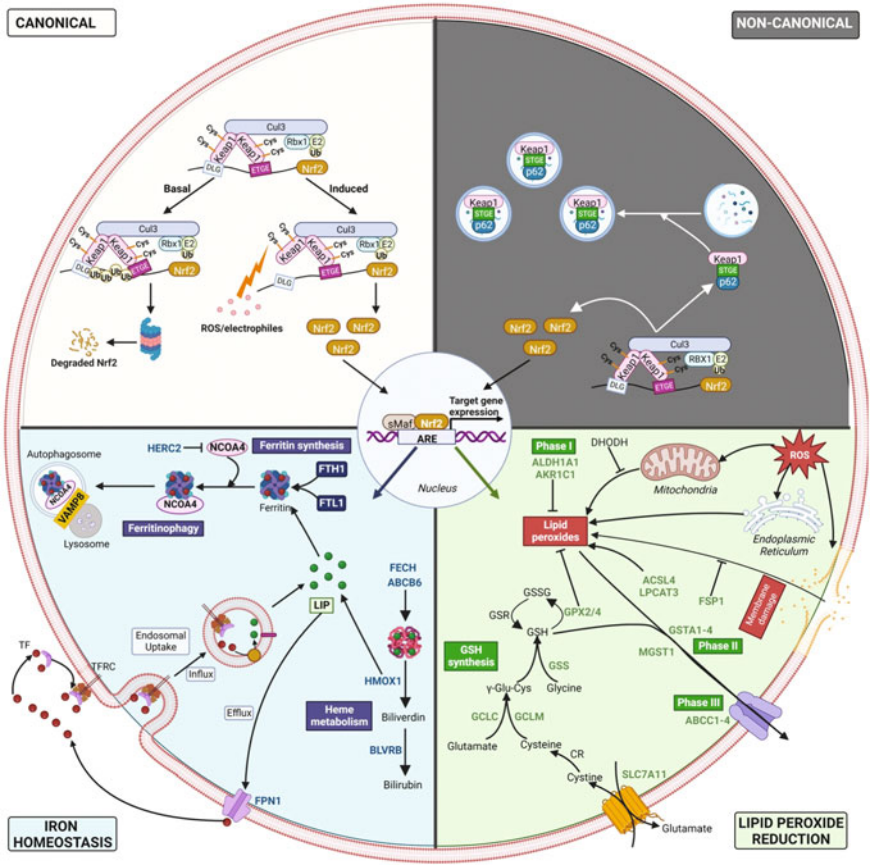

Fig. 10.2 The NRF2 signaling pathway. NRF2 expression is primarily regulated at the protein level by the CUL3-RBX1-KEAP1 E3 ubiquitin ligase complex. Under normal basal conditions, this complex targets NRF2 for degradation by the proteasome. Under stress conditions, NRF2 activation can occur via the canonical pathway (i.e., oxidative/electrophilic modification of KEAP1 cysteines, top left) or the noncanonical pathway (p62-dependent sequestration of KEAP1, top right). Once in the nucleus, NRF2 forms a heterodimer with sMAF proteins and initiates the transcription of ARE-containing genes. Two primary transcriptional programs govern NRF2-dependent regulation of ferroptosis, 1) Iron metabolism (bottom left), and 2) Lipid peroxide reduction (bottom right). Key aspects of NRF2-regulated iron metabolism, including ferritin synthesis, ferritinophagy, and heme metabolism, as well as lipid peroxide reduction, including GSH metabolism, enzymatic production, and reduction of lipid peroxides, as well as conjugation and export are all indicated. All genes indicated in the figure (in bold) are validated NRF2 target genes

a ferroptosis context (discussed in more detail below), there are several transcriptional targets of NRF2 that have been identified over the past two decades that are particularly relevant to the prevention of ferroptosis. One example is *xCT/SLC7A11*, a subunit of the system xc- antiporter, which was initially discovered as an NRF2 target gene in 2002 (Sasaki et al. 2002). In 2012, *AIFM2* (now *FSP1*) and *GPX4*

were both identified to contain putative AREs in a series of ChIP-seq studies (Chorley et al. 2012; Hirotsu et al. 2012). However, while a functional ARE in *FSP1* was later validated through additional analysis (Koppula et al. 2022), *GPX4* has yet to be verified. On the other hand, *GPX2*, which unlike its more ubiquitous counterpart *GPX4* is primarily expressed in the gastrointestinal tract and liver, is directly regulated by NRF2 and plays a role in suppressing ferroptosis (Banning et al. 2005; Wang et al. 2017a).

The first indication that NRF2 might play a role in mediating ferroptosis in cancer came from a pair of studies in 2014 and 2016, where elevated expression of the NRF2 target genes aldo-ketoreductase family 1, members C1–3 (*AKR1C1–3*), and metallothionein-1G (*MT-1G*) was correlated with resistance to xCT inhibition, and NRF2 was clearly shown to be a critical driver of ferroptosis resistance in hepatocellular carcinoma (Dixon et al. 2014; Sun et al. 2016a, 2016b). In 2021, *MGST1* was also identified as an NRF2 target gene that prevents ferroptosis in pancreatic cancer cells (Kuang et al. 2021). In a recent study conducted by our group, it was demonstrated that NRF2 plays a crucial role in regulating iron homeostasis and ferroptosis by controlling ferritin synthesis (*HERC2*) and degradation (*VAMP8*). As such, a combined treatment approach using a ferroptosis inducer and an NRF2 inhibitor has been proposed as a strategy to target ovarian cancer, a refractory malignancy (Anandhan et al. 2023). We also showed that α-synuclein can suppress NRF2 and promote ferroptosis in a mouse model of Parkinson's disease, and that high fat diet-induced nonalcoholic fatty liver disease in mice involves increased degradation of NRF2 and ferroptotic death (A et al. 2022; Liu et al. 2023). Similar to the ferroptosis discoveries indicated above, key NRF2-related ferroptosis discoveries are summarized in Fig. 10.1 (bottom portion). Overall, NRF2 clearly plays a role in mediating ferroptosis, particularly in the context of cancer cell survival. Below, we will provide a detailed overview of the key anti-ferroptotic transcriptional programs that are governed by the NRF2 signaling pathway.

10.4 NRF2 Regulation of Ferroptosis

As NRF2 has been suggested to regulate upwards of 300 target genes whose functions range from dictating the intracellular redox balance to maintaining protein homeostasis, it is not surprising that many have been linked to ferroptosis. Classifying the key pillars that constitute the NRF2 anti-ferroptotic cascade can vary depending on the ferroptotic response in question; however, it can generally be divided into two major categories: 1) Mitigating lipid peroxidation, and 2) preventing accumulation of labile iron. The following section will introduce the target genes mediated by NRF2 that have been linked to preventing ferroptotic death (summarized in Fig. 10.3).

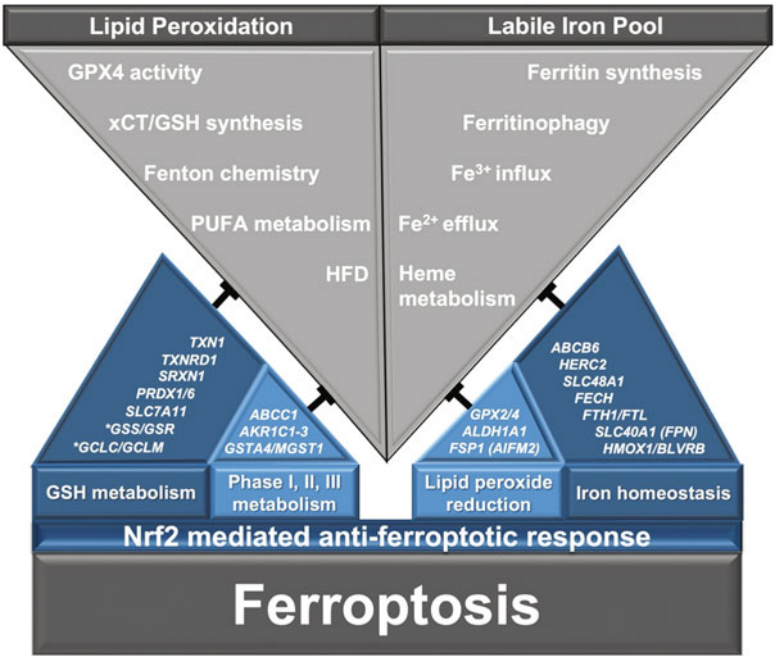

Fig. 10.3 The NRF2-anti-ferroptotic response. There are two critical drivers of ferroptosis, increased lipid peroxide production and accumulation of labile iron. Excess lipid peroxidation can occur as a result of altered GPX4 activity, decreased xCT function/GSH synthesis, increased Fenton-dependent production of hydroxyl radicals, increased catabolism/anabolism of polyunsaturated fatty acids (PUFAs), and exposure to excess exogenous lipids in the form of high fat diet (HFD). Accumulation of labile iron can result from decreased ferritin synthesis, altered ferritinophagy, changes in iron influx/efflux, or aberrant heme metabolism. Several GSH, phase I/II/III, lipid peroxide reducing, and iron-related enzymes/proteins have been specifically identified to prevent ferroptosis. *Indicates target genes involved in GSH metabolism that are also phase II genes

10.4.1 Lipid Peroxidation

Due to the reactive nature of lipid peroxides, which are capable of damaging DNA, proteins, and membrane lipids, several defense systems are in place to ensure they are properly kept in check. This starts with glutathione (GSH), which is present at millimolar concentrations in most cell types, and thus represents one of the first lines of defense against the harmful effects of reactive species. NRF2 has been shown to regulate every aspect of GSH metabolism, starting with the import of cystine, which passes through NRF2 regulated *xCT/SLC7A11* (one subunit of the system xc-glutamate-cystine antiporter) (Sasaki et al. 2002; Hirotsu et al. 2012), and gets reduced intracellularly to cysteine, the critical amino acid that gives GSH its reducing power. The genes that encode the catalytic (*GCLC*) and modulatory (*GCLM*) subunits of glutamate cysteine ligase (GCL), which joins cysteine to

glutamate in the first step of GSH synthesis, are both NRF2 target genes (Erickson et al. 2002; Yang et al. 2005; Hirotsu et al. 2012; Chorley et al. 2012). Glutathione synthetase (GSS), which adds glycine to the newly formed glutamate-cysteine dipeptide, is also regulated by NRF2 (Lee et al. 2005). Along with its synthesis, the enzyme that reduces glutathione (*GSR*, glutathione reductase), as well as several enzymatic systems that utilize GSH to reduce peroxides, including thioredoxin reductase 1/thioredoxin 1 (*TXNRD1/TXN1*), peroxiredoxins 1 and 6 (*PRDX1/6*), and sulfiredoxin 1 (*SRXN1*), are all well-established target genes (Harvey et al. 2009; Wang et al. 2007; Kim et al. 2003; Sakurai et al. 2005; Kim et al. 2007; Chowdhury et al. 2009; Soriano et al. 2009; Hirotsu et al. 2012; Chorley et al. 2012). Several components of phase, I, II, and III drug/xenobiotic detoxification, which are responsible for reducing, conjugating, and exporting reactive intermediates from the cell, are also regulated by NRF2. This includes phase I enzymes, i.e., aldo-ketoreductase family C1, members 1–3 (*AKR1C1–3*), phase II enzymes, including NADPH quinone dehydrogenase 1 (*NQO1*) and several glutathione-S-transferases (*GSTA1–4*, *MGST1*), and phase III multidrug resistance proteins 1–4 (*ABCC1–4*) (Nioi et al. 2003; Lou et al. 2006; Hirotsu et al. 2012; Kuang et al. 2021; Maher et al. 2007; Itoh et al. 1997). Importantly, many of the aforementioned targets have been linked to ferroptosis in different pathologies, including *GCLC/GCLM*, *SLC7A11*, *PRDX1/6*, *SRXN1*, *TXN1/TXRD1*, *AKR1C1*, *ABCC1*, and *GSTA4/MGST1* (Kang et al. 2021; Nishizawa et al. 2020; Lovatt et al. 2020; Lu et al. 2019; Ding et al. 2022; Bai et al. 2021; Yang et al. 2020; Gagliardi et al. 2019; de Souza et al. 2022; Ide et al. 2022; Kuang et al. 2021). Thus, NRF2 regulation of GSH metabolism and phase I, II, and III xenobiotic detoxification represent a key frontline defense in preventing the accumulation of the reactive species needed to initiate lipid peroxidation and subsequent ferroptotic cell death.

Should excessive lipid peroxidation occur, several NRF2-regulated enzymes have been shown to be critical for reducing lipid peroxides, particularly in the context of ferroptosis. Covered in detail above, glutathione peroxidase 4 (GPX4) was one of the first enzymes linked to lipid peroxidation where loss of function resulted in ferroptotic death. Intriguingly, while the majority of the NRF2 field has considered *GPX4* a target gene for the past decade, mostly due to its identification as a putative target gene in an NRF2 ChIP-seq pulldown experiment (Hirotsu et al. 2012), an actual functional ARE has never been fully validated. Thus, while clearly linked to NRF2, its definitive status as a target gene has yet to be verified. Glutathione peroxidase 2 (*GPX2*), on the other hand, has been validated as a bona fide target gene (Banning et al. 2005) and was recently indicated to be a biomarker of ferroptosis across several different cancer types (Luo et al. 2022; Tian et al. 2021; Ping et al. 2022). Aldehyde dehydrogenase family 1 member 1A (*ALDH1A1*) was also shown to be a putative target gene (Hirotsu et al. 2012), as well as several enzymes involved in lipogenesis, including peroxisome proliferator activated receptor gamma (*PPARG*) and nuclear receptor subfamily 0 group B member 2 (*NR0B2*), which could influence lipid peroxidation through excess lipid production, are also mediated by NRF2 (Cho et al. 2010; Huang et al. 2010; Chorley et al. 2012). Finally, ferroptosis suppressor protein 1 (FSP1) was recently identified to prevent ferroptosis

by controlling reduction of the critical membrane antioxidant CoQ10 (Doll et al. 2019; Bersuker et al. 2019). FSP1 was previously known as apoptosis inducing factor mitochondria-associated 2 (*AIFM2*), which was reported as a possible NRF2 target gene in 2012, and recently validated by Boyi Gan's group (Koppula et al. 2022). Overall, it is clear that NRF2 plays a critical role in not only preventing lipid peroxides from forming, but also detoxifying them should an excess of these harmful lipid species arise.

10.4.2 Iron Metabolism and Homeostasis

The other critical requirement for ferroptotic death is the accumulation of labile, reactive ferrous iron (Fe^{2+}), which drives the Fenton-dependent production of reactive hydroxyl radicals that go on to initiate lipid peroxidation in the plasma and organellar membranes. Under normal conditions, iron import, export, metabolism, and storage are all tightly regulated, in large part to prevent the toxicity associated with excess free iron. Similar to its governance of overall antioxidant maintenance and preventing lipid peroxidation, NRF2 also regulates multiple aspects of iron homeostasis. Perhaps one of the more prevalent sources of NRF2-regulated iron processing is heme metabolism, which is mediated by multiple NRF2 targets, including heme oxygenase 1 (*HMOX1*; converts heme to biliverdin), biliverdin reductase B (*BLVRB*; converts biliverdin to bilirubin), solute carrier family 48 member A1 (*SLC48A1*; heme transporter), ATP binding cassette subfamily B member 6 (*ABCB6*; heme synthesis), and ferrochelatase (*FECH*; heme synthesis) (Campbell et al. 2013; Chorley et al. 2012; Hirotsu et al. 2012). HMOX1 in particular has been linked to ferroptosis across several pathological contexts (Chiang et al. 2018; Adedoyin et al. 2018; Wang et al. 2022f; Fang et al. 2023), inferring that NRF2-dependent maintenance of proper heme metabolism could serve as a critical barrier to ferroptosis initiation.

The iron that is not incorporated into heme/iron-containing proteins or iron-sulfur clusters is stored in the critical iron storage protein ferritin. Ferritin consists of alternating repeats of the ferritin heavy (FTH1) and light (FTL) chains that form a cage with ferric iron (Fe^{3+}) at the core. Both *FTH1* and *FTL* are known target genes of NRF2 (Pietsch et al. 2003; Hintze and Theil 2005), indicating that NRF2 is a critical regulator of ferritin synthesis, and thus iron storage. Consistent with this notion, our group recently identified that ferritin stability at the protein level is also regulated by NRF2, as we demonstrated that HECT and RLD domain containing E3 protein ligase 2 (*HERC2*), an E3 ligase that controls the proteasomal degradation of NCOA4 (ferritinophagy adaptor) and F-box and leucine-rich repeat protein 5 (FBXL5; regulator of IRP1/2), is a direct target gene of NRF2 (Anandhan et al. 2023). Interestingly, while not a direct transcriptional target, we also showed that NRF2 regulated the expression of vesicle-associated membrane protein 8 (*VAMP8*; required for the final autophagy fusion step to occur) via the lysosomal-mediated transcription factor TFEB, indicating that NRF2 regulation of ferritin degradation can be both direct and indirect. This NRF2-HERC2-VAMP8 axis played a critical

role in mediating ovarian cancer cell sensitivity to ferroptosis, further supporting the notion that NRF2 and iron reliance represent targetable vulnerabilities in a cancer setting, particularly in NRF2 hyperactivated, refractory cancers (Rodriguez et al. 2022; Anandhan et al. 2020).

10.5 NRF2 and Ferroptosis in Disease

As mentioned above, the relevance of the NRF2-anti-ferroptosis axis in disease was first shown in cancer, but has since expanded to several other pathological contexts. Below, we will highlight some of the acute and chronic pathologies that have been associated with NRF2 prevention of ferroptosis (summarized in Fig. 10.4).

10.5.1 Cancer

Discussed briefly above, the first direct evidence that NRF2 plays an anti-ferroptotic role in cancer was shown in hepatocellular carcinoma, as genetic ablation, or pharmacological inhibition of NRF2 significantly enhanced sorafenib and erastin-induced ferroptotic death (Sun et al. 2016b). Intriguingly, NRF2 activation in this context was via the noncanonical pathway, i.e., p62-dependent sequestration of KEAP1, which has been shown to drive other means of cancer resistance as well (Inami et al. 2011; Saito et al. 2016; Hsu et al. 2022; Li et al. 2020; Ryoo et al. 2018). Noncanonical activation of NRF2 also mediated ferroptosis resistance in head and neck cancer, which could be reversed by treatment with the nonspecific NRF2 inhibitor trigonelline (Shin et al. 2018). NRF2-driven resistance to ferroptosis has also been reported in glioma (Fan et al. 2017; de Souza et al. 2022), gastric cancer (Fu et al. 2021), renal carcinoma (Kerins et al. 2017), colorectal cancer (Yang et al. 2021a), lung adenocarcinoma (Takahashi et al. 2020; Liu et al. 2020b; Wang et al. 2022b), esophageal squamous cell carcinoma (Feng et al. 2021; He et al. 2023), and ovarian cancer (Liu et al. 2020a; Anandhan et al. 2023), indicating that the NRF2-anti-ferroptosis axis represents a significant driver of tumor cell survival. While these studies indicated a direct link to NRF2, it is important to note that the relevance of NRF2 in an anti-ferroptotic context coincides with many of the earliest discoveries in the field, due to NRF2 regulation of *SLC7A11* and *GPX4*. Along with these two critical targets, *GCLC/GCLM*, *FTH1/FTL*, *PRDX6*, and *HMOX1* are also NRF2 target genes shown to promote ferroptosis resistance in a cancer context (Kang et al. 2021; Nishizawa et al. 2020; Yang et al. 2022a; Lu et al. 2019; Roh et al. 2017; Lu et al. 2021). Thus, NRF2-dependent prevention of ferroptosis plays a significant role in promoting cancer progression and resistance, highlighting the need for the development of specific NRF2 inhibitors, before ferroptosis inducers can be successfully applied in the clinic.

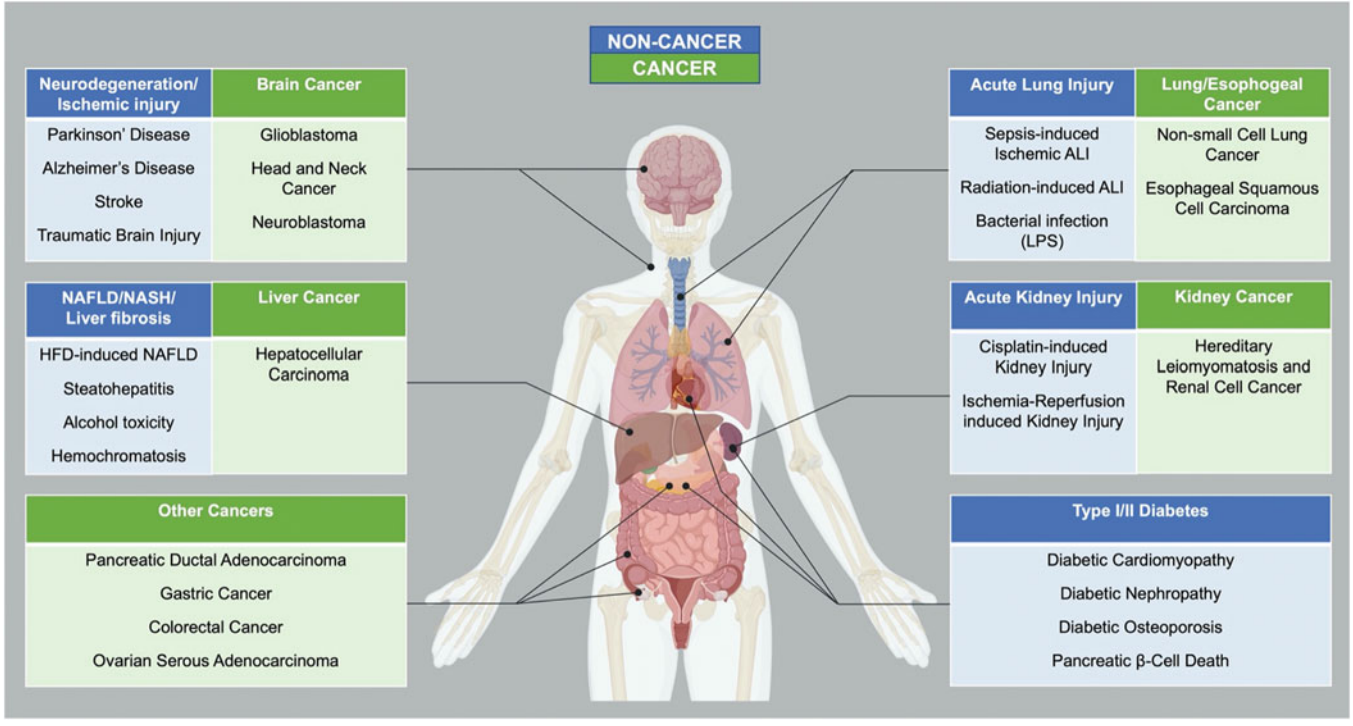

Fig. 10.4 NRF2 regulation of ferroptosis in disease. The anti-ferroptotic role of NRF2 has been demonstrated across several cancer and noncancer disease states. NRF2-ferroptosis-related cancers are indicated in green, and noncancer related pathologies are indicated in blue

10.5.2 Neurodegeneration

Though many of the early findings regarding the pathological relevance of ferroptotic cell death were made in cancer models, a large body of work continues to emerge showing the importance of this cascade in other disease states as well. In the case of neurodegenerative disorders, ferroptosis has just begun to emerge as a prominent mode of cell death in this subset of diseases (Ratan 2020). To date, only a handful of studies have demonstrated a link between NRF2 and ferroptosis in a neuropathological context. For example, noncanonical p62-dependent activation of NRF2 was shown to prevent ferroptosis in 6-hydroxydopamine-treated dopaminergic SH-SY5Y cells (Sun et al. 2020). Pharmacological activation of NRF2 using carnosic acid also protected against erastin-induced ferroptosis in PC12 cells (Cheng et al. 2021). It has also been proposed that BDNF-dependent activation of NRF2 in astrocytes can prevent ferroptosis from occurring in neighboring dopaminergic neurons, although this still needs to be fully validated in an experimental setting (Ishii et al. 2019). Importantly, all of these studies strongly suggest that NRF2 protects against ferroptosis in dopaminergic neurons, one of the primary neuronal cell types lost during the progression of Parkinson's disease (PD). Supporting this notion, work from our group demonstrated that excess α-synuclein significantly enhanced ferroptotic death in vitro, ex vivo, and in vivo using a mouse model that overexpresses human α-synuclein. We also showed that α-synuclein overexpression suppressed NRF2 at the protein level, and that loss of NRF2 increased several key markers of ferroptosis in PD-relevant brain regions in an age-dependent manner (Anandhan et al. 2022). In the context of Alzheimer's disease, several compounds have been shown to ameliorate ferroptosis in erastin- and $A\beta_{1-42}$-treated HT22 hippocampal cells, respectively (Wang et al. 2022a; Li et al. 2022a). Thus, NRF2-mediated regulation of ferroptosis represents a critical mechanism for promoting neuronal survival and slowing down the progression of neurodegenerative diseases.

10.5.3 Diabetes

Another disease state where the relevance of ferroptotic death is evolving rapidly is diabetes, as several diabetic complications have now been linked to ferroptosis. For example, both diabetic cardiomyopathy and diabetic nephropathy in diabetic mouse models were recently linked to ferroptotic cell death that could be prevented by pharmacological activation of NRF2 using sulforaphane and fenofibrate, respectively (Wang et al. 2022d; Li et al. 2021). Similarly, melatonin-dependent activation of NRF2 and its downstream targets SLC7A11 and GPX4 was shown to slow the progression of osteoporosis in diabetic rats (Ma et al. 2020). Additionally, empagliflozin was shown to activate NRF2 to attenuate renal ferroptosis in in vitro and in vivo models of diabetic kidney disease (Lu et al. 2023). Another intriguing study found that autophagy dysfunction-dependent activation of NRF2 led to a chronic increase in lipogenesis that fueled increased lipid peroxidation and cardiomyocyte ferroptotic death in a mouse diabetic cardiomyopathy model (Zang

et al. 2020). Finally, decreased expression of NRF2 was associated with increased pancreatic β-cell ferroptosis in a streptozotocin-injected mouse model of diabetes, which could be rescued by administration of the lipophilic antioxidant ferrostatin-1 (Stancic et al. 2022). These studies already provide a solid foundation indicating that ferroptosis plays an important role in diabetes progression, with more studies sure to come.

10.5.4 Liver Disease

10.5.4.1 NAFLD/NASH

NAFLD (Nonalcoholic fatty liver disease) refers to a group of conditions where there is accumulation of excess fat in the liver of people with little or no alcohol consumption. It is estimated that up to one third of the global population is affected by NAFLD (Henry et al. 2022). Nonalcoholic steatohepatitis (NASH) is recognized as the progressive form of NAFLD characterized by inflammation and liver cell damage (Kleiner et al. 2005). NAFLD increases the risk of type 2 diabetes mellitus (T2DM) (Targher et al. 2016), cardiovascular disease (Targher et al. 2016), and liver cancer, to name a few, but effective therapies are still not available. A recent study found that ferroptosis inhibition protected hepatocytes from death and suppressed the subsequent infiltration of immune cells and the inflammatory response normally observed in a choline-deficient, ethionine-supplemented (CDE) diet model of steatohepatitis. This suggested that hepatic ferroptosis may function as the trigger for initiating inflammation in steatohepatitis (Tsurusaki et al. 2019). Other studies also showed that ferroptosis accompanies the development of fatty liver. For instance, in a high fat diet-fed mouse model, induction of ferroptosis in the liver was confirmed by the presence of increased lipid peroxidation and known ferroptosis biomarkers, including SLC7A11, GPX4, COX2, and ACSL4 (Liu et al. 2023). Consistently, in the clinic, patients with NAFLD usually display increased levels of lipid peroxidation products and systemic oxidative stress markers (Madan et al. 2006). Histological analyses confirmed that patients with NAFLD exhibit a significant increase in 4-hydroxynonenal (4-HNE)-positive cells in the liver (Podszun et al. 2020). Thus, from primary cell models to clinical patient samples, ferroptosis has proved to be an active participant in the pathological development of fatty liver.

Recent studies have indicated that NRF2 activation by a variety of compounds improves NAFLD by suppressing ferroptosis. Sulforaphane, which is a well-known NRF2 activator, suppressed HFD-induced NAFLD and ferroptotic cell death in an NRF2-dependent manner (Liu et al. 2023). Similarly, Delerive's group discovered S217879 as a potent and selective compound that disrupted the KEAP1-NRF2 interaction, leading to robust NRF2 pathway activation and prevention of NASH progression in mice (Seedorf et al. 2023). RNA-sequencing analyses revealed major alterations in the liver transcriptome in response to S217879, with *GPX4* and *PRDX6* (Seedorf et al. 2023), which as mentioned above are established ferroptosis suppressors and NRF2 targets, being significantly upregulated by S217879. These

studies serve as strong evidence that targeting the NRF2-anti-ferroptosis axis could serve as a promising therapeutic strategy for fatty liver disease.

10.5.4.2 Liver Fibrosis

Liver fibrosis is characterized by the deposition of extracellular matrix (ECM) proteins and the formation of a scar in place of normal tissue. It can be caused by various factors, including viral infections, alcohol abuse, NASH, exposure to certain drugs and toxins, and obstruction of bile flow from the liver to the intestines (Kisseleva and Brenner 2021). Both hepatotoxic injury and cholestatic injury can cause inflammation and damage to the liver, leading to the activation of hepatic stellate cells (HSCs) which are engaged in the production of ECM proteins. When aggravated, hepatic fibrosis can progress to cirrhosis and hepatocarcinoma.

Administration of carbon tetrachloride (CCl_4) is one of the most used experimental models for inducing toxin-mediated liver fibrosis (Scholten et al. 2015). Notably, CCl_4-induced liver damage displays key features of ferroptosis, including increased lipid peroxidation, GSH depletion, and SAT1 induction (Zahedi et al. 2012); furthermore, these features are inhibited by prior- or coadministration of the ferroptosis inhibitors/antioxidants deferoxamine (DFO), GSH, or N-acetyl cysteine (NAC) (Cai et al. 2015; Mohammed et al. 2016). NRF2 deficiency in mice was shown to worsen CCl_4-induced liver inflammation and fibrosis (Xu et al. 2008). This study also found that these abnormalities were partly due to the reduced expression of NRF2 target genes which encode enzymes involved in the detoxification of CCl_4 and its metabolites. Further, loss of NRF2 in hepatocytes, but not myeloid lineage cells, worsened oxidative damage and increased malondialdehyde (MDA) levels in response to CCl_4 treatment, which in turn resulted in more severe liver fibrosis (Lyu et al. 2020). It is worth noting that these studies didn't focus specifically on ferroptosis, despite showing some of the key markers and reversibility, and therefore whether ferroptotic cell death is a key contributor to the exacerbated fibrosis observed in NRF2-deficient mice still requires further validation. Another group generated mice expressing a constitutively active NRF2 (caNrf2) mutant in hepatocytes to see if NRF2 overexpression could protect mice from liver injury. Surprisingly, this study found no beneficial effect of NRF2 activation on CCl_4-induced liver injury and fibrosis, at least in the presence of constitutively active NRF2 (Kohler et al. 2014). In contrast, acute pharmacological activation of NRF2 by sulforaphane reduced the levels of MDA, a marker of lipid peroxidation, and improved fibrosis in the livers of CCl_4-treated mice exposed to ethanol (Ishida et al. 2021). Overall, a better understanding of the role of NRF2 and the ferroptosis pathway in the development of liver fibrosis is still needed.

In the context of iron-related liver diseases, hereditary hemochromatosis, which is a type of disease that results from inherited mutations in iron regulatory genes, is characterized by excessive iron deposition in the liver, which eventually leads to fibrosis, cirrhosis, and hepatocellular carcinoma (Pietrangelo 2010). Iron supplementation in the form of ferric citrate potently induced ferroptosis in murine primary

hepatocytes and bone marrow-derived macrophages (Wang et al. 2017b). Further, mice fed with a high iron diet for 8 weeks, developed liver damage with increased expression of ferroptotic markers, which was reversed by ferroptosis inhibition with ferrostatin-1 (Wang et al. 2017b). NRF2 was recently shown to influence the transcriptional response to iron in sinusoidal endothelial cells. Iron-overloaded *Nrf2*$^{-/-}$ mice demonstrated elevated liver lipid peroxidation as revealed by increased MDA levels and elevated fibrotic markers (Lim et al. 2019). In the context of murine hereditary hemochromatosis, loss of NRF2 led to increased parenchymal iron deposition, higher levels of 4-HNE-protein adducts, and the development of a fibrotic phenotype (Duarte et al. 2017; Lim et al. 2019). When the NRF2 activator CDDO-Im was used to treat *Hfe*$^{-/-}$ mice, a mouse model of hereditary hemochromatosis, these mice exhibited reduced liver lipid peroxidation (Lim et al. 2019). Finally, *SLC7A11* was found to be significantly upregulated in iron-treated cells compared to untreated cells, with genetic ablation of *SLC7A11* resulting in enhanced sensitivity to ferroptosis in both hepatocytes and mouse liver (Wang et al. 2017b). Altogether, the NRF2-anti-ferroptosis axis appears to be a promising target for treating iron-overload and hemochromatosis-related liver damage.

10.6 Acute Organ Injury

The NRF2-anti-ferroptosis axis has also been reported to play a role in mediating acute injury across several different organ systems. Increased expression of *SLC7A11* and *HMOX1* was shown to mediate the anti-ferroptotic effects of NRF2 in a model of sepsis-induced ischemic lung injury (Dong et al. 2020). NRF2 activation and prevention of ferroptosis in sepsis-induced acute lung injury (ALI) may be mediated by the AU-rich element binding factor 1 (AUF1), which was shown in a separate study to dictate NRF2 mRNA stability, and thus the ferroptotic response (Wang et al. 2022e). Several other proteins with anti-ferroptotic function have also been linked to NRF2 in carrying out their protective effects in ALI, including telomerase reverse transcriptase (TERT,(Dong, 2021 #17781)) and inhibitor of apoptosis-stimulating protein of p53 (iASPP,(Li et al. 2020 #17782)). Additionally, p62-dependent, noncanonical activation of NRF2 also prevented ferroptotic death via increased expression of *HMOX1*, *NQO1*, and *FTH1* in a model of radiation-induced acute lung injury (Li et al. 2022b). In the liver, pharmacological induction of NRF2 using bardoxolone methyl or bicyclol prevented iron accumulation, lipid peroxidation, and ferroptosis in CCl_4 injured mice (Wu et al. 2022; Zhao et al. 2022). Intriguingly, overexpression of fibroblast growth factor 21 (*FGF21*) or sestrin 2 (*SESN2*) also protected from hepatic ferroptosis in an iron-overload mouse model in an NRF2-dependent manner (Wu et al. 2021; Park et al. 2019). Finally, NRF2 was also indicated to protect against ferroptosis in cisplatin-induced acute kidney injury, as pharmacological activation with leonurine (Hu et al. 2022), or dimethyl fumarate (Yang et al. 2021b), prevented ferroptotic injury, which was lost in NRF2 knockout mice. Furthermore, p62-dependent activation of NRF2 prevented ferroptosis in a mouse ischemic kidney injury model (Yang et al. 2022b).

The NRF2-anti-ferroptosis axis has also been identified to mediate ischemic stroke and traumatic brain injury. One notable recent example was a study indicating that pharmacological administration of the flavonoid icariside II ameliorated cerebral injury following ischemic stroke in an NRF2-dependent manner (Gao et al. 2023). In another study, the myokine irisin attenuated the hippocampal dysfunction and ferroptotic death cause by sepsis-associated encephalopathy via enhanced activity of NRF2 and GPX4 (Wang et al. 2022c). NRF2 mediation of ferroptosis also plays a critical role in traumatic brain injury (TBI), as pharmacological activation improved, whereas genetic ablation enhanced TBI-induced ferroptosis, and the expression of NRF2 and several of its anti-ferroptotic target genes (i.e., *SLC7A11*, *FTH1*, *FTL*, and *FSP1*) were elevated post-TBI (Cheng et al. 2023). Overall, it is clear that upregulation of NRF2 protects, whereas suppression exacerbates, ferroptotic death across multiple organ injury models.

10.7 Conclusions and Future Perspectives

It is becoming increasingly clear that NRF2 is an indispensable barrier to preventing initiation of the ferroptotic cascade. Through its regulation of almost every anti-ferroptotic mediator identified to date, NRF2 represents the first and last line of defense in preventing the iron accumulation and lipid peroxidation required for ferroptosis to occur. Perhaps the most exciting aspect of the NRF2-ferroptosis field is its recent growth, quickly expanding from a cancer context to cover pathologies ranging from acute organ injury to chronic cell loss in neurodegeneration, liver disease, and diabetes. Discussed in detail above, NRF2 primarily exerts its anti-ferroptotic effects through preventing iron accumulation, in large part through dictating ferritin stability and heme metabolism, as well as mitigating lipid peroxidation, via its governance of GSH metabolism and the reduction of peroxides once they form. Of recent interest is the ongoing indication that ferroptosis indeed occurs in normal (patho)physiological settings, as there was early concern that it represented an "artificial" mode of death that only occurred in certain specific contexts in cancer cells. This includes the demonstration of ferroptosis in patient samples across several different disease contexts and the continued expansion of known initiators and effectors of this death pathway. The emergence of NRF2 and its downstream transcriptional mediators of ferroptosis as viable therapeutic targets also holds great promise for successfully targeting this axis to treat disease. It is also worth noting that both canonical (oxidation/electrophilic modification-dependent) and noncanonical (p62-dependent) activation of NRF2 have been shown to prevent ferroptosis across several pathological contexts. This fact, coupled with the intricate network of NRF2-regulated proteins and pathways that have already been linked to ferroptosis, indicates that targeting the NRF2-anti-ferroptosis axis represents an increasingly viable approach for inducing or preventing ferroptosis depending on the disease context.

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Epigenetic Modification in Ferroptosis

11

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Abstract

Ferroptosis is a recently discovered type of cell death that is characterized by significant iron accumulation and lipid peroxidation during the cell death process. Over the past decade, epigenetics has emerged as a key player in mechanisms of disease. Epigenetic changes, whether acquired or inherited, can modulate gene expression states without altering the DNA sequence itself. While the study of epigenetics and its role in ferroptosis is still a nascent research area, it has attracted a great deal of attention and is advancing at a rapid pace, thanks to methodological improvements and important breakthroughs each year. In this chapter, we provide an overview of the interplay between ferroptosis and epigenetic modifications, which could pave the way for novel disease treatments.

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11.1 Introduction

Epigenetic modifications lead to heritable changes in gene function that occur without alterations in the DNA sequence (Zhang et al. 2020a). The primary epigenetic mechanisms include modifications of nucleic acids, histones and non-histones, non-coding RNAs (ncRNAs), chromatin remodeling, and post-translational modifications. These mechanisms play a vital role in the initiation and maintenance of epigenetic silencing and regulation of the gene expression profile and are integral to numerous cellular processes such as gene expression, (Wang et al. 2020a) X-chromosome inactivation, (Loda et al. 2022) embryogenesis, (Xu et al. 2021a) and genomic imprinting (Tucci et al. 2019).

Ferroptosis is a type of non-apoptotic cell death that is characterized by the iron-dependent overproduction of lipid hydroperoxides (Li et al. 2020). Upon depletion of intracellular glutathione (GSH) and a decrease in the activity of glutathione peroxidase 4 (GPX4), lipid peroxides cannot be metabolized by the GPX4-catalyzed reduction reaction. Fe^{2+} then oxidizes lipids in a Fenton-like manner, leading to the production of a large amount of reactive oxygen species (ROS), which promotes ferroptosis (Li et al. 2020). Ferroptosis is a biological process that is regulated by multiple factors; however, the specific regulatory mechanisms require further study.

Like ferroptosis, the epigenetic machinery is highly susceptible to oxidative stress and iron metabolism. As research in this area progresses, numerous genes have already been identified as pivotal to ferroptosis. Research has demonstrated an involvement of epigenetic regulatory mechanisms in ferroptosis. A significant number of epigenetic enzymes and modifications, such as histone lysine demethylase 3B (KDM3B), lymphoid-specific helicase (LSH), and monoubiquitination of H2A-B, have been identified as regulators of ferroptosis. This chapter aims to discuss the epigenetic changes that occur during ferroptosis.

11.2 Molecular Mechanisms of Ferroptosis

Ferroptosis is regulated by multiple metabolic events and signaling pathways. Lipogenesis involves the production of phospholipids containing polyunsaturated fatty acid chains (PUFA-PLs) mediated by acyl-CoA synthetase long-chain family member 4 (ACSL4) and various other enzymes and is required for phospholipid peroxidation and ferroptosis (Yi et al. 2020). Cellular iron is another crucial factor in ferroptosis, and processes such as transferrin receptor- (TFRC-) mediated iron import and autophagy-mediated ferritin degradation promote this process (Battaglia et al. 2020). Additionally, imbalanced cellular redox homeostasis contributes to ferroptosis. When cells lack reducing agents such as cysteine, cellular metabolism, particularly oxidative metabolism in mitochondria, causes an accumulation of ROS, which promotes ferroptosis (Badgley et al. 2020). The vital function of glutaminolysis in cysteine deprivation–induced ferroptosis can be partially explained by its anaplerotic role in supporting the mitochondrial tricarboxylic acid (TCA) cycle (Gao et al. 2019). Multiple signal transduction pathways and

transcription regulators modulate lipogenesis, iron homeostasis, cellular metabolism, and redox homeostasis, altering ferroptosis sensitivity.

11.3 Molecular Mechanisms of Epigenetic Modification

Epigenetic mechanisms lead to heritable changes in gene expression or phenotype that occur without alterations to the underlying DNA sequence (Peixoto et al. 2020). These changes can persist through multiple cell divisions and across multiple generations, without any change in the DNA sequence of the organism. The most notable epigenetic mechanisms include DNA methylation, histone modifications, and ncRNAs.

DNA methylation is a process in which cytosine is selectively methylated at CpG dinucleotides, resulting in the formation of 5-methylcytosine (Moore et al. 2013). There are two groups of DNA methyltransferases (DNMTs): DNMT1, which mainly maintains DNA methylation by methylating hemi-methylated cytosine in double-stranded DNA and may also be involved in the methylation of the newly synthesized DNA strand during replication and DNMT3a and DNMT3b, which are primarily responsible for de novo methylation on unmethylated double-stranded DNA (Qureshi et al. 2022). DNA methylation is generally associated with gene silencing, while DNA demethylation is usually associated with gene activation.

Histone modification is a complex process involving the post-translational modification of histone proteins by enzymes. These post-translational modifications, including methylation, acetylation, phosphorylation, and ubiquitination, (Zhang et al. 2021a) and play a critical role in gene expression by regulating the degree of chromatin compaction. Histone methylation frequently occurs on specific lysine and arginine residues on histones H3 and H4. Histone lysine methylation can lead to either activation or inhibition of gene expression, depending on the location of the modification. For example, methylation events at H3K9, H3K27, and H4K20 are well-known "inactivation" markers and repressive marks; methylation at these sites is associated with heterochromatin formation (Barski et al. 2007). In contrast, the methylation of H3K4 and H3K36 is considered an "activation" mark. Acetylation of histone proteins mostly occurs on N-terminal conserved lysine residues. Acetylation of lysine residues 9 and 14 of histone H3 and of lysines 5, 8, 12, and 16 of histone H4 is associated with chromatin activation or opening (Dhar et al. 2017). Deacetylation of lysine residues leads to chromatin compression and gene transcription inactivation. Different histone modifications can affect each other and can interact with DNA methylation.

ncRNAs can be categorized into two types: housekeeping and regulatory ncRNAs. Regulatory ncRNAs can be further divided on the basis of size into short non-coding RNAs, such as siRNAs, microRNAs (miRNAs), and circular RNAs (circRNAs), and long non-coding RNAs (lncRNAs) (Dhar et al. 2017). Recent studies have demonstrated that ncRNAs play a critical role in epigenetic modification and can regulate gene and chromosome expression, ultimately regulating multiple cellular and biological processes.

11.4 Association Between Epigenetics and Ferroptosis

11.4.1 DNA Modification

DNA modifications play important roles in multiple biological processes and diseases, including cancer, (Skvortsova et al. 2019) cardiovascular diseases, (Corsi et al. 2020) and hemorrhagic injury (Ma et al. 2022). These modifications do not interfere with Watson-Crick pairing but affect the DNA-protein interaction within the major groove of the double helix. Recent studies have begun to reveal the epigenetic mechanisms involved in ferroptosis. Critical epigenetic modifications that regulate ferroptosis are described below.

Hydroxyl radicals are produced during Fe^{2+}-dependent Haber–Weiss reactions that occur in ferroptotic cells (Ma et al. 2022). Research suggests that hydroxyl radicals can generate methyl radicals and cause non-enzymatic methylation of cytosine and guanidine residues in DNA, directly impacting the methylome (Kawai et al. 2010). Erastin, an inhibitor of cystine import, triggers activation of the transsulfuration pathway, limiting the availability of methyl donors required for DNA and histone methylation (Liu et al. 2020). JmjC-domain-containing histone demethylases (JHDMs) and ten-eleven translocation (TET) DNA demethylases are highly dependent on the availability of iron (Cyr and Domann 2011; Wang et al. 2018). DNA modifications also influence ferroptosis. Homocysteine promotes the DNA methylation of GPX4 in the nucleus pulposus, inducing ferroptosis (Zhang et al. 2020b).

11.4.2 RNA Modifications

Cellular RNA is subject to a wide range of diverse chemical modifications that participate in all aspects of RNA metabolism. These modifications include N6-adenosine methylation (m^6A), 5-methylcytidine (m^5C), 2′-O-ribose-methylation (Nm), pseudouridine (Ψ), N7-methylguanosine (m^7G), and N1-adenosine methylation (m^1A) and are found in tRNA, rRNA, mRNA, and other ncRNAs. These modifications play key roles in mechanisms in various diseases such as cancer, metabolic syndrome, heart failure, coronary heart disease, and hypertension. Better understanding of the participation of miRNA, lncRNA, and other types of RNA in disease progression is critical to determine their potential clinical relevance as therapeutic targets or disease biomarkers (Barbieri and Kouzarides 2020; Wu et al. 2021).

11.4.2.1 m^6A

m^6A modification, an N6-methylation that occurs on adenosine in RNA, has been the subject of many epitranscriptomic studies (Oerum et al. 2021). The close relationship between m^6A and ferroptosis has been extensively investigated in various pathological contexts. Several m^6A writers, readers, and erasers have been

studied as potential direct or indirect targets for mediating ferroptosis (Yang et al. 2022; Shen et al. 2021).

The m^6A modification can affect ferroptotic signaling and ferroptosis. For instance, methyltransferase-like 14 (METTL14) catalyzes m^6A modification of the lncRNA KCNQ1OT1, which inhibits miR-7-5p activity. Reduced expression of miR-7-5p leads to increased levels of transferrin receptor, which promotes the uptake of iron and the production of lipid reactive oxygen species (Zhuang et al. 2021). Another study found that the m^6A writer methyltransferase-like 3 (METTL3) was negatively regulated by miR-4443 overexpression, leading to lower levels of m^6A on ferroptosis-suppressing protein 1 (FSP1) and inhibiting FSP1 activity in suppressing ferroptosis (Song et al. 2021). Bioinformatics analyses on lncRNAs have also revealed that m^6A regulators such as fragile X mental retardation 1 (FMR1), heterogeneous nuclear ribonucleoprotein C (HNRNPC), METTL3, and METTL5 are expressed at higher levels in high-risk ferroptosis groups (Zheng et al. 2021).

11.4.2.2 m^5C

The investigation of m^5C is ongoing and is the second only to m^6A in terms of the level of exploration. m^5C is the C5 methylation of cytosine in RNA and is catalyzed by enzymes of the NOL1/NOP2/SUN domain (NSUN) family and the DNA methyltransferase homologue DNMT2. One of the most recent discoveries linking m^5C and ferroptosis involves the role of the exclusive writer of m^5C, NOP2/Sun RNA methyltransferase 5 (NSUN5). Depletion of NSUN5 in bone marrow mesenchymal stem cells reduces the levels of m^5C in ferritin heavy chain 1 (FTH1) and ferritin light chain (FTL) RNA and leads to an increase in intracellular iron concentrations. This in turn results in the downregulation of GPX4, the accumulation of ROS, and lipid peroxidation products, all of which mediate ferroptosis (Liu et al. 2022a).

11.4.2.3 Other RNA Modifications

Other RNA modifications have received less research attention. N4-acetylcytidine (ac4C) modification of mRNA is mediated by N-acetyltransferase 10 (NAT10), which is important for mRNA stability and translation efficiency. Zheng et al. showed that NAT10 plays a crucial role in the development of colon cancer by influencing the stability of FSP1 mRNA and ferroptosis. These findings suggest that NAT10 may be a promising marker and target for the prognosis and treatment of colon cancer.

ncRNAs

ncRNAs are RNA molecules that do not encode proteins and include intronic RNAs, miRNAs, lncRNAs, circRNAs, and extracellular RNAs (Li and Wang 2022). ncRNAs play crucial roles in many biological processes related to ferroptosis, including regulating the mRNA or protein levels of ferroptosis-associated genes, regulating iron metabolism, and altering the Fenton reaction (Fig. 11.1). In this

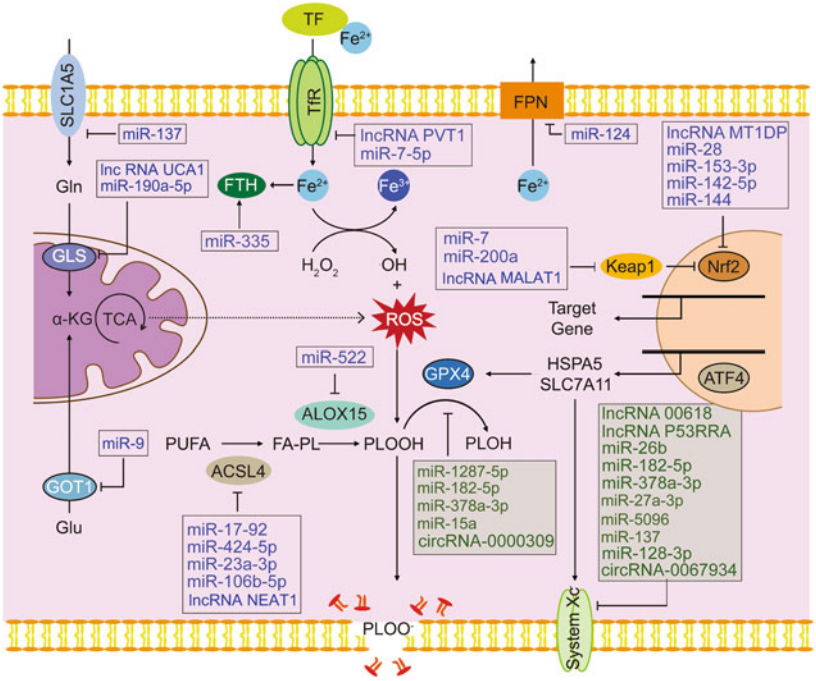

Fig. 11.1 Regulation of ferroptosis by ncRNAs

section, we will discuss three ncRNA groups that have been associated with ferroptosis.

lncRNAs

lncRNAs are RNA transcripts longer than 200 nucleotides with limited protein-coding potential (van Heesch et al. 2019). These lncRNAs are regulators that play important roles in gene expression and diverse physiological and pathological processes, including ferroptosis, and are evolutionarily conserved.

GPX4 maintains redox homeostasis and protects cells against ferroptosis by reducing highly reactive lipid hydroperoxides (LOOH) to non-reactive lipid alcohols and is a target of lncRNAs (Jia et al. 2020). The lncRNA UCA1 upregulates the expression of GSH and NADPH, which allows GPX4 to play an antioxidant role and reduce the production of ROS, ultimately inhibiting ferroptosis (Li et al. 2015; Gao et al. 2015). Other lncRNAs, such as MALAT1, regulate ROS generation by modulating the Kelch-like ECH-associated protein 1 (KEAP1)/nuclear factor erythroid 2-related factor 2 (NRF2) pathway, which in turn affects GPX4 activity (Gao et al. 2015; Radhakrishnan and Kowluru 2021). Additionally, the lncRNA NEAT1 inhibits the expression of ACSL4, increases the expression of SLC7A11 and GPX4, and reduces the iron content in cells, further regulating ferroptosis (Jiang et al. 2020).

lncRNAs also play key roles in regulating iron metabolism, which is an important mechanism in ferroptosis. Fe^{2+} provides electrons to oxygen through the Fenton reaction, leading to ROS formation, which catalyzes the formation of lipid radicals and ultimately ferroptosis (Li et al. 2020). Knock down of the lncRNA PVT1 significantly reduced the expression of TFR1 and increased the expression of FTL, ultimately reducing the uptake of iron and inhibiting ferroptosis (Xu et al. 2018). The lncRNA MT1DP stabilizes miR-365a-3p, which inhibits the expression of NRF2, increases intracellular iron levels, and increases oxidative stress (Gai et al. 2020).

Cysteine metabolism, another important component of ferroptosis, is also regulated by lncRNAs. Cysteine is the most limiting amino acid for GSH synthesis, and inhibition of its import through the cystine-glutamate transport receptor (system Xc-) is sufficient to trigger ferroptosis in vitro (Doll and Conrad 2017). Mao et al. found that the lncRNA P53RRA increased the retention of p53 in the nucleus, inhibiting the transcription of SLC7A11 and reducing the uptake of cysteine, ultimately increasing the sensitivity of cells to ferroptosis (Mao et al. 2018). Furthermore, lncRNA00618 directly induced ferroptosis by reducing the transcriptional activity of SLC7A11 (Wang et al. 2021a). lnc00336 acts as a sponge for miRNA6852 to regulate the expression of cystathionine-β-synthase, a surrogate marker of ferroptosis (Wang et al. 2019a).

lncRNAs also regulate the function of small molecule compounds associated with ferroptosis. Erastin, one of the most common ferroptosis inducers, mediates ferroptosis through a variety of mechanism, including the system Xc−, the voltage-dependent anion channel (VDAC), and p53 (Zhao et al. 2020). Erastin upregulates the lncRNA GABPB1-AS1, which downregulates GABPB1 protein levels by blocking GABPB1 translation, leading to the downregulation of the gene encoding peroxiredoxin-5 (PRDX5) peroxidase and the eventual suppression of the cellular antioxidant capacity (Qi et al. 2019).

miRNAs

miRNAs are a major class of small ncRNAs found in animals, plants, and some viruses, and negatively regulate gene expression at the mRNA level (Saliminejad et al. 2019). They not only have profound effects on metabolism but also play critical functions in the regulation of ferroptosis.

System Xc−, located in the cell membrane, mediates the transport of cysteine and glutamate. Glutamate is transported outside the cell, while cystine is imported. Cystine participates in the generation of GSH, which is used by GPX4 to detoxify lipid peroxidation and plays an essential role in inhibiting ferroptosis. Overexpressing miR-27a-3p leads to SLC7A11 suppression, thereby reducing erastin-caused ferroptosis (Lu et al. 2021). Similarly, miR-5096 induces ferroptotic cell death in human breast cancer cells by reducing the mRNA and protein levels of SLC7A11, resulting in the simultaneous increase of ROS, OH^-, lipid ROS, and iron levels and decreased GSH and mitochondrial membrane potential (Yadav et al. 2021). Zhang et al. demonstrated that lncRNA OIP5-AS1 serves as an endogenous sponge of miR-128-3p to regulate the expression of SLC7A11 and decrease cell viability by enhancing ferroptosis (Zhang et al. 2021b). SLC7A11 is a direct target

of miR-26b, and its expression is increased in both breast cancer cell lines and clinical samples (Liu et al. 2011). Another study showed that miR-1287-5p directly binds to the 3′-UTR region of GPX4 mRNA to inhibit its protein level and activity, leading to ferroptotic induction and tumor suppression (Xu et al. 2021b). miR-15a also interacts with the 3′-UTR region of GPX4 mRNA, which suppresses cell proliferation, elevates lactate dehydrogenase (LDH) release, accumulates intracellular ferrous iron and ROS, disrupts mitochondrial membrane potential (MMP), and increases malondialdehyde (MDA) levels (Xu et al. 2022). miR-182-5p and miR-378a-3p are upregulated in ferroptosis and regulate the expression of GPX4 and SLC7A11 negatively by directly binding to the 3′-UTR (Ding et al. 2020).

Iron is an essential element for ferroptosis, and its homeostasis is strictly regulated. TFRC is a cell surface receptor necessary for cellular iron uptake by receptor-mediated endocytosis. After binding with transferrin (TF), iron is transported into the cell by TFRC and combined with ferritin, a hollow iron storage protein composed of 24 FTH and FTL subunits. Ferroportin (FPN), a cell membrane protein, functions in iron export and removes excess iron. A lack of miR-7-5p expression increases the levels of TFRC, promoting the uptake of iron and production of lipid ROS (Zhuang et al. 2021). Iron storage and release are altered by miRNAs, which target FTH or FPN, respectively. Administration of miR-124 antagomir enhanced FPN expression and attenuated iron accumulation in aged mice models (Bao et al. 2020). Moreover, miR-335 enhanced ferroptosis through the degradation of FTH1 to increase iron release, lipid peroxidation, and ROS accumulation and decrease MMP (Li et al. 2021).

The initiation and execution of ferroptosis are controlled by glutamine metabolism, which is crucial for the synthesis of TCA metabolites, nonessential amino acids, nucleotides, fatty acids, antioxidants, and ATP energy (Huang et al. 2021). Glutamine transport is mediated by SLC5A1. In mitochondria, glutamine is converted to glutamate by glutaminase (GLS) and subsequently deaminated to α-ketoglutarate (α-KG), which is incorporated into the TCA cycle and provides O^{2-} to redox signaling. miR-137 negatively regulates ferroptosis by directly targeting the glutamine transporter SLC1A5, leading to decreased glutamine uptake and MDA accumulation (Luo et al. 2018). Similarly, miR-190a-5p was shown to negatively regulate ferroptosis in rat cardiomyocyte H9c2 cells by directly targeting GLS2, resulting in the downregulation of ROS, MDA, and Fe^{2+} accumulation (Zhou et al. 2021).

Ferroptosis is a form of lipid peroxidation–driven cell death. ACSL4 is an isozyme that catalyzes polyunsaturated fatty acid–containing lipid biosynthesis, promoting the accumulation of lipid peroxidation products and leading to ferroptosis (Wang et al. 2022). Arachidonic acid 15-lipoxygenase-1 (ALOX15) plays a key role in mediating the production of lipid ROS in various types of tissues and tumors (Friedmann Angeli et al. 2014). miR-424-5p negatively regulates ferroptosis by directly targeting ACSL4 in ovarian cancer cells (Ma et al. 2021). miR-23a-3p modulates ferroptosis by directly targeting the 3′-UTR of ACSL4 mRNA (Lu et al. 2022). ACSL4 is also a target gene of miR-106b-5p, and overexpression of miR-106b-5p reverses the effects of the lncRNA H19 on cell viability and

ferroptosis in BMVECs (Chen et al. 2021a). Exosome-miR-522 is a potential inhibitor of ALOX15, leading to ALOX15 suppression and decreased lipid-ROS accumulation in cancer cells, ultimately resulting in decreased chemo-sensitivity (Zhang et al. 2020c).

The KEAP1-NRF2 pathway is the principal protective mechanism and response to oxidative and electrophilic stress. Under normal conditions, KEAP1 is part of the E3 ubiquitin ligase that regulates the levels and activity of NRF2, a transcription factor that is regulated by ubiquitination and proteosomal degradation. When the cell is under stress, KEAP1 undergoes a complex molecular process involving sensor cysteines that facilitates NRF2 to evade ubiquitination, accumulate within the cell, and move into the nucleus. Nuclear NRF2 promotes an antioxidant transcription program, which helps to combat oxidative stress (Baird and Yamamoto 2020). In human neuroblastoma cells, miR-7, which is highly expressed in the brain, represses KEAP1 expression by targeting the 3′-UTR of KEAP1 mRNA. This results in increased NRF2 activity, which decreases intracellular hydroperoxide levels and increases the level of reduced glutathione, indicating relief from oxidative stress. miR-200a has also been reported to inhibit KEAP1 and activate NRF2 signaling (Wang et al. 2020a). Conversely, miR-28 targets the 3′-UTR of NRF2 mRNA and decreases NRF2 expression (Yang et al. 2011). miR-153-3p also suppresses the expression of NRF-2 by binding to its 3′-UTR, while miR-142-5p negatively regulates NRF2 expression in esophageal squamous cell carcinoma (Zuo et al. 2020; Xiao et al. 2021). In obesity, suppression of miR-144 leads to a decrease in fumarate production, which consequently inhibits NRF2 activity (Azzimato et al. 2021).

circRNAs

circRNAs are endogenous biomolecules found in eukaryotes with cell-specific and tissue-specific expression patterns; their biogenesis is regulated by specific cis-acting elements and trans-acting factors (Kristensen et al. 2019). CircRNAs are more stable than other ncRNAs because of their covalently closed loop structure.

An important role for circRNAs in ferroptosis has recently been recognized. circRNAs play a crucial role in regulating lipid peroxidation, a key step in driving ferroptosis. cir93 interacts with and increases the expression of fatty acid–binding protein 3, which transports arachidonic acid and facilitates its reaction with taurine, thereby reducing ferroptosis in lung adenocarcinoma cells (Zhang et al. 2022a). circPtpn14 (mmu_circ_0000130) acts as a miR-351-5p sponge to upregulate the expression of the ferroptosis-related 5-lipoxygenase (Wu et al. 2022). Moreover, mmu_circRNA_0000309 competitively sponges miR-188-3p and upregulates the lipid repair enzyme GPX4, which inactivates ferroptosis-dependent mitochondrial damage (Jin et al. 2022). In thyroid cancer cells, circ_0067934 upregulates the expression of SLC7A11, a ferroptosis-negative regulator, by sponging and inhibiting miR-545-3p (Wang et al. 2021b). Similarly, circ0097009 acts as a competing endogenous RNA to regulate the expression of SLC7A11 by sponging miR-1261 in hepatocellular carcinoma (Lyu et al. 2021).

circRNAs also affect iron homeostasis. CircRAPGEF5 interacts with the Fox-1 C-terminal domain of RBFOX2 and causes a targeted exclusion of a specific exon in TFRC by blocking RBFOX2's binding to pre-mRNA. This ultimately results in a reduction of the labile iron pool and decreased production of lipid peroxides. Elevated levels of circRAPGEF5 result in ferroptosis resistance (Zhang et al. 2022b).

11.5 Chromatin Remodeling Factors

Chromatin is a highly dynamic and plastic structure that exhibits compositional diversity, providing it with a high potential to modify genome topology and orchestrate gene regulation across various cellular processes, including ferroptosis (Michael and Thomä 2021). LSH, a member of the chromatin remodeling ATPase SNF2 family, is a chromatin remodeling protein that plays a stabilizing role by maintaining an appropriate DNA methylation level (Han et al. 2020). Recent studies have demonstrated that LSH also participates in metabolic pathways associated with ferroptosis. By interacting with WDR76, LSH inhibits ferroptosis by activating lipid metabolism-associated genes such as glucose transporter type 1 (GLUT1), stearoyl-CoA desaturase (SCD1), and fatty acid desaturase 2 (FADS2) through DNA methylation and histone modification (Jiang et al. 2017).

11.6 Histone Modifications

Histones serve as the framework for DNA packaging. The tails of the four core histones (H2A, H2B, H3, and H4) are subject to chemical modifications including methylation, acetylation, ubiquitination, and glycosylation, which alter the interactions between histones and nuclear proteins including the transcriptional machinery, ultimately changing the expression of targeted genes (Lawrence et al. 2016). In this section, we summarize the roles of various histone modifications in ferroptosis (Fig. 11.2).

DNA methylation is an important epigenetic modification that regulates gene expression and is implicated in many cellular processes (Martisova et al. 2021). The di- or tri-methylation of H3K9 is a well-established epigenetic mark of heterochromatin and associated with transcriptional silencing (Becker et al. 2016). Deficiency of suppressor of variegation 3–9 homologue 1 (SUV39H1) modulates the H3K9me3 status of the dipeptidyl-peptidase-4 (DPP4) gene promoter, resulting in upregulation of its expression and contributes to ferroptosis (Wang et al. 2021c). The histone methyltransferase SET domain bifurcated 1(SETDB1) significantly promotes the expression of E-cadherin, which reduces lipid ROS and ferrous ions and inhibits ferroptosis (Liu et al. 2022b). Conversely, KDM3B, a histone H3 lysine 9 demethylase, can protect against ferroptosis induced by erastin, an inhibitor of SLC7A11 (Wang et al. 2020b). Lysine demethylase 3B (KDM3B) upregulates the expression of SLC7A11 through cooperation with the transcription factor activating

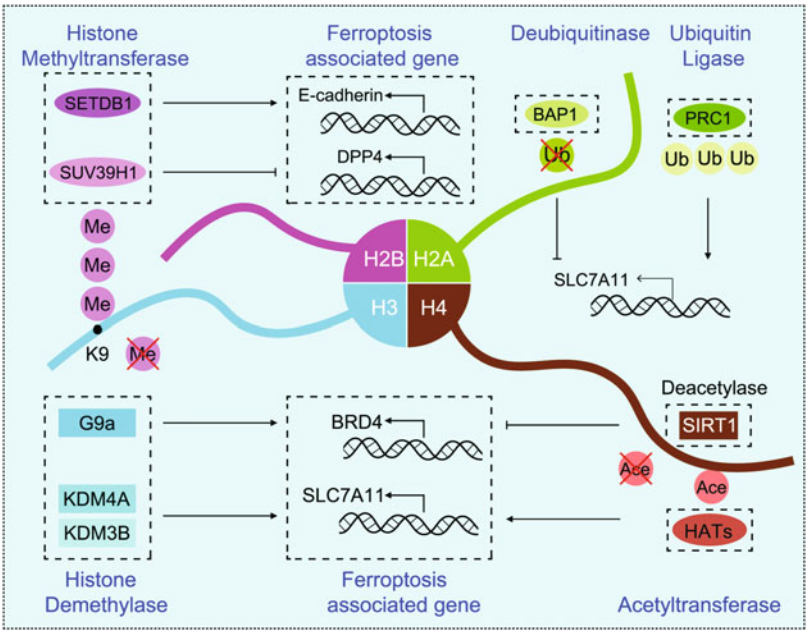

Fig. 11.2 Histone modifications and the regulation of ferroptosis

transcription factor 4 (ATF4) by decreasing histone H3 lysine 9 methylation (Wang et al. 2020b). KDM4A regulates SLC7A11 transcription and cells ferroptosis by controlling H3K9me3 demethylation in the promoter region of SLC7A11 (Chen et al. 2021b). Multiple lysine and arginine residues of histones (such as H3R2, H3K4, H3K27, H3K79, H4R3, H4K20, and H2BK5) also undergo methylation/demethylation and exhibit pleiotropic roles in gene transcription (Jambhekar et al. 2019). Therefore, determining whether methylation of histones and their writers, readers, and erasers also participate in the regulation of ferroptosis should be explored.

Acetylation is a widespread, reversible post-translational protein modification that is regulated by two classes of enzymes: lysine acetyltransferases and lysine deacetylases (Shvedunova and Akhtar 2022). Over 40 different lysine residues in the four canonical core histones, H2A, H2B, H3, and H4, are modified by acetylation (Zhao and Garcia 2015). NRF2 activates the transcription of the SLC7A11 gene partially through recruiting HAT, a histone acetyltransferase (Chen et al. 2017). Moreover, (+)-JQ1 regulates ferritinophagy and the expression of ferroptosis-associated genes via epigenetic inhibition of bromodomain-containing protein 4 (BRD4) by suppressing the expression of the histone methyltransferase G9a or enhancing the expression of the histone deacetylase silent information regulator Sirtuin 1 (SIRT1) (Sui et al. 2019).

Histone ubiquitination is distinct from other post-translational modifications of histones, which usually involve small chemical groups. Ubiquitination involves the

covalent binding of a 76-amino acid protein, ubiquitin, to lysine residues on proteins or to ubiquitin itself, resulting in the formation of different types of polyubiquitin chains. Ubiquitination involves the sequential activity of E1 activating, E2 conjugating, and E3 ligase enzymes (Mattiroli and Penengo 2021). The regulation of SLC7A11 expression and erastin-induced ferroptosis is critically dependent on H2A ubiquitination/de-ubiquitination. BRCA1-associated protein (BAP1), a tumor suppressor and H2A deubiquitinase, suppresses SLC7A11 expression by reducing H2A ubiquitination (H2Aub) on the SLC7A11 promoter (Zhang et al. 2019). Conversely, the phosphorylating protein regulator of cytokinesis 1 (PRC1) complex, a well-known ubiquitin ligase of H2Aub, is responsible for establishing H2A ubiquitination on the SLC7A11 promoter (Zhang et al. 2019). These findings suggest that dynamic regulation of H2Aub is crucial for SLC7A11 repression in cancer cells (Zhang et al. 2019). Studies showed that histone H2B monoubiquitination (H2Bub1) negatively regulates the Warburg effect and tumorigenesis in human lung cancer cells by controlling the expression of multiple mitochondrial respiratory genes essential for oxidative phosphorylation (Jing et al. 2020). H2Bub1 also regulates the expression of SLC7A11 and a group of ion-binding genes involved in various metabolism-related processes, highlighting the role of H2Bub1 as a novel epigenetic regulator of ferroptosis. Moreover, H2Bub1 epigenetically activates SLC7A11 expression, thereby inhibiting cellular sensitivity to ferroptosis (Wang et al. 2019b).

11.7 Non-histone Modifications

Multiple pathways participate in ferroptosis, including the iron metabolism pathway, lipid metabolism pathway, and system Xc- (Li et al. 2020). Epigenetic modifications of these pathways play critical functions in ferroptosis.

Acetylation is catalyzed by a series of acetyltransferases that transfer acetyl groups (-CO-CH_3) from acetylated molecules, including acetyl-coenzyme A, to specific amino acid residues of protein substrates (Polevoda and Sherman 2002). Acetylation plays a vital role in many biological phenomena, including ferroptosis. Acetylation of p53 has a significant impact on its influence on apoptosis and ferroptosis. The p53 acetylation-defective mutant (3KR) fails to induce cell cycle arrest, senescence, and apoptosis but it fully retains the ability to regulate SLC7A11 expression and induce ferroptosis upon ROS-induced stress (Jiang et al. 2015). Activation of spermidine/spermine N1-acetyltransferase 1 (SAT1) induces lipid peroxidation and sensitizes cells to undergo ferroptosis upon ROS-induced stress, which suppresses tumor growth in xenograft tumor models (Ou et al. 2016).

Protein glycosylation is the covalent attachment of single sugars or glycans, including multi-sugar polysaccharides or complex oligosaccharides, to specific residues of target proteins (Eichler 2019). The role of glycosylation modification of proteins in ferroptosis has not been well discussed. Downregulation of N-acetylgalactosaminyltransferase-14 (GALNT14) suppressed the activity of the mTOR pathway by modifying O-glycosylation of EGFR, resulting in inhibiting

both apoptosis and ferroptosis of ovarian cancer cells (Li et al. 2022). Deficiency of lysosome-associated membrane protein-2, a highly glycosylated lysosomal membrane protein, reduced the concentration of cytosolic cysteine, resulting in low GSH, inferior antioxidant capability, and mitochondrial lipid peroxidation and leading to ferroptosis (Lee et al. 2020).

Ubiquitination is vital for multiple cellular processes and regulates multiple proteins related to cell growth, proliferation, and survival (Han et al. 2022). Among the ubiquitination system components, E3 ubiquitin ligases and deubiquitinases have the most prominent roles in modulating ferroptosis. Iron is necessary for ferroptosis, and FPN is the only iron-exporting protein on the cytoplasmic membrane (Hassannia et al. 2019). Ubiquitination of FPN is a key mechanism that regulates its function. The ubiquitin ligase RNF217 mediates the degradation of FPN and regulates iron homeostasis (Jiang et al. 2021). The key regulatory factors of ferroptosis are also regulated by ubiquitination. The reduction of ubiquitin specific peptidase 11 (USP11), a deubiquitinase of NRF2, contributes to the suppression of cell proliferation and induction of ferroptotic cell death from ROS-mediated stress (Meng et al. 2021). Quiescin sulfhydryl oxidase homolog (QSOX1) restrains EGF-induced EGFR activation by promoting ubiquitination-mediated degradation of EGFR and accelerating its intracellular endosomal trafficking, leading to the suppression of NRF2 activity (Sun et al. 2021). The ubiquitin hydrolase OTUB1 maintains SLC7A11 stability through direct interaction with SLC7A11, and this process does not depend on the participation of p53 (Liu et al. 2019). Depletion of NEDD4, a HECT domain E3 ubiquitin ligase, limits the protein degradation of VDAC, which increases the sensitivity of cancer cells to erastin, a ferroptosis inducer (Yang et al. 2020). SCD1, an enzyme that catalyzes the rate-limiting step in monounsaturated fatty acid synthesis, inhibits ferroptosis by increasing coenzyme Q-binding protein 10 (CoQ10) (Tesfay et al. 2019). CoQ10 is an endogenous membrane antioxidant whose depletion has been linked to ferroptosis.

11.8 Selenium

Selenium (Se) is an essential micronutrient that plays a crucial role in development and a wide variety of physiological processes, including immune responses (Avery and Hoffmann 2018). Selenoproteins are proteins with a selenocysteine (Sec, U, Se-Cys) and include five antioxidant ATF glutathione peroxidases and three thioredoxin reductases (TrxR/TXNRD), both of which contain only one Se (Santesmasses et al. 2020). As mentioned above, the glutathione-dependent lipid hydroperoxidase GPX4 prevents ferroptosis by converting lipid hydroperoxides into non-toxic lipid alcohols (Bersuker et al. 2019). However, the upstream regulatory mechanisms are unknown. Augmentation of the selenome gene via selenium-mediated epigenetic regulation inhibited ferroptosis and protected against neuronal cell death after brain hemorrhage. Pharmacological selenium augments GPX4 and other genes in this transcriptional program, the selenome, via coordinated activation of the transcription factors TFAP2c and Sp1, to protect neurons (Alim et al. 2019).

High levels of GPX4 expression are critical for an adaptive response and reduce cell death after intracerebral hemorrhage by inhibiting ferroptosis. Furthermore, Tat-linked SelP peptide (Tat SelPep), a selenocysteine-containing peptide created artificially, has the same function to improve outcomes after stroke in an Sp1-dependent manner, providing a wider therapeutic window. Pharmacological selenium may be a novel therapeutic strategy to block ferroptosis and promote cell survival.

11.9 Conclusion

The present review provides an overview of epigenetics and its involvement in ferroptosis, a recent but rapidly growing research field. Recent methodological advancements have increased interest and led to important progress in understanding the relationship between epigenetics and ferroptosis. While current research has focused on several key ferroptosis genes, the mechanisms by which epigenetic regulation affects other ferroptosis-related genes and signaling pathways remain largely unknown. A more comprehensive and systematic study of the epigenetic regulatory network involved in ferroptosis is needed.

Given the close association of ferroptosis with diseases such as cancer, cardiovascular disease, and neurotoxicity, investigating the epigenetic mechanisms underlying altered ferroptosis sensitivity in different pathological contexts is of great interest. Advances in this cross-disciplinary research may offer new insights and treatment options for these diseases. Although the precise role of epigenetic mechanisms in ferroptosis is not yet fully understood, ongoing studies in this area hold great promise for overcoming current barriers. Further investigation into epigenetic molecules and their role in ferroptosis will contribute to the development of novel treatments for ferroptosis-related diseases.

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Organelle-specific Mechanisms of Ferroptosis

12

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Abstract

Ferroptosis, which occurs due to the peroxidation of unsaturated lipids, is associated with several pathological conditions. The fate of a ferroptotic cell is determined by the extent of damage and repair of biological membranes. In recent years, it has been discovered that multiple organelles are involved in regulating intracellular signaling pathways that either promote or inhibit ferroptosis. This chapter is dedicated to discussing the regulation of ferroptosis by various organelles, such as mitochondria, lipid droplets, endoplasmic reticulum, lysosomes, ribosomes, peroxisomes, and the Golgi apparatus. Given the crucial role of lipid peroxidation in this process, we emphasize the importance of various cellular events, including ROS production, antioxidant systems, iron metabolism, and lipid metabolism, in different organelles.

12.1 Introduction

Ferroptosis is a form of cell death triggered by excessive lipid peroxidation-mediated membrane damage (Dixon et al. 2012; Xie et al. 2016). The onset of lipid peroxidation in ferroptosis is primarily caused by the oxidation of polyunsaturated fatty acids (PUFAs) in membrane lipids, which is dependent on the presence of iron. The key antioxidant system that counteracts lipid peroxidation is composed of solute carrier family 7 member 11 (SLC7A11), glutathione (GSH), and glutathione peroxidase 4 (GPX4) (Dixon et al. 2012; Yang et al. 2014). SLC7A11, a plasma membrane

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antiporter, plays a critical role in maintaining GSH synthesis by facilitating the transport of cystine. Within cells, cystine is converted to cysteine, which serves as a building block for GSH synthesis. GPX4, a GSH-dependent enzyme, reduces lipid peroxides to their alcohol form, thus limiting lipid peroxidation. Inhibition of SLC7A11-mediated cystine uptake or GSH-dependent GPX4 activity can result in excessive lipid peroxidation and subsequent ferroptosis (Dixon et al. 2012; Yang et al. 2014). GPX4 is found in the cytosol, mitochondria, and nucleus. While the roles of cytosolic and mitochondrial GPX4 in defending against ferroptosis are well established, the function of nuclear GPX4 remains incompletely understood. In addition to the GSH-dependent pathway, cells possess a GSH-independent mechanism to resist ferroptosis. For instance, in the cytosol, apoptosis-inducing factor mitochondria-associated 2 (AIFM2/FSP1) reduces Coenzyme Q (CoQ) to its active form, $CoQH_2$. Similarly, in the mitochondria, dihydroorotate dehydrogenase (DHODH) or glycerol-3-phosphate dehydrogenase 2 (GPD2) are responsible for this reduction process (Doll et al. 2019; Bersuker et al. 2019; Mao et al. 2021b; Wu et al. 2022). $CoQH_2$ functions as a lipidic antioxidant, effectively limiting lipid peroxidation in ferroptosis. Lipid peroxidation-induced disruption of the cell membrane can be repaired by the endosomal sorting complex required for transport-III (ESCRT-III) machinery (Dai et al. 2020b; Pedrera et al. 2020).

Biological membranes play a crucial role in maintaining cellular architecture and physiology. The plasma membrane acts as a barrier, separating the cell's interior from the external environment, and regulates the transport of substances into and out of the cell. The nucleus is surrounded by the nuclear envelope, which serves as a repository of genetic information and controls cell metabolism and heredity. In eukaryotic cells, membrane-bound organelles provide compartments that partially restrict the free exchange of proteins and metabolites. Autophagy, an intracellular membrane trafficking pathway, functions to clear damaged or unnecessary cytoplasmic material. It has a dual role in cell death, contributing both to protection and destruction (Kroemer and Jaattela 2005). The regulation of ferroptosis involves various regulators in different organelles, including mitochondria, lipid droplets (LDs), endoplasmic reticulum (ER), lysosomes, ribosomes, peroxisomes, and the Golgi apparatus (Chen et al. 2021e, f).

12.2 Role of Mitochondria in Ferroptosis

Mitochondria are dynamic organelles involved in energy generation, metabolism, reactive oxygen species (ROS) production, and cell death. In the context of ferroptosis, both mitochondria-dependent and mitochondria-independent pathways have been reported. Mitochondrial depletion has been shown to promote ferroptosis induced by cystine starvation or the inhibition of SLC7A11 (e.g., erastin), while it does not affect cellular sensitivity to GPX4 inhibitors (e.g., RSL3 and FIN56) (Gao et al. 2019; Gaschler et al. 2018). Several regulators of mitochondrial metabolism, iron, ROS, mitochondrial DNA (mtDNA), and proteins have been implicated in the regulation of the ferroptotic response (Fig. 12.1).

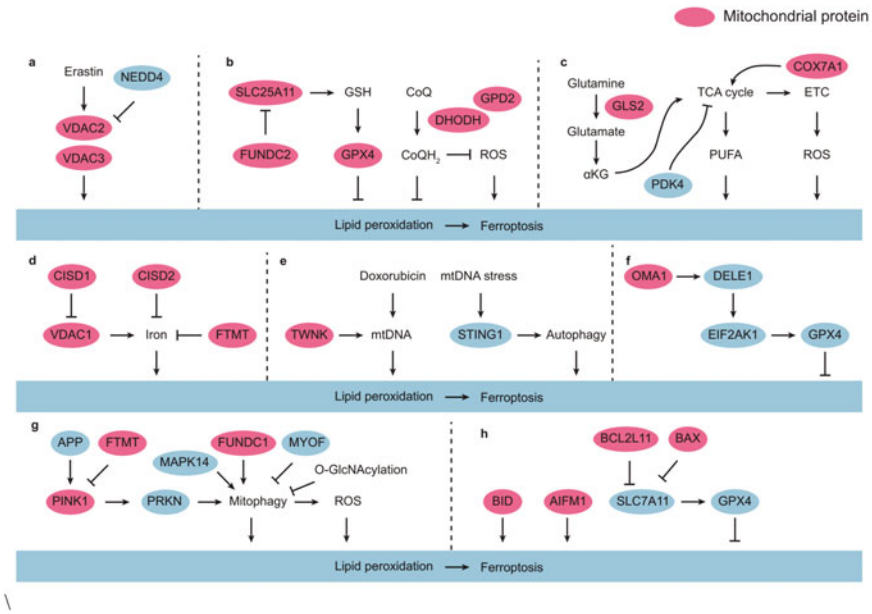

Fig. 12.1 Role of mitochondria in ferroptosis. Ferroptotic response is regulated by mitochondrial VDACs (**a**), ROS production and antioxidant system (**b**), TCA cycle and ETC (**c**), iron (**d**), mtDNA stress (**e**), proteases OMA1 (**f**), mitophagy (**g**), and apoptosis-related proteins (**h**)

12.2.1 Morphological Morphology in Ferroptosis

In electron microscopic studies, ferroptotic cells exhibit intact nuclei and show no alterations in nuclear morphology, which distinguishes them from apoptotic cells (Yagoda et al. 2007). However, the most prominent mitochondrial morphological changes observed in ferroptosis include increased membrane densities, decreased volume, and a reduction in the number of cristae (Dixon et al. 2012; Friedmann Angeli et al. 2014). These changes suggest that ferroptosis may induce mitochondrial dysfunction. However, the precise mechanisms underlying these alterations in mitochondrial morphology remain largely unclear.

12.2.2 Mitochondrial VDACs as Targets for Erastin

The voltage-dependent anion channel (VDAC) is a mitochondrial porin located in the outer membrane of mitochondria. It facilitates the exchange of various ions and metabolites between the mitochondria and cytosol. Additionally, VDAC serves as a platform for numerous proteins such as hexokinase (HK) or BCL2-associated X, apoptosis regulator (BAX), which may be involved in cell survival or cell death processes (Shoshan-Barmatz et al. 2008). Studies utilizing affinity-based target identification have identified VDACs as important targets for erastin (Yagoda

et al. 2007). Knockdown of VDAC2 or VDAC3 has been shown to inhibit erastin-induced ferroptosis. However, overexpression of VDAC3 does not affect erastin sensitivity, suggesting that while VDACs are necessary for erastin-induced ferroptosis, they are not sufficient on their own (Yagoda et al. 2007). Consistently, an inhibitor of VDAC oligomerization called VBIT-12 has been found to alleviate ferroptosis-induced liver injury in mice with acetaminophen-induced liver injury (Niu et al. 2022). Furthermore, ubiquitination plays a significant role in the regulation of VDAC during ferroptosis. Erastin induces the degradation of VDAC2 or VDAC3 through the ubiquitin-proteasome system in melanoma cells (Yang et al. 2020). NEDD4 has been identified as an essential E3 ligase for the ubiquitination and degradation of VDAC2 or VDAC3 induced by erastin (Yang and Stockwell 2008). Inhibition of NEDD4 enhances the stabilization of VDAC2 or VDAC3 and increases sensitivity to erastin (Yang and Stockwell 2008). Interestingly, erastin has also been shown to induce ROS-dependent apoptosis in colorectal cancer cells through its interaction with VDAC1 (Huo et al. 2016), suggesting that VDAC serves as a regulatory hub for multiple types of cell death. However, GPX4 inhibitors (e.g., RSL3) appear to induce ferroptosis in a manner independent of VDAC and NEDD4 (Yang and Stockwell 2008; Yang et al. 2020). It remains unclear whether the inhibition of cysteine uptake by erastin affects the function of VDAC isoforms.

12.2.3 Mitochondrial ROS

Mitochondria are intracellular organelles that generate a significant amount ROS. While earlier articles dismissed the involvement of mitochondrial ROS in ferroptosis (Dixon et al. 2012), recent studies have revealed that mitochondrial ROS can act as a crucial source of ROS-mediated ferroptotic cell death in specific contexts (Liang et al. 2022). In erastin-treated mouse embryonic fibroblasts (MEFs), lipid peroxidation in mitochondria occurs earlier than in the plasma membrane (Gao et al. 2019). Ferroptosis can lead to an increase in hypochlorous acid (HClO), a reactive oxygen species, within mitochondria (Fang et al. 2022). Consistently, mitoquinone, a mitochondria-targeted antioxidant, has been shown to reverse erastin- or RSL3-induced ferroptosis in liver cancer cells or mouse hippocampal cells (Jelinek et al. 2018; Oh et al. 2022). Similarly, the mitochondria-targeted antioxidant MitoTEMPO effectively scavenges mitochondrial ROS to inhibit ferroptosis in doxorubicin-induced cardiomyopathy, whereas non-mitochondria-targeted antioxidants have weaker effects (Fang et al. 2019). Additionally, malondialdehyde (MDA), one of the end products of lipid peroxidation, exhibits higher levels within the mitochondria of doxorubicin-induced ferroptotic cardiomyocytes compared to the non-mitochondrial fraction (Tadokoro et al. 2020). These findings highlight the significant role of mitochondrial ROS in ferroptosis-related diseases.

Mitochondria, being inherently producers of ROS, also possess powerful antioxidant systems to detoxify both endogenous and exogenous ROS. Antioxidant enzymes such as GPX4 and DHODH, as well as endogenous substances like CoQ and GSH, located within mitochondria, play a critical role in suppressing ferroptosis.

GPX4, in addition to its cytoplasmic distribution, is also present in mitochondria and protects them from ferroptosis-mediated doxorubicin cardiotoxicity (Tadokoro et al. 2020). CoQ, synthesized at the mitochondrial inner membrane, acts as a GSH-independent endogenous molecule with strong anti-ferroptotic activity. Within mitochondria, DHODH or GPD2 promotes the reduction of CoQ to $CoQH_2$, a lipid-soluble antioxidant that effectively counteracts lipid peroxidation and inhibits ferroptosis (Mao et al. 2021a; Wu et al. 2022). Inactivation of GPX4 increases the flux through DHODH, leading to enhanced generation of $CoQH_2$ to neutralize lipid peroxidation and inhibit ferroptosis (Mao et al. 2021a). Consequently, inhibition of both mitochondrial GPX4 and DHODH results in the robust accumulation of mitochondrial lipid peroxidation and promotes ferroptosis.

Moreover, the mitochondrial protein presenilin-associated rhomboid-like (PARL) contributes to cellular protection against ferroptosis by facilitating the transport of CoQ from mitochondria to the plasma membrane (Deshwal et al. 2023). GSH, synthesized in the cytosol, is also localized within mitochondria at a concentration similar to that in the cytosol (Mari et al. 2009). Inhibition of the GSH transporters solute carrier family 25 member 10 (SLC25A10) and solute carrier family 25 member 11 (SLC25A11) depletes mitochondrial GSH and exacerbates ferroptosis in H9c2 cardioblasts (Jang et al. 2021). Specifically, FUN14 domain-containing protein 2 (FUNDC2), a mitochondrial outer membrane protein, promotes ferroptosis by interacting with SLC25A11 to decrease mitochondrial GSH levels (Ta et al. 2022). Thus, the mitochondrial antioxidant systems play a crucial role as important regulators of ferroptotic cell death.

12.2.4 Mitochondrial Tricarboxylic Acid Cycle and Oxidative Phosphorylation

In eukaryotic systems, the tricarboxylic acid (TCA) cycle and oxidative phosphorylation (OXPHOS) occur within mitochondria and are closely linked to ATP production. Under normal conditions, glucose, as the primary energy source, is converted to pyruvate via glycolysis in the cytoplasm. Pyruvate then enters the mitochondria to produce acetyl CoA, which fuels the TCA cycle and OXPHOS for complete metabolization. As discussed earlier, ROS generated by the electron transport chain (ETC) within the OXPHOS system may promote ferroptosis. Consequently, inhibitors of the ETC are known to suppress cystine starvation or erastin-induced ferroptosis (Gao et al. 2019). There is evidence suggesting that the TCA cycle is required for ferroptosis. Cytochrome c oxidase subunit 7A1 (COX7A1), a subunit of the mitochondrial ETC, enhances sensitivity to ferroptosis induced by cysteine deprivation through the promotion of mitochondrial TCA cycle activity (Feng et al. 2022). The activity of α-ketoglutarate dehydrogenase (KGDH), an enzyme within the TCA cycle that catalyzes the conversion of α-ketoglutarate (αKG) to succinyl-CoA, is increased during ferroptosis induced by cysteine deprivation or sulfasalazine (Shin et al. 2020). Intermediate products in the TCA cycle, such as αKG, succinic acid, and fumaric acid, can enhance ferroptosis induced by cysteine

depletion (Gao et al. 2019). Interestingly, loss-of-function mutations in fumarate hydratase (FH), another TCA cycle enzyme responsible for the conversion of fumarate to malate, inhibit cystine starvation-induced ferroptosis in renal cancer cells (Gao et al. 2019). Paradoxically, another study shows that FH knockout cells are more sensitive to erastin-induced cell death (Kerins et al. 2018). Furthermore, downregulation of isocitrate dehydrogenase (NADP(+)) 2 (IDH2), which catalyzes the conversion of isocitrate to αKG, promotes erastin-induced ferroptotic cell death (Kim et al. 2020). However, metabolic reprogramming towards a pro-ferroptotic phenotype occurs upon interfering with the expression of a single enzyme remains to be elucidated.

During glutaminolysis, glutamine is converted to glutamate, which can be further converted to αKG to fuel the TCA cycle. Glutaminases (GLSs) are rate-limiting enzymes that control glutaminolysis by catalyzing the hydrolysis of glutamine to glutamate. Interestingly, glutamine supplementation in an amino acid-starved medium induces ferroptotic cell death in PC3 cells (Ogor et al. 2021). In contrast, pharmacological and genetic inhibition of mitochondrial GLS2, but not the cytoplasmic isoform GLS1, inhibits ferroptotic cell death (Gao et al. 2015; Suzuki et al. 2022). Moreover, GLS2 is a transcriptional target of the tumor protein p53 (TP53) and may be required for TP53-mediated ferroptosis induction (Jennis et al. 2016). Additionally, microRNA 190a (MIR190A) targets GLS2, resulting in the downregulation of ferroptosis in cardiomyocytes (Zhou et al. 2021). Furthermore, besides its coupling with oxidative phosphorylation (OXPHOS), the TCA cycle acts as a metabolic engine that can influence various metabolic pathways, including fatty acid synthesis. Pyruvate dehydrogenase kinase 4 (PDK4) suppresses glucose-mediated ferroptosis, partially by inhibiting the TCA cycle and subsequent fatty acid synthesis (Song et al. 2021). Thus, the mitochondrial TCA cycle and OXPHOS provide a fine-tuning mechanism in modulating ferroptosis.

12.2.5 Mitochondrial Iron

Intracellular iron promotes the production of ROS through the Fenton reaction, leading to lipid peroxidation during ferroptosis. Thus, iron metabolism plays a crucial role in the process of ferroptosis (Chen et al. 2020). Chelatable iron is present in mitochondria with a concentration of 4-12 μM, similar to that in the cytosol and nuclei (Petrat et al. 2001). Mitochondrial iron overload induces inhibition of the antioxidant system, contributing to ferroptosis-mediated nephrotoxicity induced by aristolactam (Deng et al. 2020). Iron-sulfur-containing CISD family proteins on the outer mitochondrial membrane are involved in maintaining various mitochondrial functions. CISD1/mitoNEET may affect mitochondrial iron levels by regulating the function of VDAC1, a potential iron ion channel (Lipper et al. 2019). Systemic knockdown of CISD1 induces mitochondrial iron overload in adipocytes and the liver, resulting in increased mitochondrial lipid peroxidation (Kusminski et al. 2012). Consistently, genetic inhibition of CISD1 increases mitochondrial lipid peroxidation and erastin-induced ferroptosis in liver cancer cells (Yuan et al.

2016). Similarly, disruption of CISD2 function leads to the accumulation of mitochondrial iron and ROS (Karmi et al. 2021). Accordingly, CISD2 promotes resistance to sorafenib- or sulfasalazine-induced ferroptotic cell death in hepatocellular carcinoma or head and neck cancer cells (Kim et al. 2018; Li et al. 2021a; Li et al. 2022c). CISD2 overexpression also alleviates ferroptosis-mediated neuronal injury following intracerebral hemorrhage in mice (Ruihao Li et al. 2023). Other iron-sulfur cluster assembly enzymes, such as frataxin (FXN), NFS1 cysteine desulfurase, and iron-sulfur cluster assembly enzyme (ISCU), inhibit ferroptosis by controlling iron metabolism and mitochondrial function (Alvarez et al. 2017; Terzi et al. 2021; Du et al. 2020; Du et al. 2019).

Ferritin has the capacity to store iron and protect cells from iron-mediated ferroptosis (Hou et al. 2016; Gao et al. 2016). As a predominantly cytoplasmic protein, it can also be localized in mitochondria, known as mitochondrial ferritin (FTMT). Overexpression of FTMT inhibits erastin-induced neuronal cell death (Wang et al. 2016). Additionally, hypoxia increases mitochondrial ferritin, leading to the inhibition of ferroptosis in primary human macrophages (Fuhrmann et al. 2020).

12.2.6 Mitochondrial DNA

Mitochondrial DNA (mtDNA), which encodes essential OXPHOS-associated proteins, regulates ferroptotic cell death in a context-dependent manner. Initial studies showed that mtDNA-depleted (ρ^0) 143B cells and mtDNA-wild-type 143B cells exhibited similar sensitivity to erastin- and RSL3-induced ferroptosis (Dixon et al. 2012). However, subsequent studies demonstrated that SK-Hep1 ρ^0 cells were more resistant to erastin-induced ferroptosis compared to matched mtDNA-wild-type cells, due to upregulation of mitochondrial GPX4 expression (Oh et al. 2022). In contrast, mtDNA depletion induced by a mutant of deoxyguanosine kinase (DGUOK), an enzyme involved in mtDNA replication, enhanced ferroptosis sensitivity in hepatocytes by inducing iron overload (Jingyi Guo et al. 2021). ρ^0 cells appear to produce more lipid peroxidation, possibly due to mitochondrial dysfunction (Takashi et al. 2020). Moreover, doxorubicin can intercalate into mtDNA and induce ferroptosis in cardiomyocytes (Abe et al. 2022). Silencing the twinkle mtDNA helicase (TWNK) decreases the accumulation of doxorubicin in mitochondria and prevents doxorubicin-induced ferroptosis (Abe et al. 2022). However, the exact role of mtDNA-mediated mitochondrial function in ferroptosis remains unclear.

The release of double-stranded mtDNA from mitochondria to the cytosol can trigger inflammatory responses by activating the DNA-sensing cyclic GMP-AMP synthase (CGAS)-cGAMP-stimulator of interferon response cGAMP interactor 1 (STING1) pathway. In addition to initiating a type I interferon response, activation of STING1 also promotes autophagy in an interferon response-independent manner (Gui et al. 2019). An antiviral compound zalcitabine induces mtDNA stress and causes ferroptosis in human pancreatic cancer cells (Li et al. 2020). Mechanistically,

mtDNA stress activates the STING1 pathway, which promotes autophagy-dependent ferroptosis (Li et al. 2020). Additionally, the fatty acid ethanolamide oleoylethanolamide inhibits mtDNA stress, thereby alleviating ferroptosis in hyperlipidemia-mediated vascular calcification (Chen et al. 2023d).

12.2.7 Mitochondrial Proteases

OMA1 zinc metallopeptidase is an inner membrane protease that can be activated in response to mitochondrial stress, including mitochondrial depolarization and oxidative stress. In the presence of mitochondrial dysfunction, OMA1 cleaves DAP3 binding cell death enhancer 1 (DELE1), leading to the accumulation of DELE1 in the cytosol. This accumulation activates the downstream eukaryotic translation initiation factor 2 alpha kinase 1 (EIF2AK1/HRI), thereby inducing the integrated stress response (Guo et al. 2020). The OMA1-dependent integrated stress response plays a protective role against ferroptosis-induced cardiomyopathy by promoting the expression of GPX4 (Ahola et al. 2022).

12.2.8 Mitophagy and Ferroptosis

Mitophagy refers to the selective removal of mitochondria by autophagy. As a quality control mechanism, mitophagy eliminates defective mitochondria that are detrimental to cellular homeostasis. Initial studies showed that upon mitophagy, cells become more resistant to ferroptosis induced by cystine starvation or erastin, suggesting that mitochondria are essential for the initiation of ferroptosis (Gao et al. 2019). However, increasing evidence shows that mitophagy may function to promote ferroptotic cell death. First, several mitophagy agonists have been reported to promote autophagy-dependent ferroptosis. For instance, mitochondrial complex I inhibitor BAY 87-2243 triggers ferroptosis in melanoma cells by inducing mitophagy-dependent ROS production (Basit et al. 2017). Activation of mitophagy by carbonyl cyanide-m-chlorophenyl hydrazine (CCCP) also increases ferroptosis in osteoblasts (Wang et al. 2022). The organophosphorus flame retardant TDCPP induces neurotoxicity through the activation of mitophagy-related ferroptosis in vivo and in vitro (Qian et al. 2022). Additionally, the co-treatment of the ferroptosis inducer erastin and celastrol induces mitochondrial dysfunction and subsequent mitophagy through the activation of mitogen-activated protein kinase 14 (MAPK14/p38)-mediated mitochondrial fission (Liu et al. 2021). Secondly, FUN14 domain-containing 1 (FUNDC1), a mitophagy receptor that interacts with microtubule-associated protein 1 light chain 3 (MAP1LC3) to mediate mitophagy, is required for ferroptosis (Peng et al. 2022). Thus, FUNDC1 deletion protects against paraquat-induced cardiotoxicity by inhibiting ferroptosis (Peng et al. 2022). Thirdly, mitophagy-dependent ferroptosis can also be regulated by amyloid-beta precursor protein (APP) (Li et al. 2022a), myoferlin (MYOF) (Rademaker et al. 2022), and FTMT (Wang et al. 2022) under different conditions. APP, extensively studied for

its role in Alzheimer's disease, induces mitophagy-dependent ferroptosis in pericytes through the CD36-PTEN-induced kinase 1 (PINK1)-parkin RBR E3 ubiquitin protein ligase (PRKN) pathway, which may contribute to blood-brain barrier dysfunction induced by APP (Li et al. 2022a). Pharmacological targeting of MYOF, an emerging oncoprotein, triggers mitophagy-dependent ferroptosis in pancreatic cancer cells (Rademaker et al. 2022). Silencing of FTMT, a mitochondrial iron storage protein, induces mitophagy-dependent ferroptosis in human osteoblast cells through the activation of the ROS-PINK1-PRKN pathway (Wang et al. 2022), indicating a close connection between mitochondrial iron and mitophagy. Lastly, inhibition of O-GlcNAcylation modification increases mitophagy, resulting in an accumulation of intracellular labile iron that enhances ferroptosis (Yu et al. 2022). Given the complex role of mitochondria in ferroptosis, caution should be exercised when drawing conclusions about whether mitophagy mediates ferroptosis.

12.2.9 Mitochondrial Crosstalk between Ferroptosis and Apoptosis

Mitochondria play a central role in apoptotic cell death. Various stimuli induce apoptosis by triggering mitochondrial outer membrane permeabilization (MOMP). BH3 interacting domain death agonist (BID), cleaved by caspase 8 (CASP8), translocates to mitochondria where it induces MOMP through the activation of BAX and/or BCL2 antagonist/killer 1 (BAK1). Following MOMP, mitochondrial pro-apoptotic proteins, such as cytochrome c and apoptosis-inducing factor mitochondria associated 1 (AIFM1, also known as AIF), are released from mitochondria to activate apoptosis. AIFM1 induces caspase-independent apoptosis by causing chromatin condensation and DNA fragmentation in the cell nucleus. Interestingly, BID and AIFM1 have also been implicated in certain contexts of ferroptosis induction. The ferroptosis inducer RSL3 induces mitochondrial fragmentation and MOMP in neuronal HT22 cells and MEFs (Jelinek et al. 2018). Unexpectedly, pharmacological and genetic inhibition of BID prevents cells from succumbing to ferroptosis induced by RSL3 (Jelinek et al. 2018; Neitemeier et al. 2017). Deficiency of GPX4 increases lipid peroxidation and induces nuclear translocation of AIFM1 in MEFs (Seiler et al. 2008). Importantly, knockdown of AIFM1 limits GPX4-deficient or GPX4 inhibitor-induced ferroptotic cell death (Seiler et al. 2008; Jelinek et al. 2018). Moreover, BCL2 like 11 (BCL2L11/BIM) and BAX may also act upstream of SLC7A11 and GPX4 to enhance ferroptosis in certain circumstances (Tang et al. 2022). However, the mechanism of mitochondrial pro-apoptotic proteins in ferroptosis is still not fully understood. Nevertheless, combining ferroptosis inducers with apoptosis-inducing drugs could be an innovative approach in cancer therapy (Lee et al. 2020a).

12.3 Role of Lipid Droplets (LDs) in Ferroptosis

LDs (lipid droplets) are dynamic organelles involved in the storage of neutral lipids, primarily triacylglycerides and cholesterol esters. The biogenesis and degradation of LDs are closely linked to cellular lipid metabolism and play a key role in buffering toxic lipid species (Olzmann and Carvalho 2019). In ferroptotic cells triggered by erastin and RSL3, LDs exhibit increased viscosity and polarity (Dong et al. 2021; Wang et al. 2021), suggesting a connection between ferroptosis and LDs. Growing evidence shows that LDs function as negative regulators of ferroptosis (Bai et al. 2019). The formation of LDs is increased during the early stages of treatment with ferroptosis inducers, potentially serving as a negative feedback mechanism (Bai et al. 2019). Similarly, tumor protein D52 (TPD52)-mediated lipid storage prevents ferroptotic cell death (Bai et al. 2019). In contrast, the induction of ferroptosis is partially dependent on RAB7A, a protein required for the autophagic breakdown of LDs (lipophagy) (Bai et al. 2019). Mechanistically, lipophagy enhances intracellular polyunsaturated fatty acids (PUFA) and subsequent lipid peroxidation. Additionally, progesterone receptor membrane component 1 (PGRMC1), a heme-binding protein, promotes ferroptosis through lipophagy in paclitaxel-tolerant persister cancer cells (You et al. 2021). Thus, the activation of lipophagy-dependent ferroptosis might be an attractive strategy to eliminate resistant cancer cells.

12.4 Role of Endoplasmic Reticulum (ER) in Ferroptosis

ER plays a critical role in ferroptosis by regulating lipid peroxidation, ER stress, lipid synthesis, protein trafficking, and ion transport.

12.4.1 Lipid Peroxidation in ER

ER is a continuous membrane system surrounding the perinuclear space and closely associated with other organelles, such as the Golgi, mitochondria, peroxisomes, and lysosomes. In ferroptosis, the ER plays a significant role as an important source of lipid peroxidation. First, ferrostatin-1 and its analogs (ferrostatins) are distributed in the ER, lysosomes, and mitochondria, but it appears that ER-localized ferrostatins are crucial for effective suppression of ferroptosis, although direct evidence is still lacking (Gaschler et al. 2018). Second, deuterated polyunsaturated fatty acids at their bis-allylic sites (D-PUFAs) act as ferroptosis inhibitors by accumulating in the ER membrane (von Krusenstiern et al. 2023), indicating that the ER is an important regulatory organelle for ferroptosis induction. Third, FINO2, a lipophilic ferroptosis inducer, localizes to the ER and Golgi (von Krusenstiern et al. 2023). Moreover, ferroptosis induced by various inducers (including RSL3, FIN56, and FINO2) leads to lipid peroxidation in the ER (von Krusenstiern et al. 2023). Fourth, GSH levels in the ER decrease during erastin-induced ferroptosis (Chen et al. 2023b), which may contribute to increased lipid peroxidation in the ER. Fifth, iron overload in the ER of

cardiomyocytes induces ferroptosis during ischemia-reperfusion injury (Miyamoto et al. 2022). These findings indicate that lipid peroxidation in ER can cause ferroptosis.

12.4.2 ER Stress

ER is the principal organelle responsible for protein folding and maturation. The ER stress response is activated when protein folding in the ER is disrupted, triggered by various stimuli including oxidative stress. Accumulation of unfolded proteins in the ER can lead to ER stress. While adaptive responses to ER stress aim to restore ER homeostasis, excessive ER stress can induce cell death. The transcription factor activating transcription factor 4 (ATF4) plays a crucial role in the response to ER stress and is upregulated in various stress states, including ER stress. During ferroptosis, ER stress can increase ER viscosity and alter pH (Silswal and Koner 2023; Song et al. 2022; Hao et al. 2021). Treatment with erastin induces phosphorylation of the eukaryotic translation initiation factor 2A (EIF2A) and subsequent upregulation of ATF4 in HT-1080 cells (Dixon et al. 2014). However, the role of ER stress-induced ATF4 in ferroptosis is controversial. ATF4 can upregulate the expression of HSPA5, which stabilizes GPX4, or transcriptionally activate the expression of SLC7A11, driving resistance to ferroptosis in cancer cells (Zhu et al. 2017; Chen et al. 2019; Chen et al. 2017a). Constituently, ER stress may activate endosomal sorting complexes required for transport (ESCRT)-III, which promotes membrane repair in ferroptosis (Dai et al. 2020b). Accordingly, ER stress induced by the flavonoid resveratrol protects cells forms ferroptosis in MIN6 cells (Zhang et al. 2022b). In contrast, ATF4 triggers the transcriptional activation of GSH-degrading enzyme ChaC glutathione specific gamma-glutamylcyclotransferase 1 (CHAC1), which accelerates ferroptosis by degradation of GSH (Wang et al. 2019a; Chen et al. 2017b). Furthermore, ER stress contributes to ferroptosis induced by artesunate or erastin through the activation of autophagy or ferritinophagy (Lee et al. 2020b; He et al. 2022). The physical and biochemical connections between the ER and mitochondria can also influence ferroptosis. Dysfunction of the mitochondria-associated ER membrane may contribute to arsenic-induced pulmonary ferroptosis (Li et al. 2022b). These findings suggest a dual role of ER stress in the regulation of ferroptotic cell death.

12.4.3 ER-resident Proteins

Solute carrier family 39 member 7 (SLC39A7, also known as ZIP7) is an ER-located zinc transporter that plays an essential role in ferroptosis (Chen et al. 2021a). The potential involvement of zinc in ferroptosis has been investigated, revealing that exogenous zinc promotes ferroptosis, while TPEN, a zinc chelator, inhibits ferroptosis (Chen et al. 2021a). Mechanically, inhibition of the ZIP7 protects cells against ferroptosis through activation of ER stress (Chen et al. 2021a). The induction

of the homocysteine inducible ER protein with ubiquitin like domain 1 (HERPUD1) by ER stress may be responsible for the protective effects of zinc chelation on ferroptosis (Chen et al. 2021a). Given that iron and copper ions are required for ferroptosis (Xue et al. 2023), the process of ferroptosis likely involves the coordinated action of these metal elements.

STING1, a transmembrane protein located on the ER, acts as an adaptor protein for the interferon response and a positive regulator of autophagy. The CGAS-STING1 pathway is activated in response to cytosolic DNA, leading to robust innate immune responses. In ferroptotic pancreatic cells, the release of oxidized nucleobase 8-hydroxyguanine (8-OHG) triggers the activation of STING1 (Dai et al. 2020a). STING1-dependent inflammation promotes pancreatic tumorigenesis by enhancing macrophage activation and infiltration (Dai et al. 2020a). Furthermore, zalcitabine induces mtDNA stress, leading to the activation of STING1-mediated autophagy and subsequent induction of ferroptosis in pancreatic cancer (Li et al. 2020). However, lipid peroxidation induced by GPX4 knockout inhibits STING1 function through carbonylation in myeloid cells (Jia et al. 2020). It appears that lipid peroxidation directly inhibits STING1 activity, while lipid peroxidation products (such as 8-OHG) or lipid peroxidation-induced mtDNA stress can activate the STING1 pathway, promoting autophagy-dependent ferroptosis. Additionally, Mn^{2+} facilitates ferroptosis by activating CGAS-STING1 signaling in tumor cells, which downregulates DHODH function (Zhang et al. 2022a).

Stearoyl-CoA desaturase (SCD) is an ER membrane-bound enzyme that catalyzes the rate-limiting step of monounsaturated fatty acids synthesis (MUFA). Distinct from PUFA, MUFA suppresses ferroptosis possibly due to its ability to compete with PUFA to incorporate into phospholipids. Thus, SCD acts as a negative regulator of ferroptosis (Magtanong et al. 2019; Tesfay et al. 2019). Signaling pathway that regulates SCD may be linked to ferroptosis in various kinds of cancer. Firstly, sterol regulatory element-binding protein 1 (SREBP1) is one of the key transcription factors of SCD and contributes to ferroptosis resistance (Yi et al. 2020; Zhao et al. 2020). In several types of cancer cells, constitutive activation of phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha (PIK3CA/PI3K)-AKT-mechanistic target of rapamycin kinase (MTOR) signaling or loss of phosphatase and tensin homolog (PTEN) decreases ferroptosis sensitivity by promoting SREBP1-mediated transcription of SCD (Yi et al. 2020; Zhao et al. 2020). Moreover, co-mutation of serine/threonine kinase 11 (STK11) and Kelch like ECH associated protein 1 (KEAP1) may result in elevated expression of SCD in lung adenocarcinoma (Wohlhieter et al. 2020). F-Box and WD repeat domain containing 7 (FBW7) inhibits the expression of SCD, while hypoxia and nutrient starvation microenvironment upregulate SCD, thus affecting ferroptosis sensitivity in pancreatic cancer (Ye et al. 2021; Gao et al. 2021). Combined treatment with SCD inhibitors and ferroptosis inducers provides a potential anticancer therapeutic strategy. Collectively, these findings suggest an optimal strategy for treating cancers by inhibition of anti-ferroptotic SCD.

12.4.4 ER-phagy

ER-phagy, also known as reticulophagy, is a selective autophagy process that targets the degradation of the ER. One of the key regulators of ER-phagy is reticulophagy regulator 1 (RETREG1/FAM134B), an ER-phagy receptor that interacts with LC3 to facilitate ER degradation through autophagy (Khaminets et al. 2015). In hepatocellular carcinoma cells, sorafenib induces ER-phagy (Liu et al. 2022c). However, when FAM134B is knocked down, ER-phagy is blocked, leading to increased cell sensitivity to sorafenib-induced ferroptosis (Liu et al. 2022c). These findings suggest that ER-phagy may act as a protective mechanism inhibiting sorafenib-induced ferroptosis.

12.5 Role of Lysosomes in Ferroptosis

Lysosomes are organelles surrounded by a single membrane and contain an acidic environment with a pH ranging from 4.5 to 5. They are often referred to as "suicide bags" due to their role in releasing various digestive enzymes during accidental or unregulated cellular processes. Lysosomes not only contribute to apoptosis, necrosis, and autophagic cell death but also play a role in initiating ferroptotic signaling.

12.5.1 Lysosomal Cathepsins

Lysosomes contain a variety of hydrolases that can be released into the cytosol when lysosomes are disrupted due to severe cellular stimuli. Inhibition of lysosomal function by various inhibitors, such as bafilomycin A1, ammonium chloride, and pepstatin A, can block ferroptotic cell death induced by erastin or cystine starvation (Torii et al. 2016; Gao et al. 2016). Among the numerous lysosomal hydrolases, cathepsins, a group of proteases involved in protein turnover, have been extensively studied. Pharmacological inhibition of cathepsins, specifically by CA-074Me, limits erastin-induced ferroptosis (Kuang et al. 2020; Nagakannan et al. 2021), highlighting the essential role of cathepsins in ferroptosis. In particular, the release of lysosomal cathepsin B (CTSB) is a critical step during the execution of ferroptotic cell death (Gao et al. 2018; Kuang et al. 2020; Nagakannan et al. 2021). Ferroptosis inducers enhance the expression of CTSB in human pancreatic cancer cells through the activation of the MEK-ERK-signal transducer and activator of transcription 3 (STAT3) pathway (Gao et al. 2018). He released CTSB that not only induces DNA damage (Kuang et al. 2020), but also cleaves histone H3, contributing to the execution of ferroptotic cell death (Nagakannan et al. 2021). It appears that lipid peroxidation serves as an upstream activator of the release of lysosomal CTSB. These findings underscore the critical role of lysosomal cathepsin release as a pivotal step in ferroptosis.

12.5.2 Lysosomal Iron and ROS

Lysosomes have the capacity to accumulate significant amounts of iron through the degradation of ferritin or other organelles. The presence of Fe^{2+} in lysosomes can generate reactive hydroxyl radicals, which can compromise lysosomal membrane integrity and induce ferroptosis. For example, a lysosome-targeted ROS inducer can accelerate the iron-mediated Fenton reaction, leading to ferroptosis in cancer cells (Chen et al. 2022b). Additionally, dichloroacetate induces ferroptosis in colorectal cancer cells by increasing and sequestering iron within lysosomes (Sun et al. 2021a). Knockdown of the lysosomal protein prosaposin (PSAP) results in the accumulation of iron in lipofuscin, leading to ferroptosis in neurons (Tian et al. 2021). However, lysosomal stress may also have a suppressive effect on ferroptosis through the upregulation of superoxide dismutase 1 (SOD1) mediated by the transcription factor EB (TFEB) (Li et al. 2019). The specific mechanisms by which lysosome stress induces either protective or damaging responses in the context of ferroptosis are still unclear.

12.5.3 Lysosomal Nitric Oxide

Nitric oxide is generated endogenously from L-arginine by nitric oxide synthase (NOS) in a wide variety of organisms. It acts as a signaling molecule in various physiological and pathological conditions. Non-thermal plasma (NTP) is a promising technology for generating reactive species. Non-thermal plasma-activated Ringer's lactate (PAL) induces ferroptosis in malignant mesothelioma cells by increasing lysosomal nitric oxide levels (Jiang et al. 2021). This leads to the subsequent accumulation of nitric oxide in lysosomes, which may induce lysosomal membrane permeabilization and lysosomal lipid peroxidation (Jiang et al. 2021).

12.5.4 Lysosome in Autophagy

Autophagy is a proteolytic degradation pathway that relies on the lysosome's degradative capabilities. During autophagy, autophagosomes merge with lysosomes to form autolysosomes, where the sequestered contents are degraded by lysosomal hydrolases. This highly conserved process is executed by a set of autophagy-related (ATG) genes and proteins. Several ATGs, including ATG3, ATG5, BECN1 (also known as ATG6), ATG7, and ATG13, are essential for ferroptosis in specific contexts (Hou et al. 2016; Gao et al. 2016; Park and Chung 2019). Ferroptosis has been sometimes recognized as an autophagic cell death process (Gao et al. 2016; Zhou et al. 2020; Liu et al. 2022b; Chen et al. 2023a). Besides the discussed mitophagy, lipophagy, and ER-phagy, the autophagic degradation of ferroptosis modulators or organelles can also impact ferroptosis sensitivity. First, autophagic degradation of ferritin (ferritinophagy) (Hou et al. 2016; Gao et al. 2016) and solute carrier family 40 member 1 (SLC40A1) (Li et al. 2021b) promote, while autophagic

degradation of TFRC (Xiong et al. 2021) inhibits, ferroptosis by regulating intracellular Fe^{2+} levels. Second, autophagic degradation of aryl hydrocarbon receptor nuclear translocator like (ARNTL/BMAL1, known as clockophagy) (Yang et al. 2019) facilitates ferroptosis by increasing intracellular free fatty acid levels. Third, autophagy- or chaperone-mediated autophagy (CMA)-mediated GPX4 degradation results in the accumulation of lipid peroxidation and ferroptosis (Wu et al. 2019; Xue et al. 2023; Sun et al. 2021b). Fourth, hippocalcin like 1 (HPCAL1) acts as a specific autophagy receptor for ferroptosis by mediating the autophagic degradation of cadherin 2 (CDH2) (Chen et al. 2023c). HPCAL1-mediated CDH2 degradation decreases membrane tension to favor lipid peroxidation (Chen et al. 2023c). These findings demonstrate the critical role of autophagy as a common regulatory mechanism in mediating ferroptosis. However, the extent of interdependency between autophagy and lysosomal function in ferroptosis-related diseases is still unclear. Further research is needed to elucidate their interconnectedness.

12.6 Role of Ribosome in Ferroptosis

The ribosome is the main organelle responsible for protein translation, converting the genetic code into amino acid sequences. In addition to free ribosomes, a portion of ribosomes is attached to the ER membranes. Ribosome-mediated protein translation tightly regulates ferroptosis. Treatment with the protein synthesis inhibitor cycloheximide (CHX) or knockdown of ribosomal protein L8 (RPL8) inhibits erastin-induced ferroptosis (Dixon et al. 2012), suggesting that de novo protein synthesis may be required for the induction of ferroptosis. Interestingly, eukaryotic translation initiation factor 4E (EIF4E), a key component of protein synthesis, promotes ferroptosis by limiting the metabolism of 4-hydroxynonenal (4HNE) mediated by aldehyde dehydrogenase 1 family member B1 (ALDH1B1) in a translation-independent manner (Chen et al. 2022a). The translational regulation of GPX4 has been a focus of research. Firstly, the protein synthesis of GPX4 can be regulated by the availability of selenium or cyst(e)ine. Selenium is utilized in the translation of certain selenoproteins, including GPX4. LDL receptor-related protein 8 (LRP8) acts as a receptor for the uptake of the selenium-rich protein selenoprotein P (SELENOP/SEPP1), and its breakdown by the lysosome releases selenium. Thus, LRP8 inhibits ferroptosis by enhancing cellular selenium levels and subsequent GPX4 protein translation (Li et al. 2022d). Cystine or cysteine promotes GPX4 protein translation by activating the mTOR complex 1 (MTORC1)-eukaryotic translation initiation factor 4E binding protein 1 (EIF4EBP1) signaling pathway (Zhang et al. 2021). Accordingly, inhibition of SLC7A11 mediated-cystine uptake decreases not only GSH synthesis, but also GPX4 protein levels (Zhang et al. 2021). Secondly, a specific selenocysteine tRNA is required for the insertion of selenocysteine into GPX4. Isopentenyl diphosphate (IPP), a product of the mevalonate (MVA) pathway, enhances GPX4 synthesis by promoting the maturation of selenocysteine tRNA. Thus, disruption of the MVA pathway by statins can

downregulate GPX4 protein synthesis, thereby increasing ferroptosis sensitivity (Viswanathan et al. 2017).

12.7 Role of Peroxisomes in Ferroptosis

The discovery of various ROS-producing enzymes (e.g., urate oxidase/UOX or xanthine dehydrogenase/XDH) and ROS-metabolizing enzymes (e.g., catalase/CAT and SOD1) in peroxisomes supports the key role of peroxisomes in both the production and scavenging of ROS. The involvement of peroxisomal biogenesis proteins in ether phospholipid (ePL) biosynthesis has established a direct relationship between peroxisomes and ferroptosis (Zou et al. 2020). ePL is a unique class of phospholipids characterized by an ether bond at the sn-1 position of the glycerol backbone. Similar to PUFA-phosphatidylethanolamine (PE) (Kagan et al. 2017), excessive oxidation of PUFA-ePL also leads to ferroptosis. Knockdown of peroxisomal biogenesis factors family proteins (e.g., PEX3 and PEX10) reduces the abundance of peroxisomes and protects cells from ferroptosis (Zou et al. 2020; Cui et al. 2021). Additionally, several peroxisomal enzymes such as fatty acyl-CoA reductase 1 (FAR1) and alkylglycerone phosphate synthase (AGPS), as well as ER-resident enzymes like 1-acylglycerol-3-phosphate O-acyltransferase 3 (AGPAT3) and transmembrane protein 164 (TMEM164), which catalyze the biosynthesis of ePL and PUFA-ePL, promote ferroptosis in a context-dependent manner (Zou et al. 2020; Cui et al. 2021; Reed et al. 2023). The positive role of TMEM164 in autophagy highlights another mechanism that promotes ferroptosis (Liu et al. 2023). Conversely, deficiency of these enzymes may decrease sensitivity to ferroptosis (Zou et al. 2020; Cui et al. 2021; Reed et al. 2023). Thus, peroxisome-driven accumulation of PUFA-ePL increases susceptibility to ferroptotic cell death. However, peroxisome proliferator-activated receptor delta (PPARD/PPARδ) inhibits ferroptosis by upregulating the expression of peroxisomal CAT, which helps quench hydrogen peroxide in peroxisomes (Hwang et al. 2021). Collectively, these results emphasize a critical role of peroxisome in modulating ferroptosis (Tang and Kroemer 2020).

12.8 Role of Golgi in Ferroptosis

The Golgi apparatus functions to process, sort, and transport proteins synthesized by the ER. Golgi stress induced by compounds like brefeldin A and golgicide A can trigger ferroptosis (Alborzinia et al. 2018). However, it is worth noting that brefeldin A can also induce ER stress, which actually protects cells from erastin-induced ferroptosis (Lin et al. 2021a). Therefore, it appears that Golgi stress leads to ferroptosis only when a death threshold is reached. Additionally, UbiA prenyltransferase domain containing 1 (UBIAD1), which catalyzes CoQ10 biosynthesis in the Golgi, has been found to attenuate ischemic/reperfusion-mediated ferroptosis in neuronal cells by restoring impaired mitochondria and Golgi (Huang

et al. 2022). Although there is currently limited evidence linking Golgi stress to ferroptosis, further research is needed to explore the specific mechanisms in detail.

12.9 Conclusions and Perspectives

Research in the field of ferroptosis is rapidly expanding and has been investigated in almost every organ system of the body (Tang et al. 2021). When functioning properly, ferroptosis significantly contributes to human health, including tumor suppression and immune surveillance (Jiang et al. 2015; Wang et al. 2019b; Chen et al. 2021d). Induction of ferroptosis has emerged as a promising strategy for anti-tumor therapy (Chen et al. 2021c). As discussed in this review, our understanding of the role of organelles in the ferroptosis process is still in its early stages. Current evidence suggests that most organelles play a significant role in ferroptosis, although their contributions may vary (Chen et al. 2021e). The origins and dynamic changes of lipid peroxidation in ferroptosis are not yet fully understood. While some studies have suggested a direct involvement of specific organelles such as mitochondria and the ER in lipid peroxidation, further research is needed to elucidate the specific contributions of organelles in lipid peroxidation using transgenic animal models or in clinical settings (Lin et al. 2021b). The regulatory functions of different organelles in ferroptosis provide promising opportunities for the treatment of ferroptosis-related diseases. However, our understanding of how organelles contribute to specific ferroptosis-related diseases is limited. Exploring the role of ferroptosis in the spectrum of human diseases may lead to unprecedented opportunities for the clinical application of ferroptosis-based therapies or biomarkers (Chen et al. 2021b). Additionally, developing organ-specific ferroptosis inducers or inhibitors poses a challenge in therapeutically targeting specific organs. Moving forward, it is important to expand our understanding of the crosstalk between different organelles, as this may uncover a more complex regulatory network involved in ferroptosis (Liu et al. 2022a).

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Ferroptosis: A Promising Therapeutic Target for Cardiovascular Diseases

13

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Abstract

Cardiovascular diseases (CVDs) refer to a group of conditions that affect the heart and blood vessels and are a leading cause of death worldwide. Ferroptosis is an iron-dependent regulated cell death process that occurs due to unlimited lipid peroxidation and subsequent plasma membrane rupture. Impaired ferroptosis has been linked to the pathophysiology of various CVDs, including

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cardiomyopathies, myocardial infarction and ischemia, coronary atherosclerosis, and heart failure. Excessive iron accumulation can trigger phospholipid hydroperoxide accumulation in the cell membrane and ferroptosis, ultimately causing CVD. Conversely, iron deficiency, which often develops under conditions of malnutrition, negatively affects cardiac metabolism and function in humans. This chapter delves into the role of ferroptosis in the pathophysiology of CVD and explores therapeutic targets and compounds for preventing ferroptosis-related CVD.

13.1 Introduction

Proper function of the cardiovascular system requires a fine-tuned homeostasis of many trace elements including iron. Ferroptosis refers to highly regulated iron-dependent cell death, which is implicated in the pathophysiology of a broad range of cardiovascular diseases (CVD) such as in cardiomyopathies, myocardial infarction and ischemia, coronary atherosclerosis, and heart failure (Del Re et al. 2019; Ajoolabady et al. 2021). Iron deficiency frequently develops under malnutrition and negatively impacts cardiac metabolism and function in humans (Bi et al. 2021). Conversely, iron overload is associated with intracellular oxidative stress, contributing to cardiovascular pathologies (Bi et al. 2021; Berdoukas et al. 2015). In cardiomyocytes, excessive iron triggers ferroptosis via phospholipid hydroperoxide accumulation in the cell membrane, ultimately causing CVD (Fang et al. 2019). In this chapter, we discuss the role of ferroptosis and its underpinning mechanisms in the pathophysiology of CVD, as well as highlighting potential pre-clinical targets and therapeutic compounds for the prevention of ferroptosis in CVD.

13.2 Role and Signaling Pathways of Ferroptosis in CVD and Potential Therapeutic Targets

13.2.1 Myocardial Infarction and Heart Ischemia

Multiple pieces of evidence have identified ferroptosis in the pathogenesis of myocardial infarction (MI) and ischemia (Zhao et al. 2021). During acute and subacute stages of MI, *GPX4* downregulation leads to lipid peroxidation and induction of ferroptosis in H9c2 cardiomyocytes under conditions of hypoxia-reperfusion (H/R) (Park et al. 2019). Meanwhile, cysteine deprivation aggravates ferroptosis due in part to effects on glutathione (GSH) synthesis (Park et al. 2019). Moreover, upregulation of the long noncoding RNA (lncRNA) *Erdr1y/Gm47283* in a murine model of MI blocks microRNA *Mir706*, leading to upregulation of *Ptgs2* mRNA, induction of ferroptosis, and ultimately, exacerbation of MI (Gao et al. 2022). Conversely, inhibition of *Erdr1y* lncRNA and/or overexpression of *Mir706* alleviate myocardial injury in this model (Gao et al. 2022). Hence, modulation of the *Erdr1y*

lncRNA-*Mir706*-PTGS2 axis may alleviate ferroptosis and cardiac injury. In most cell types, SLC11A2/DMT1 takes up non-heme iron and its upregulation in cardiomyocytes triggers ferroptosis (Song et al. 2021). In line with this, mesenchymal stem cells (MSCs) of human umbilical cord release exosomes containing *MIR23A-3p* that target/block *SLC11A2*, resulting in the inhibition of ferroptosis and alleviation of myocardial injury (Song et al. 2021). Therefore, MSCs-exosomes through activation of the *MIR23A-3p*-SLC11A2 pathway may confer protection against ferroptosis upon MI.

A large body of evidence highlights the importance of ferroptosis in the onset and development of myocardial ischemia-reperfusion (I/R) injury. In vitro, cardiac myocyte ischemia induces ALOX15-mediated peroxidation of polyunsaturated fatty acids/PUFAs, resulting in ferroptosis induction and cell damage. These findings suggest that targeted inhibition of ALOX15 might be a promising strategy to combat ferroptosis during I/R injury (Ma et al. 2022b). In H9c2 cells exposed to H/R, *Mir190a-5p* reduces reactive oxygen species (ROS), malondialdehyde, and Fe^{2+} accumulation by sponging *Gls2* mRNA, thus antagonizing ferroptosis (Zhou et al. 2021). These findings indicate that the *Mir190a-5p*-GLS2 axis could serve as a potential target for the prevention of ferroptosis and myocardial damage. Besides, inducing *Fndc5* overexpression or administration of its cleaved form (namely irisin) attenuates ferroptosis and mitochondrial impairment through activation of the NFE2L2/NRF2-HMOX1 signaling cascade in hypoxic cardiomyocytes (Cao et al. 2022a). Therefore, inducible activation of the FNDC5-NFE2L2-HMOX1 signaling axis may represent a promising strategy to block ferroptosis under H/R settings in cardiomyocytes. Moreover, myocardial I/R injury is accompanied by generation of oxidized phosphatidylcholines (OxPCs) which elicit ferroptosis and cardiac dysfunction in rats (Stamenkovic et al. 2021). Mechanistically, OxPCs-induced ferroptosis involves a dramatic reduction of GPX4 activity in rat cardiomyocytes. Additionally, OxPCs contribute to ferroptosis by dampening calcium transients and mitochondrial bioenergetics (Stamenkovic et al. 2021). Neutralizing antibodies against OxPCs blocks ferroptosis during the perfusion stage. Both in vivo and in vitro, myocardial I/R upregulates *Elavl1* via FOXC1. ELAVL1 then binds and stabilizes *Becn1* mRNA, inciting autophagy-dependent ferroptosis and myocardial injury (Chen et al. 2021), which can be rescued by *Elavl1* knockout. These observations indicate that the FOXC1-ELAVL1-BECN1 signaling cascade plays an essential role in autophagy-mediated ferroptosis in the context of myocardial I/R injury. Furthermore, upon myocardial I/R injury, bone marrow MSCs-exosomes containing *Mir9-3hg* lncRNA reduces ferroptosis by promoting cell proliferation, GSH content, and by reducing ROS and iron overload in mice and HL-1 cardiomyocytes (Zhang et al. 2022a). From the mechanistic point of view, *Mir9-3hg* lncRNA binds and inhibits PUM2, thereby activating *Prdx6* transcription, mitigating ferroptosis (Zhang et al. 2022a). Thus, the *Mir9-3hg* lncRNA-PUM2-PRDX6 pathway renders protection against ferroptosis and myocardial I/R injury. Similar to *Mir9-3hg* lncRNA, MTOR protects against ferroptosis and iron overload by suppressing mitochondrial ROS in mice subject to myocardial I/R (Baba et al. 2018). In early reperfusion, upregulation of *ATF3* leads to regression of ferroptosis

and myocardial I/R injury (Liu et al. 2022a). The underlying mechanism appears to be linked to binding of ATF3 to the *FANCD2* promoter, leading to its transactivation and thus inhibition of ferroptosis in AC16 human cardiomyocytes (Liu et al. 2022a). Conversely, *ATF3* ablation retrieves ferroptosis and aggravates I/R injury. These findings imply that the ATF3-FANCD2 axis could serve as a potential target for prevention of ferroptosis in ischemic hearts.

KMT2B induces histone H3 methylation and upregulation of *Rfk* to activate TNF/TNF-α-CYBB/NOX2 signaling, resulting in ferroptosis and cardiac injury in a rat myocardial I/R model and H9c2 cardiomyocytes (Cao et al. 2022b). Hence, *Kmt2b* ablation significantly curbs ferroptosis and infarct size (Cao et al. 2022b). In this sense, targeted inhibition of KMT2B-RFK-TNF-CYBB/NOX2 signaling or genetic ablations of its key components might suppress ferroptosis upon myocardial I/R. Furthermore, NFE2L2 upregulates SLC40A1, thereby limiting iron overload and ferroptosis, ultimately alleviating myocardial I/R injury in diabetic rats and H9c2 cardiomyocytes (Tian et al. 2022). These observations indicate that the NFE2L2-SLC40A1 axis endows protection against ferroptosis under conditions of diabetic myocardial I/R injury. Finally, it is worth mentioning that ferroptosis genes including *CAT*, *HMOX1*, *RTN3*, *GPX4*, and *SLC25A1* are correlated with other biochemical risk factors in patients with coronary artery disease/CAD expression of oxidative stress, suggesting a cardinal role for ferroptosis in the progression of this disease (Ozuynuk et al. 2022) (Figs. 13.1 and 13.2).

13.2.2 Cardiomyopathy

Ferroptosis is thought to be critical in the pathogenesis of cardiomyopathy (Li et al. 2022a). In the *cox10*$^{-/-}$ mouse model of mitochondrial cardiomyopathy, the mitochondrial peptidase OMA1 induces mitochondrial fragmentation and integrated stress response/ISR via activation of OMA1-DELE1-ATF4 signaling, resulting in improved GSH metabolism and inhibition of lipid peroxidation and ferroptosis (Ahola et al. 2022). Therefore, the OMA1-DELE1-ATF4 axis plays a protective role against ferroptosis. In contrast, palmitic acid (PA) downregulates *Hsf1* and *Gpx4* in a dose- and time-dependent manner, leading to lipid peroxidation and ferroptosis in mice and H9c2 cardiomyoblasts in vitro (Wang et al. 2021). However, inducing *Hsf1* expression alleviates these effects, renormalizes iron metabolism, and upregulates *Gpx4* (Wang et al. 2021). These findings indicate an anti-ferroptotic role of HSF1 in PA-induced cardiomyopathy. In an in vitro model of doxorubicin (DOX)-induced cardiomyopathy, *Carm1/Prmt4* overexpression triggers ferroptosis and aggravates cardiomyocyte injury (Wang et al. 2022b). Mechanistically, PRMT4 methylates NFE2L2 thus dampening its nuclear translocation and reducing *Gpx4* transcription, ultimately, resulting in ferroptosis (Wang et al. 2022b). Therefore, NFE2L2 activation, administration of Fer-1, and genetic ablation or pharmacological inhibition of CARM1/*Carm1* have all been shown to alleviate ferroptosis and cardiomyopathy in vitro (Wang et al. 2022b). These data suggest that forcible

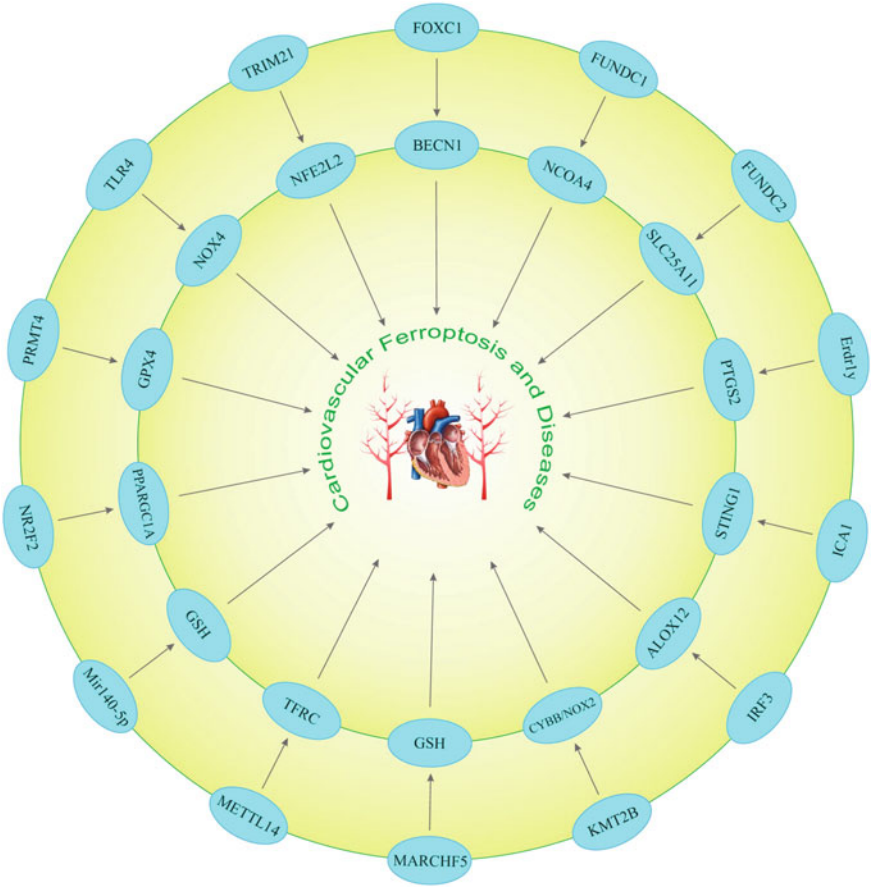

Fig. 13.1 Signaling cascades inducing ferroptosis in CVD. The underpinning mechanisms of ferroptosis in various CVDs constitute complex signaling pathways demanding immense work by scientists in the field to develop proper therapeutic strategies

inhibition of CARM1-NFE2L2-GPX4 signaling could be a potent approach for suppression of ferroptosis and alleviation of DOX-induced cardiomyopathy.

Similarly, the presence of ferroptosis in the pathophysiology of diabetic cardiomyopathy has also been reported. Also, in db/db mice and glucose-challenged cardiomyocytes, the lncRNA *Zfas1* binds and inhibits *Mir150-5p*, leading to downregulation of *Ccnd2*, ferroptosis, and progression of diabetic cardiomyopathy, all of which can be alleviated by inhibition of *Zfas1* (Ni et al. 2021). Furthermore, ferroptosis contributes to obesity-associated cardiomyopathy. In obese mice, exosomes derived from adipose tissue macrophages impair mitochondria, provoking upregulation of malondialdehyde and 4-hydroxynonenal (lipid peroxides) in cardiomyocytes (Zhao et al. 2022). *Mir140-5p* in these exosomes targets and sponges *Slc7a11*, causing loss of GSH and induction of ferroptosis (Zhao et al.

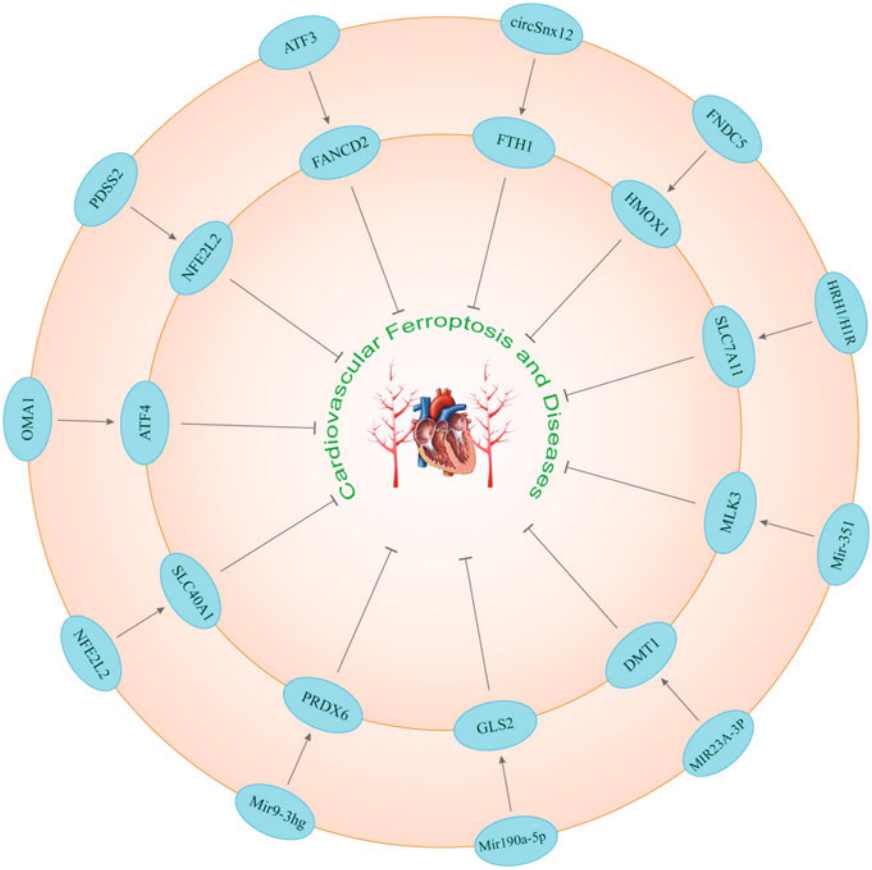

Fig. 13.2 Signaling cascades resisting ferroptosis in CVD. Despite the complexity of ferroptosis in CVD, a glimpse of hope exists due to the promising potential of anti-ferroptosis mechanisms in cardiomyocytes capable of alleviating CVD

2022). Inhibition of exosomal *Mir140-5p* can retard ferroptosis and cardiac injury in obesity-associated cardiomyopathy (Zhao et al. 2022). These findings indicate the possible role of the *Mir140-5p*-SLC7A11-GSH signaling cascade as a promising target for neutralization of ferroptosis and mitigation of obesity-induced cardiomyopathy.

Likewise, in septic cardiomyopathy, *ica1* knockout overtly augments cardiac function through suppression of inflammatory cytokines, oxidative stress, and ferroptosis in lipopolysaccharide (LPS)-challenged mice (Kong et al. 2022). The underlying mechanism appears to involve *Ica1* upregulation to turn on STING1 signaling, resulting in lipid peroxidation, ferroptosis, and cardiotoxicity (Kong et al. 2022). Moreover, septic patients exhibit increased ICA1 levels in plasma and mononuclear cells (Kong et al. 2022). Therefore, the ICA1-STING1 pathway may serve as a key target for inhibition of ferroptosis and septic cardiomyopathy. In mice

with sepsis/LPS-induced cardiac injury and H9c2 cardiomyocytes, *tmem43* ablation aggravates ferroptosis and iron overload (Chen et al. 2022b). Conversely, inducing *Tmem43* overexpression blocks lipid peroxidation and ferroptosis, thereby ameliorating cardiac dysfunction and injury (Chen et al. 2022b). Mechanistically, LPS elicits ferroptosis through upregulation of *Trp53/p53* and ferritin as well as downregulation of *Slc7a11* and *Gpx4* (Chen et al. 2022b). TMEM43 overexpression reverses these effects and protects against sepsis-induced cardiac injury (Chen et al. 2022b), indicating a possible role of TMEM43 as a novel target for ferroptosis in cardiomyocytes (Figs. 13.1 and 13.2).

13.2.3 Cardiotoxicity

Proper use of anti-cancer drugs is frequently limited by their cardiotoxic side effects (Yarmohammadi et al. 2021). Substantial evidence implicates ferroptosis in drug-induced cardiotoxicity. For instance, histamine deficiency or inhibition of its receptor, HRH1/H1R, ignites ferroptosis in cardiomyocytes, to exacerbate DOX-induced cardiotoxicity in mice and cultured hiPSC-CMs and HL-1 models (Zhu et al. 2022). Mechanistically, disruption of histamine-HRH1/H1R signaling inactivates STAT3, leading to *Slc7a11* downregulation and ferroptosis, which can be rescued by histamine administration (Zhu et al. 2022). Thus, forcible activation of HRH1/H1R-STAT3-SLC7A11 signaling (e.g., using histamine) alleviates DOX-induced cardiotoxicity.

Binding of APELA peptide hormone to its receptor, APLNR, provokes pronounced activation of KLF15-GPX4 signaling and upregulation of GSH, SLC7A11, and NFE2L2, to alleviate ROS production, ferroptosis, and DOX-induced cardiotoxicity in rat aortic fibroblasts (Zhang et al. 2022b). These findings favor the idea that APELA could be a promising target for abrogation of cardiotoxic ferroptosis. The outer mitochondrial membrane-located protein FUNDC2 interacts with and destabilizes SLC25A11 in the inner membrane, resulting in the reduction of mitochondrial GSH, which fosters ferroptosis (Ta et al. 2022). Additionally, FUNDC2 is capable of modulating GPX4 stability (Ta et al. 2022). Hence, the FUNDC2-SLC25A11-GPX4 signaling pathway is a major contributor to mitochondria-initiated ferroptosis. In mice and H9c2 cells, the E3 ubiquitin ligase TRIM21 ubiquitinates SQSTM1/p62 and interferes with the SQSTM1-KEAP1-NFE2L2 signaling cascade, causing lipid peroxidation and ferroptosis (Hou et al. 2021). Genetic ablation of *Trim21* rescues DOX-induced cardiotoxicity (Hou et al. 2021). These data imply that targeted inhibition of the TRIM21-SQSTM1-KEAP1-NFE2L2 axis may rescue ferroptosis and cardiotoxicity under DOX challenge. In rat neonatal and AC16 cardiomyocytes, DOX exposure initiates *Mettl14* upregulation, which in turn, methylates and activates lncRNA *Kcnq1ot1*, leading to the inhibition of *Mir7-5p* and subsequent upregulation

of TFRC/TFR1, iron overload, lipid peroxidation, and ultimately, induction of ferroptosis (Zhuang et al. 2021). Importantly, IGF2BP1 enhances the stability of the lncRNA *Kcnq1ot1* thus inducing its activation and subsequent blockade of *Mir7-5p* (Zhuang et al. 2021). Therefore, the METTL14-*Kcnq1ot1* lncRNA-*Mir7-5p*-TFRC cascade plays an indispensable role in DOX-induced ferroptosis. Hence, targeted inhibition of METTL14-*Kcnq1ot1* lncRNA-*Mir7-5p*-TFRC may abate cardiotoxicity.

Under DOX exposure, *Marchf5* downregulation leads to upregulation of *Chac1* that overtly degrades GSH and downregulates *Gpx4*, thereby favoring lipid peroxidation and ferroptosis in rat neonatal cardiomyocytes (Kitakata et al. 2021). This finding suggests that the MARCHF5-CHAC1-GSH axis incites ferroptosis in cardiomyocytes, which may be a novel target for maneuvering ferroptosis and DOX cardiotoxicity. Triptolide is another anti-cancer medication with its clinical application limited by its cardiotoxicity (Liu et al. 2022c). Cardiotoxic effects of triptolide are partially attributed to induction of ferroptosis through accumulation of lipid peroxides (e.g., malondialdehyde and 4-hydroxynonenal), Fe^{2+} overload, GSH reduction, ferritin degradation, and ROS generation, as well as blockade of the NFE2L2-HMOX1 axis in human AC16 cardiomyocytes (Liu et al. 2022c). Importantly, activation of the TF-TRFC-SLC11A2 pathway accounts for triptolide-induced Fe^{2+} overload (Liu et al. 2022c). Furthermore, triptolide interrupts the SLC7A11-GPX4 axis through a direct binding with SLC7A11 (Liu et al. 2022c). Likewise, herceptin (trastuzumab) renders cardiotoxicity and heart failure (HF) through elevation of mitochondrial/intracellular ROS and downregulation of *Gpx* and *Slc7a11*, resulting in ferroptosis in H9c2 cardiomyocytes (Sun et al. 2022a). Administration of the iron chelator deferoxamine, or Fer-1, reverses these effects (Sun et al. 2022a). Conceivably, safe treatment of ERBB2/$HER2^+$ breast cancer with herceptin could be improved by blocking ferroptosis. Imatinib mesylate-associated cardiotoxicity is linked to ROS production, iron overload, NFE2L2 downregulation, and ultimately, induction of ferroptosis in mice and H9c2 cardiomyocytes (Song et al. 2022). Conversely, findings from our group indicated that paraquat triggers cardiotoxicity and contractile dysfunction through downregulation of SLC7A11, GPX4, and ferritin, and activation of a FUNDC1-MAPK/JNK-NCOA4 axis, ultimately, leading to lipid peroxidation and ferroptosis in mice (Peng et al. 2022). Not surprisingly, *fundc1* ablation confers resistance against ferroptosis and alleviates myocardial toxicity and dysfunction (Peng et al. 2022). This study illustrates that inhibition of FUNDC1-MAPK/sJNK-NCOA4 signaling could avert ferroptosis and cardiotoxicity upon paraquat exposure (Figs. 13.1 and 13.2).

13.2.4 Cardiac Remodeling and Hypertrophy

Strong evidence support the role of ferroptosis in cardiac remodeling and hypertrophy (Wu et al. 2021). In mice, when a high-fat diet (HFD) is fed, *Fundc1* deficiency elicits ACSL4-induced ferroptosis and cardiac remodeling (Pei et al. 2021). Therefore, targeted inhibition of the FUNDC1-ACSL4 axis might reverse cardiac remodeling owing to the handicap of ferroptosis. In the early stage of angiotensin II-mediated cardiac hypertrophy, *Slc7a11* downregulation triggers ferroptosis, thereby promoting interstitial fibrosis, cardiac hypertrophy, and cardiac contractile dysfunction (Zhang et al. 2022c). Thus, suppression of ferroptosis using Fer-1 or upregulation of *Slc7a11* reverts these effects (Zhang et al. 2022c). Given the anti-ferroptotic role of SLC7A11, it could be a promising target for retardation of cardiac hypertrophy. Pressure overload leads to downregulation of *Irf3*, resulting in endothelial ferroptosis and cardiac injury in rats (Shi et al. 2022). Mechanistically, *Irf3* downregulation culminates in SLC7A11 downregulation and enhanced ALOX12 activity, leading to lipid peroxidation and ferroptosis in rat microvascular endothelial cells (Shi et al. 2022). However, docosahexaenoic acid treatment interrupts ferroptosis through upregulation of IRF3, thereby protecting against endothelial damage and cardiac hypertrophy (Shi et al. 2022). Based on these findings, the IRF3-SLC7A11-ALOX12 axis should play a cardinal role in cardiac hypertrophy and ferroptosis, and thus its targeting merits attention. Moreover, in a mouse model of angiotensin II-mediated hypertension, reduction of APELA elicits ferroptosis, myocardial fibrosis, and hypertrophy due to induction of ferroptosis in microvascular endothelial cells (Zhang et al. 2022d). APELA addition or ferroptosis inhibition using Fer-1 retards myocardial dysfunction and remodeling owing to the attenuation of iron overload and lipid peroxidation, and activation of GPX4 (Zhang et al. 2022d). These data indicate that APELA antagonizes ferroptosis upon hypertension, therefore, resulting in alleviated cardiac hypertrophy and remodeling (Figs. 13.1 and 13.2).

13.2.5 Atherosclerosis

Increasing clinical and experimental evidence has delineated a critical role of ferroptosis in atherosclerosis (Ouyang et al. 2021). During atherosclerosis, *PDSS2* overexpression prevents ROS generation and ferroptosis through activation of NFE2L2 signaling, thereby alleviating atherosclerosis in vivo and reducing ferroptosis in human coronary artery endothelial cells/HCAECs (Yang et al. 2021). Consistent with, atherosclerotic patients exhibit reduced levels of PDSS2 and NFE2L2 in plasma as compared to healthy controls (Yang et al. 2021). Hence, the PDSS2-NFE2L2 pathway appears to play an anti-ferroptotic role and inducing its activation might alleviate atherosclerosis. Moreover, *Nod1* deficiency reduces iron level in murine spleen, liver, and heart. In line with this, splenic deficiency of *Nod1*

induces ferroptosis and CXCR2 signaling in ADGRE1/F4/80^{+} macrophages, leading to their recruitment to atherosclerotic plaques (Fernández-García et al. 2022). Tangibly, *Nod1* upregulation inhibits ferroptosis by restraining macrophage migration and raising GPX4 and anti-ferroptosis proteins in macrophages, ultimately, resulting in alleviation of atherosclerotic plaques (Fernández-García et al. 2022). These data indicate that NOD1 is a favorable target for retardation of plaque growth through inhibition of ferroptosis in macrophages/splenic cells.

In addition, endothelial progenitor cells (EPCs)-released extracellular vesicles induce attenuation of ROS, iron content, GSH consumption, and lipid peroxidation, thereby favoring ferroptosis in aortic endothelial cells and atherosclerotic mice (Li et al. 2021). Mechanistically, EPCs-exosomes contain *Mir199a-3p* that is transferred into endothelial cells and then targets/inhibits *Sp1* mRNA, leading to mitigation of ferroptosis and atherosclerosis (Li et al. 2021). Hence, inducing activation of *Mir199a-3p*-SP1 signaling via administration of EPCs-exosomes might regress atherosclerosis. Conversely, ferroptosis contributes to the development of diabetic atherosclerosis. In this regard, HMOX1 upregulation promotes ferroptosis by inducing ROS generation, Fe^{2+} overload, and lipid peroxidation, thus promoting diabetic atherosclerosis in human endothelial cells (Meng et al. 2021). Hence, inhibition of ferroptosis using ferrostatin-1 (Fer-1) or genetic ablation of *HMOX1* ameliorates diabetic atherosclerosis, indicating that HMOX1 could be a potential target for suppression of ferroptosis in diabetic atherosclerosis (Meng et al. 2021). Importantly, in hyperlipidemic $Jak2^{V617F}$ mice, increased hematocrit induces ferroptosis in plaque macrophages through excessive phagocytosis of red blood cells, thereby leading to macrophage ferroptosis and aggravation of atherosclerosis (Liu et al. 2022b) (Figs. 13.1 and 13.2).

13.2.6 Heart Failure

HF entails ferroptosis in the frontline of its pathogenesis (Yang et al. 2022b). In mouse cardiomyocytes, *Fth1* deficiency induces *Slc7a11* downregulation, leading to ferroptosis and development of HF (Fang et al. 2020). However, specific overexpression of *Slc7a11* in cardiomyocytes increases GSH levels and retards ferroptosis (Fang et al. 2020). In rat HF, activation of TLR4-NOX4 signaling contributes to ferroptosis. However, ablation of *Tlr4* or *Nox4* averts ventricular remodeling and ferroptosis (Chen et al. 2019). Hence, the TLR4-NOX4 axis favors ferroptosis and thus could be a potential target for its suppression upon HF. In diabetic mice, cardiomyocyte upregulation of *Nr2f2* aggravates HF by inducing ferroptosis, mitochondrial dysfunction, and oxidative stress through activation of PPARGC1A/PGC-1α signaling (Miao et al. 2022). Unsurprisingly, in vitro ablation of *Nr2f2* blocks ferroptosis and rescues HF (Miao et al. 2022). These findings

delineate the NR2F2-PPARGC1A signaling cascade as a potential target to foil ferroptosis upon diabetic HF. Under pressure overload stress, a circular RNA, *circSnx12*, binds and sponges *Mir224-5p* thus activating *Fth1* mRNA, leading to hinderance of iron overload and ferroptosis in cardiomyocytes (Zheng et al. 2021). In this sense, induction of *Mir224-5p* upregulation and downregulation of *circSnx12* ignites ferroptosis due to intracellular overload of Fe^{2+} (Zheng et al. 2021). Therefore, augmentation of the *circSnx12-Mir224-5p*-FTH1 pathway might be a potent strategy to curtail elevated ferroptosis upon HF.

Furthermore, in the early stage of chronic HF, *Map3k11*/*Mlk3* initiates NFKB-NLRP3 signaling, culminating in inflammation, pyroptosis, and cardiac fibrosis in mice (Wang et al. 2020). Surprisingly, in the advanced stages, MAP3K11 activates the MAPK/JNK-TRP/p53 axis, leading to oxidative stress, ferroptosis, and myocardial fibrosis. Interestingly, inducing *Mir351* upregulation blocks ferroptosis and pyroptosis by suppressing *Map3k11* expression, thereby improving cardiac function (Wang et al. 2020). In compliance with these findings, inducing activation of the *Mir351*-MAP3K11 cascade might be a potential approach for regression of ferroptosis upon HF (Figs. 13.1 and 13.2).

13.3 Concluding Remarks and Therapeutic Directions

To date, multiple lines of evidence have substantiated a vital role for ferroptosis in the pathophysiology of CVD. Ferroptosis functions either as an underscoring mechanism or as a contributing factor for the pathogenesis of CVD. Either way would involve commencement of complicated signaling pathways and gene expression modulations, altering antioxidant capacity, lipid peroxidation status, and iron metabolism in cardiomyocytes, ultimately culminating in massive ROS generation, iron overload, and the induction of ferroptosis. Owing to the complexity of ferroptosis mechanisms and gene expression patterns, the intervention of ferroptosis in various types of CVD requires targeted inhibition or activation of several signaling cascades along with robust modulation of multiple genes. Hence, despite the advances in targeted therapy of ferroptosis in pre-clinical studies using natural or pharmaceutical compounds (Table 13.1), clinical inertia still runs deeper than achieving desirable therapeutics for targeting ferroptosis in CVD. Nonetheless, future discoveries may reveal master key mechanisms encompassing the large portion of ferroptosis incidence and origination in CVD and, thereby, facilitating and simplifying pharmacological or genetic intervention of ferroptosis. Moreover, advances in drug development and targeted therapy approaches are pending to match the clinical demands. However, parallel advances in biotechnology and nanotechnology techniques might be game changers in our combat against ferroptosis in the context of CVD.

Table 13.1 Pre-clinical therapeutics for suppression of ferroptosis in CVD

Compound	Description	Mechanism of action	Ref
Atorvastatin	A type of statins medication	Inhibits oxidative stress, Fe^{2+} overload, lipid peroxidation, and ferroptosis through upregulation of *Gsh*, *Slc7a11*, and *Gpx4*, thereby augmenting cardiac function and alleviating its remodeling in mice and H9c2 cells	Ning et al. (2021)
Berberine	A protoberberine alkaloids	Suppresses lipid peroxidation, ROS generation, and ferroptosis in murine neonatal cardiomyocytes and H9c2 cardiomyoblast cells under RSL3 and erastin treatment	Yang et al. (2022a)
Britanin	Sesquiterpene compound	Inhibits ferroptosis due to activation of AMPK-GSK3B-NFE2L2 axis and upregulation of *Gpx4*, resulting in alleviation of myocardial I/R injury and reduction of infarct size in rat and H9c2 cells	Lu et al. (2022)
Canagliflozin	Anti-diabetic C-glycosyl compound	Blocks ferroptosis by reducing iron overload and lipid peroxidation, thereby ameliorating HF with preserved ejection fraction (HFpEF) in rats	Ma et al. (2022a)
Curcumin	A natural phenolic compound	Neutralizes ferroptosis by promoting NFE2L2 nuclear transfer and upregulation of *GPX4* and *HMOX1*, leading to alleviation of glucose-induced cardiomyopathy in diabetic rabbit	Wei et al. (2022)
Dexrazoxane	Bisdioxopiperazine	Prevents ferroptosis through downregulation of HMGB1, resulting in DOX detoxification in rats and H9c2 cells	Zhang et al. (2021)
Etomidate	Anesthetic agent	Alleviates myocardial I/R injury, cardiac dysfunction, and fibrosis via upregulation of the NFE2L2-HMOX1 axis and inhibition of ferroptosis in rats	Lv et al. (2021)
Ferulic acid	Synthesized in plants	Blunts ferroptosis by plummeting ROS and malondialdehyde levels, elevation of GPX4, CAT, and SOD1, as well as activation of PRKAA2/AMPKα2 signaling upon myocardial I/R injury	Liu et al. (2022e)
Icariin	A type of chemical flavonoid	Protects against H/R-induced ferroptosis and oxidative stress through upregulation and activation of NFE2L2-HMOX1 signaling in H9c2 cells	Liu et al. (2021)

(continued)

Table 13.1 (continued)

Compound	Description	Mechanism of action	Ref
LCZ696	A type of neprilysin inhibitors medication	Reduces ROS and lipid peroxides, increases GPX4, and ceases ferroptosis through activation of AKT1-SIRT3-SOD2 signaling, alleviation of DOX cardiotoxicity, and augmentation of ventricular function in Wistar rats and H9c2 cells	Liu et al. (2022d)
Melatonin	A natural hormone found in animals and plants	Averts DOX-induced ferroptosis and myocardial injury via downregulation of *Yap1* in SD rats and H9c2 cardiomyocytes	Sun et al. (2022b)
Puerarin	Natural isoflavone glycoside	Hinders lipid peroxidation, ROS generation, and ferroptosis, thereby alleviating HF in rats and H9c2 myocytes	Liu et al. (2018)
Resveratrol	Natural nonflavonoid polyphenol	Suppresses oxidative stress, Fe^{2+} overload, and inhibits ferroptosis due to downregulation of *Tfrc* and upregulation of *Gpx4* and *Fth1* in myocardial I/R rats and in vitro H9c2 cells	Li et al. (2022b)
Ruscogenin	Natural anti-inflammatory steroid	Significantly mitigates myocardial ischemia and infarct size by suppressing oxidative stress and ferroptosis through upregulation of *Gpx4*, downregulation of *Ftl/Flc* and *Acsl4*, and upregulation of BCAT1 and BCAT2, leading to activation of KEAP1-NFE2L2-HMOX1 signaling in mice	Fu et al. (2022a)
		Alleviates MI and infarct size, and inhibits oxidative stress and ferroptosis through downregulation of *Acsl4* and upregulation of *Gpx4*, *Bcat1* and *Bcat2*, resulting in activation of the KEAP1-NFE2L2-HMOX1 axis in mice and in vitro	Fu et al. (2022b)
Salidroside	Phenylpropanoid glycoside derived from *Rhodiola rosea*	Ameliorates cardiac dysfunction and fibrosis via inhibition of ferroptosis through reduction of Fe^{2+} and ROS levels, GPX4 upregulation, and retardation of lipid peroxidation, as well as activation of AMPK signaling in mice under DOX treatment	Chen et al. (2022a)
Salvianolic acid B	Natural anti-oxidant derived from *Salvia miltiorrhiza Bunge*	In a dose-dependent pattern mitigates iron overload, ferroptosis, lipid peroxidation, and mitochondrial injury, mainly through upregulation of NFE2L2 signaling in MI rats	Shen et al. (2022)

(continued)

Table 13.1 (continued)

Compound	Description	Mechanism of action	Ref
Tanshinone IIA	A natural compound in Salvia miltiorrhiza plant	Markedly reduces ROS production and lipid peroxidation, restores GSH level, and upregulates NFE2L2, thereby regressing atherosclerotic ferroptosis in human coronary artery endothelial cells	He et al. (2021)
Tongxinluo	Traditional Chinese medicine used for the treatment of atherosclerosis	Shuts off oxidative stress and ferroptosis, hence, improving microvascular barrier, leading to retardation of chronic pulmonary dysfunction and its impact on atherosclerosis development in mice and in vitro	Wang et al. (2022a)
Xanthohumol	A flavonoid derived from *Humulus lupulus*	Rescues from ferroptosis through reduction of lipid peroxidation, ROS generation, Fe^{2+} overload, and modulation of GPX4 and NFE2L2 levels in cardiomyocytes	Lin et al. (2022)

Conflict of Interest Statement None of the authors declare any conflict of interest.

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Ferroptosis in Central Nervous System Hypoxia–Ischemia

14

Namood-e Sahar and Eric S. Peeples

Abstract

Millions of people suffer from acute hypoxic–ischemic brain injuries (HIBIs) such as stroke and neonatal HIBI worldwide each year, resulting in significant mortality and lifelong morbidity. Therapeutic options for HIBI remain limited, but many investigators are actively seeking out novel therapies based on our evolving understanding of the pathophysiology. Ferroptotic cell death has recently been demonstrated to play a significant part in the complex cellular injury resulting after HIBI. This chapter will discuss the current literature evaluating the role of ferroptosis-related reactive oxygen species release and lipid peroxidation in the pathophysiology of HIBI. Based on the current mechanistic understanding, several investigators have begun to develop interventions for HIBI that target the ferroptosis pathways. As such, this chapter will also discuss many of the ferroptosis inhibitors that have shown neuroprotective effects in cell culture and animal models of HIBI.

14.1 Introduction

Hypoxic–ischemic brain injury (HIBI) affects nearly nine million people worldwide each year, ranging in ages from the over 1.1 million newborns suffering from hypoxia–ischemia-related neonatal encephalopathy to approximately 7.6 million older children and adults suffering from ischemic stroke (Lee et al. 2013; World Stroke Organization 2022). Regardless of the patient's age, however, HIBI results in high mortality and nearly 50% of survivors suffer significant neurologic and/or

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developmental impairment (Jacobs et al. 2013; Donkor 2018). These impairments range from alterations in mental health, mobility, or disrupted cognition in adults to lifelong sequelae of neonatal HIBI such as epilepsy and impairments in neurodevelopment such as cerebral palsy and cognitive deficits. Therapeutic options for HIBI remain incredibly limited, but many investigators are actively seeking out novel therapies based on our evolving understanding of the pathophysiology. In addition to the immediate cellular injury, there is delayed secondary injury driven by various molecular pathways leading to neuronal death, astrocytic gliosis, and microglial activation, more commonly affecting gray matter (Huang and Castillo 2008; Loane and Faden 2010). These pathways include both immediate/irreversible cell death as well as various types of regulated cell death.

Regulated cell death is a means to ensure tissue homeostasis, allowing for a physiologic balance between cell division and proliferation. Many forms of regulated cell death are triggered as an adaptive response in stress and trauma to preserve equilibrium (Galluzzi et al. 2018) and potentially provide effective targets for cell death modulation through pharmacologic manipulation of genes, proteins, or enzymes involved in the pathways. In addition to apoptosis, necrosis, pyroptosis, and necroptosis, another form of cell death occurring due to distress in the cellular microenvironment is ferroptosis (Dixon et al. 2012). Ferroptosis is a form of cell death involving iron- and reactive oxygen species (ROS)-dependent lipid peroxidation. The cell injury resulting from lipid peroxidation has been studied for many years (Maellaro et al. 1990), but the term "ferroptosis" was first described in 2012 by Dixon and colleagues (Dixon et al. 2012), supported in large part by the discovery of a non-apoptotic iron-dependent method of cell death induced by erastin, RSL3, and other small molecules (Dolma et al. 2003; Yang and Stockwell 2008). Ferroptosis can be activated through several different mechanisms, but ultimately results in the development of lipid oxidation-dependent pores that can lead to osmotic rupture of membranes and may propagate ferroptosis to adjacent cells via calcium flux (Hiller and Broz 2021; Pedrera et al. 2021). The structural integrity of nuclei, including chromatin, is preserved in ferroptosis; however, mitochondrial abnormalities such as loss of cristae, shrinkage, and increased density of the mitochondrial membrane are common (Xie et al. 2016).

To develop novel interventional therapies and enhance the translation of these therapies to patients suffering from HIBI, investigators must understand the different mechanisms of brain cell injury. To this aim, this chapter will provide an account of ferroptosis mechanism, regulation, and targets that are involved in the pathogenesis of HIBI. Although there are some differences in pathophysiology between the different etiologies and forms of HIBI, to maximize applicability the chapter will cover HIBI across the spectra of age and etiologies but will specify whenever possible if the data reviewed were obtained within a specific injury group.

14.1.1 Triggers of Ferroptosis

As indicated by the term ferroptosis (from the Latin word *ferrum* for iron), this unique method of cell death is usually iron-regulated. This process initiates with the transport of iron across the cell membrane by the transferrin receptor system (including transferring receptor 1, TRF1) and reduction from Fe^{3+} to Fe^{2+} before being transported to the intracellular iron pool (Gao et al. 2015; Hou et al. 2016). Aberrant accumulation of free iron activates the Fenton reaction where the pools of free intracellular iron form toxic reactive species and facilitate lipid peroxidation leading to cell death (Dixon et al. 2012).

In order to result in ferroptosis, the reactive species generated by the Fenton reaction require substrate, which generally comes in the form of esterified plasma membrane polyunsaturated fatty acids (PUFAs) (Gaschler and Stockwell 2017). The free PUFAs are modified by Acyl-CoA synthetase long-chain family member 4 (ACSL4) and lysophosphatidylcholine acyltransferase 3 (LPCAT3) into PUFA-containing phosphatidylethanolamines in order to incorporate them into membranes (Doll et al. 2017). These esterified PUFAs are highly susceptible to oxidation, and without ACSL4 and LPCAT3, cells become highly resistant to ferroptosis (Doll et al. 2017; Kagan et al. 2017). In addition to the Fenton reaction, iron also plays a role in ferroptosis through the iron-dependent lipoxygenases (LOXs) that are responsible for ultimately altering the esterified PUFAs into lipid peroxides to compel cytotoxic lipid peroxidation (Shah et al. 2018), resulting in a porous or thin membrane and ultimately cell death (Agmon et al. 2018).

14.1.2 Suppressors of Ferroptosis

Counter to the pathways above, cells also contain balancing pathways to protect against ferroptotic injury. Glutathione peroxidase 4 (GPX4) is a selenocysteine-dependent enzyme that helps to decrease lipid peroxides, thereby reducing the propagation of lipid peroxidation. The reduction of lipid peroxides by GPX4 relies on the antioxidant glutathione (GSH) as a cofactor, and the depletion of GSH or GPX4 can trigger ferroptosis (Shah et al. 2017). GSH is a tripeptide containing cysteine, glycine, and glutamic acid, and the intracellular availability of cysteine is important for GSH synthesis (Forman et al. 2009). Cystine, the oxidized form, is transported into the cell through the cystine/glutamate antiporter (system Xc-) and reduced to cysteine for the synthesis of GSH. This heteromeric system Xc- transporter is regulated by multiple genes and modulates sensitivity to ferroptotic cell death (Jyotsana et al. 2022). In fact, both of the classic ferroptosis-inducing chemicals, 1S,3R-RAS selective lethal 3 (RSL-3) and erastin, target this system Xc-/GPX4 pathway.

Lastly, given that the lipid peroxidation is induced through reactive oxygen species, antioxidants such as α-tocopherol and CoQ10 (which can be generated endogenously through the NADPH-FSP1-CoQ10 axis) can prevent the peroxidation of PUFAs and inhibit ferroptosis (Imai et al. 2017; Santoro 2020).

14.2 Ferroptosis in Non-HI Brain Disorders

There is growing evidence that ferroptosis plays a significant role in both acute and chronic brain disorders, including tauopathies and traumatic injury. Tau protein, vital in tauopathies, assists in neuronal iron efflux, and as such Tau can prevent ferroptosis by promoting iron export (Tuo et al. 2017). In tauopathies, there is neuronal iron accumulation due to inhibition of ferroxidase and ferroportin activity, both of which are required to export iron from the cell. Devoid of efficient iron removal systems, iron collects in neurons and stimulates the generation of ROS. The ROS accumulation in cells results in GSH depletion and induces ferroptosis (Lei et al. 2012). As an example, one of the secondary tauopathies, Alzheimer's disease, has been shown to have components such as iron dyshomeostasis, impaired glutathione metabolism, and lipid peroxidation (Ashraf et al. 2020). Deferiprone, a brain-penetrant iron chelator, has been shown to improve neuronal survival and hence the phenotype in tauopathies (Rao et al. 2020).

Similarly, some acute central nervous system injuries like traumatic brain injury (TBI) are also driven in large part by oxidative stress. Mechanisms which activate ferroptosis, including ROS production, GPX system inactivation, and dysregulated iron metabolism, have been reported in TBI (Tang et al. 2021). As such, the ferroptosis inhibitor and radical-trapping synthetic antioxidant ferrostatin-1 may significantly attenuate injury lesions in TBI (Xie et al. 2019). In addition, baicalein (12/15-lipoxygenase inhibitor) has also been shown to reduce ferroptosis in TBI and improve prognosis (Kenny et al. 2019).

In-depth discussion of the role of ferroptosis in non-HI brain disorders, however, is outside of the scope of this chapter. More information can be found in any of several reviews that have been written on ferroptosis in those disorders (Shen et al. 2020; Tang et al. 2020; Jakaria et al. 2021).

14.3 Ferroptosis after HIBI

14.3.1 HIBI Pathophysiology

The brain is highly metabolically active and is therefore particularly sensitive to hypoxia–ischemia. Adenosine triphosphate (ATP), glycogen, glucose, and phosphocreatine show a rapid decrease within first ten minutes after ischemia (Wagner 4th and Lanier 1994). HIBI arises due to disruption of blood flow and nutrients and oxygen delivery to the brain cells and generally consists of three main phases of injury. The primary phase, which occurs during the first few minutes to hour immediately after injury, is characterized by necrotic cell death and cytotoxic edema. In this initial phase, there is dysregulation of transmembrane ion exchange due to lack of ATP. Consequently, intracellular concentrations of sodium and calcium ions increase, triggering edema and accumulation of ROS and excitatory glutamate (Bernardo-Castro et al. 2020). During the secondary stage, injury continues to progress, resulting in increased blood–brain barrier permeability,

inflammatory activation of microglia and astrocytes, and organelle dysfunction (Johnston et al. 2001; Perlman 2006; Vannucci 2000; Bernardo-Castro et al. 2020). The secondary phase is also characterized by regulated cell death, including ferroptosis.

Generalized HIBI may be seen in older children and adults due to interruption of cerebral oxygen delivery and blood circulation during cardiac arrest (Sekhon et al. 2017). Neonatal HIBI can also be acquired postnatally due to cardiac arrest but is most commonly the result of perinatal injuries such as placental abruption, umbilical cord compression or injury, or severe maternal hypoxia or cardiac compromise (Fatemi et al. 2009; Fajersztajn and Veras 2017; Li et al. 2017). As compared to adults, the neonatal brain has an increased oxygen demand (Blomgren et al. 2003) and lower levels of endogenous antioxidants (Lafemina et al. 2006). Additionally, there are higher levels of PUFAs, especially in the preterm brain, and the PUFA content renders preterm neonates more susceptible to lipid peroxidation injury during ischemia, even more than term newborns (Millar et al. 2017). Altogether, these factors make the newborn brain particularly susceptible to ferroptosis.

Focal HIBI, such as that resulting from stroke, can occur across the age spectrum, though the risk increases significantly with age and results in high mortality or chronic disability regardless of the age of injury. Similar to generalized HIBI, ischemic stroke results in the interruption of blood and nutrients to the brain, resulting in iron accumulation (Park et al. 2011; DeGregorio-Rocasolano et al. 2018), oxidative stress, and inflammation (George and Steinberg 2015).

The remainder of this section will review the literature demonstrating the association between HIBI and the different aspects of ferroptotic cell death: ROS generation, ACSL4- and LOX-mediated lipid peroxidation, iron accumulation, antioxidants, and inflammation.

14.3.2 ROS Generation after HIBI

HIBI is associated with various pathological processes, including excessive ROS generation and subsequent lipid peroxidation. The molecular mechanisms of ferroptosis involve the redox imbalance between pro- and antioxidant processes. The rapid increased ROS generation during the primary phase of HIBI disrupts the barrier function of the cell membrane (Chan 2001; El Bana et al. 2016) and leads to microglial activation and neuroinflammation (Liu et al. 2017). ROS production is not restricted to the primary phase, however, as the rapid reperfusion that occurs as part of the latent phase has also been associated with significant ROS release after HIBI (Rodrigo et al. 2013). As such, preserving cellular homeostasis by inhibiting ROS may result in neuroprotection after HIBI (Bonfante et al. 2020), but matching the timing of the intervention to the timing of peak ROS release will be important in optimizing therapeutic benefit.

ROS in the cell are generated through multiple pathways, including NADPH oxidase family (NOX)-mediated, nitric oxide synthase (NOS)-mediated, and calcium-mediated pathways. NOX enzymes generate ROS, including

hydroperoxides (Bedard & Krause 2007), and catalyze oxidative injury in HIBI (Choi et al. 2015; Liu et al. 2015). NOX is overexpressed in injured brain tissue after HIBI, resulting in the death of vulnerable striatal neurons (Yoshioka et al. 2011). NOS has a more complex role after HIBI (Liu et al. 2015), which appears to be mostly dependent on the specific type of NOS being expressed. Endothelial NOS (eNOS) may play a neuroprotective role primarily through the regulation of the vascular bed (Huang et al. 1996; Zhang and Iadecola 1994), while both the calcium-independent inducible NOS (iNOS) and calcium-dependent neuronal NOS (nNOS) release nitric oxide, resulting in significant mitochondrial injury (Chen et al. 2017; Sims and Anderson 2002; Zhao et al. 2000). Mitochondria also participate in intracellular oxidant production by taking up cellular calcium load during reperfusion, resulting in a surge in ROS production (Siesjö et al. 1999). Given the multiple pathways involved in the generation of ROS after HIBI, it is not surprising that interventions focused on individual ROS-generating pathways have not always shown promise for neuroprotection (Doverhag et al. 2008; Muramatsu et al. 2000).

14.3.3 ACSL4- and LOX-Mediated Lipid Peroxidation after HIBI

The ACSL4 enzyme is widely expressed in brain tissues and is involved in the linkage of long-chain fatty acids with CoA for the synthesis of PUFA-containing phospholipids. Consistent with its role in the pathway that generates oxidation-sensitive PUFA-containing phospholipids, overexpression of ACSL4 has been shown to sensitize cells for ferroptosis and ACSL4 deletion renders cells resistant to ferroptotic cell death (Doll et al. 2017; Cui et al. 2021). After ACSL4 adds CoA to the PUFA, the enzyme lysophosphatidylcholine acyltransferase 3 (LPCAT3) then synthesizes the PUFA-CoA to its corresponding phospholipid-PUFA (Kagan et al. 2017). Although the ACSL4 inhibitor rosiglitazone has been shown to reduce injury and protect neurological function after HIBI (Sayan-Ozacmak et al. 2012; Han et al. 2015), little is known about the overall contribution of ACSL4 and LPCAT3 to HIBI.

The classical hypoxia-inducible factor (HIF) transcription factor dimer consisting of HIF1α and HIF1β is known to regulate more than 200 genes implicated in hypoxia (Ratan 2020). Pro-ferroptotic ACSL4 expression can be suppressed by HIF1α to inhibit neuronal cell loss and microglial cytokine production (Cui et al. 2021). Conversely, the HIF-regulating proline hydroxylases (PHDs) inhibit HIF1α, and as such have been shown to increase ferroptosis. PHD inhibition through administration of specific PHD inhibitors or iron chelators can protect against ferroptosis and suppress the pro-ferroptotic gene activating transcription factor 4 (ATF4) (Appelhoff et al. 2004; Karuppagounder et al. 2016; Siddiq et al. 2005).

LOXs—specifically 12/15-lipoxygenase—are essential enzymes catalyzing oxidation of PUFA-containing phospholipids as one of the final steps in lipid peroxidation and ultimately ferroptosis. These enzymes are overexpressed, especially in neurons, following cerebral ischemia (van Leyen et al. 2006; Yigitkanli et al. 2013). Inhibiting the LOX pathway through baicalein administration protects against

neuronal oxidative stress in a middle cerebral artery occlusion model of HIBI (van Leyen et al. 2006). The LOX enzymes are facilitated in part by several supporting molecules, including the scaffolding protein phosphatidylethanolamine-binding protein 1 (PEBP1) (Wenzel et al. 2017). During HIBI, PEBP1 is elevated in the injured tissue. PEBP1 overexpression increased infarct size and neuronal inflammation, which can be prevented by increasing Ser153 phosphorylation of PEBP1 (Wang et al. 2017). Another protein that may facilitate LOX function is spermidine/spermine N1-acetyltransferase 1 (SSAT1) which is also upregulated following HIBI and interacts with arachidonate 15-LOX (ALOX15) (Zhao et al. 2022). SSAT1 knockdown decreases ROS and cortical iron concentration after HIBI, and activation of the SSAT1/ALOX15 axis exacerbates neuronal ferroptosis (Zhao et al. 2022).

14.3.4 Iron Accumulation after HIBI

One of the primary ROS-generating pathways in ferroptosis is iron-mediated ROS production. Iron homeostasis is vital in the normal function of the brain, but excess iron accumulation and redistribution is a key component of ferroptosis and has been implicated in exacerbation of HIBI. An increase in the level of unbound iron has been reported in adult animal models of cerebral HI (Chi et al. 2000) and blood plasma from human neonates (Dorrepaal et al. 1996). This abundant metal is present in the body in two forms: transferrin-bound and non-transferrin-bound iron (McCarthy and Kosman 2015). Microglia and infiltrating macrophages can engulf iron and metabolize it into unbound ferrous/ferric iron, which can induce the formation of ROS through the non-enzymatic Fenton reaction and eventually lead to ferroptosis (Conrad and Pratt 2019; Liu et al. 2022). Moreover, the enzymes responsible for oxidation of PUFAs, including the LOXs and P450 oxidoreductase (POR), also use iron for catalyzing peroxidation (Yosca et al. 2013; Dufrusine et al. 2019).

Ferritin, a key factor in iron homeostasis, stores iron and protects cells from redox-active free iron overload. Ferritin is downregulated after middle cerebral artery occlusion (Chen et al., 2021; Wang et al. 2016). The decrease in ferritin promotes p53 expression, suppressing SLC7A11 and triggering ferroptosis in hippocampal neurons, and overexpressing ferritin reverses the ferroptotic effects and results in improved locomotor and cognitive competence (Chen et al. 2021a, b). In addition to cytoplasmic ferritin, the elevated expression of mitochondrial ferritin (FtMt) in neuronal cells increases erastin-induced ferroptosis. As such, FtMt-knockout mice experience severe neurological deficits with labile iron pools and ROS following HIBI and, conversely, FtMt overexpression reverses ferroptotic activation and therefore protects against HIBI-induced cell death (Wang et al. 2016). Lastly, intracellular free iron can be reduced by iron efflux driven by factors like Tau protein or through the administration of iron chelators. Due to the iron efflux effects, increased Tau protein may improve outcome in age-related ischemic stroke (Tuo et al. 2017). Similarly, in transient focal brain ischemia, administration of the

iron chelators deferoxamine or 2,2-dipyridyl results in reduced ROS generation, perfusion deficits, astrocyte activation, cerebral infarct volume, and swelling in the basal ganglia (Xing et al. 2009; Demougeot et al. 2004).

14.3.5 Antioxidants and Ferroptosis after HIBI

Most cells throughout the human body contain intrinsic antioxidant systems to prevent or resist the pro-ferroptotic processes noted above. Exhaustion or suppression of these antioxidant defense systems, however, can exacerbate lipid peroxidation, thereby facilitating ferroptotic cell death. In fact, although erastin also increases ROS by binding to mitochondrial voltage-dependent anion channel 2, both of the classic ferroptosis inducers used in preclinical studies—erastin and RSL3—act in large part through inhibition of the GPX4-GSH antioxidant system (Han et al. 2020; Kuang et al. 2020).

GPX4 is a selenocysteine-containing phospholipid hydroperoxidase that is a key mediator in ferroptosis. A decrease in GPX4 activity has been documented after HIBI (Zhu et al. 2021), exacerbating ferroptosis (Lan et al. 2020), and GPX4 overexpression is associated with decreased tissue injury after neonatal HIBI (Sheldon et al. 2004). GPX4 catalyzes the reduction of fatty acids hydroperoxide groups through the reducing substrate GSH. GSH is therefore vital in maintaining GPX4's antioxidant function. GSH is itself a free radical scavenger (Galano and Alvarez-Idaboy 2011), and its levels diminish during HIBI. Restoring GSH concentration, or treatment with its derivative glutathione monoethyl ester, ameliorates HIBI-induced lipid peroxidation in the hippocampus (Li et al. 2019; Anderson et al. 2004), potentially in part due to elevated expression of claudin-5 junction protein, thereby protecting against blood–brain barrier disruption (Song et al. 2015). N-acetyl cysteine (NAC) administration can also aid in maintaining cellular GSH levels after injury, resulting in a decrease in HIBI-related neuronal death (Won et al. 2015).

The amino acid trio cysteine, glycine, and glutamic acid form the tripeptide GSH, and the availability of cysteine and glutamate-cysteine ligase in cells is rate-limiting substrates for GSH production (Forman et al. 2009). The system Xc^- is responsible for cystine import and is known to exhibit increased activity in neural stem cells after hypoxia in vitro (Sims et al. 2012). Solute carrier family 3 member 2 (SLC3A2) and SLC7A11 are subunits of the system Xc^- heterodimer transporter and are therefore crucial genes upstream of GPX4. Through the transporter, the cell exchanges intracellular glutamate (an excitatory neurotransmitter) for extracellular cystine (Koppula et al. 2018). The association between HIBI and regulation of Xc^- transporter is complex, however, as the increase in activity of system Xc^- after HIBI results in a balance of excitatory cell death due to glutamate toxicity (Fogal et al. 2007; Hsieh et al. 2017; Soria et al. 2014) and inhibition of ferroptosis due to increased cystine intake (Lan et al. 2020).

Transcription of both SLC3A2 and SLC7A11 is induced by activated transcription factors (ATFs) such as ATF4 (Lewerenz et al. 2013) which is a stress-induced

transcription factor. ATF4 also induces expression of several cell death-related genes, such as Chac1, Trib3, CHOP, and cysteinyl tRNA synthetase (Ratan 2020). Hence, ATF4 is thought of as a pro-death transcriptional activator that propagates death responses to neuronal oxidative stress in vitro and to stroke in vivo, and ATF4 knockout animals have been shown to be less susceptible to HIBI (Lange et al. 2008). Nrf2 is another transcription factor that is constitutively degraded by Kelch-like ECH-associated protein 1 (Keap1) (Fan et al. 2017). Inhibiting Keap1 stabilizes Nrf2 and results in the activation of genes involved in glutathione synthesis (Kraft et al. 2004). Nrf2 knockout has been shown to increase brain infarct size in a mouse model of neonatal HIBI, and several Nrf2 inducers have demonstrated neuroprotective effects after ischemic stroke (Liu et al. 2019; Zhang et al. 2020).

14.3.6 Inflammation and Ferroptosis after HIBI

After HIBI, the injured brain releases high mobility group box 1 (HMGB1) and peroxiredoxins which act as danger-associated molecular patterns (DAMPs) and are potent inducers of cytokine production and systemic immune response (Liesz et al. 2015; Shichita et al. 2012). Release of DAMPs, including HMGB1, is common in cells undergoing ferroptosis (Wen et al. 2019). HMGB1 is a potent inducer of TLR4 expression, which is increased after HIBI. TLR4 inhibition by TAK-242 results in downregulation of ferroptosis-related genes ATP5G3, PTGS2, CS, IREB2, and RPL8, and increased expression of SLC7A11 and GPX4 after HIBI both in vitro and in vivo (Zhu et al. 2021). Additionally, TLR4 has been shown to play a key role in the inflammation after ischemic stroke (Caso et al. 2007).

14.4 Pharmacological Inhibition of Ferroptosis after HIBI

To counteract oxidative stress, therapeutic hypothermia has used clinically to prevent neuronal cell death after neonatal HIBI (Jacobs et al. 2013). Despite the benefits of hypothermia, outcomes for these patients remain poor, which is why many investigators continue to evaluate supplemental therapies that could target ferroptotic cell death (Fig. 14.1).

GPX4 and other selenoproteins activate the adaptive transcriptional response against ferroptosis and improve functional recovery following stroke. Increasing selenoprotein production by pharmacological supplementation of the substrate selenium has been shown to induce the transcription of selenoprotein mRNAs, including GPX4, and results in decreased cell death and enhanced recovery after stroke (Alim et al. 2019). Additionally, organic selenium compounds such as methylselenocysteine and selenocystamine have also been reported to boost GPX4 activity and protect primary neurons from experimental ischemia (Tuo et al. 2021).

In addition to promoting GPX4 production by improving substrate availability, GPX4 production can also be upregulated by other methods. For example, carvacrol, a monoterpenic phenol that increases GPX4, also inhibits neuronal ferroptosis in

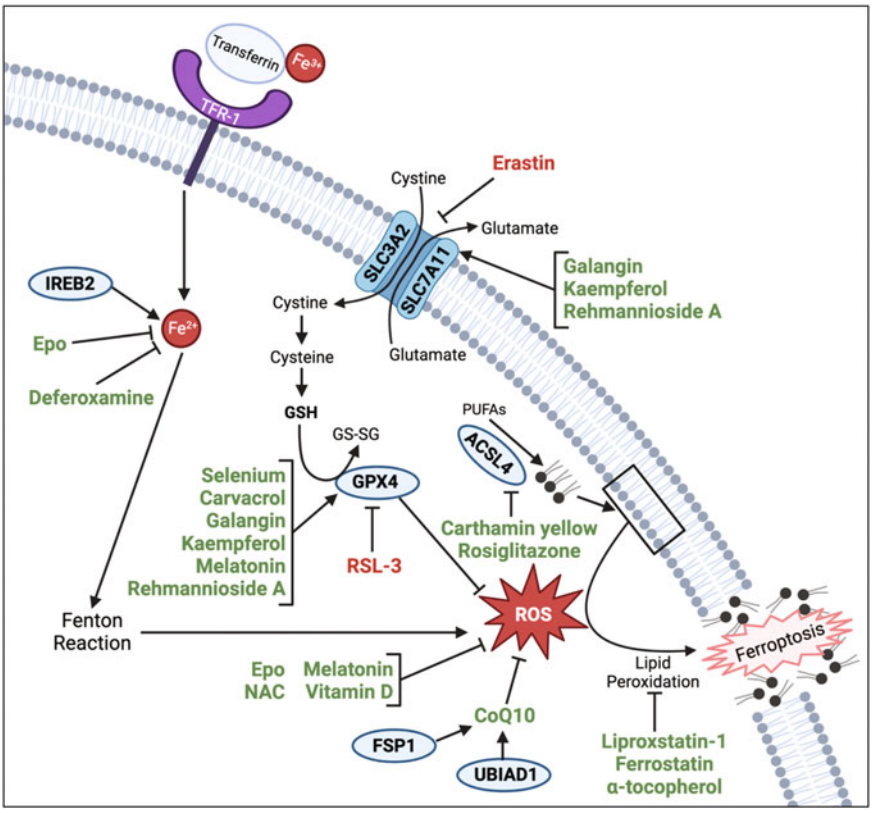

Fig. 14.1 Mechanistic pathways of ferroptotic cell death and examples of targets for pharmacologic modulation of ferroptosis. The classic ferroptosis-inducing chemicals 1S,3R-RAS selective lethal 3 (RSL-3) and erastin are shown in red, and several examples of potential ferroptosis-attenuating interventions are shown in green. ACSL4, acyl-CoA synthetase long-chain family member 4; Epo, erythropoietin; IREB2, iron-responsive element binding protein 2; FSP1, ferroptosis suppressor protein (also known as apoptosis-inducing factor 2 or AIFM2); GSH, glutathione; GS-SG, glutathione disulfide; GPX4, glutathione peroxidase 4; PUFA, polyunsaturated fatty acid; ROS, reactive oxygen species; SLC3A2, solute carrier family 3 member 2; SLC7A11, solute carrier family 7 member 11; TFR-1, transferrin receptor 1; UBIAD1, UbiA prenyltransferase domain containing 1. Figure created with BioRender.com

animal model of cerebral ischemia (Guan et al. 2019). Similarly, rehmannioside A activates SLC7A11/GPX4 and PI3K/AKT/NRF2 signaling pathways in a rat middle cerebral artery occlusion model, reducing brain infarct volume and improving spatial learning in rats (Fu et al. 2022). Galangin, kaempferol, and neuregulin1β are other natural compounds which also inhibit ferroptotic cell death through the SLC7A11/GPX4 axis during brain HI (Guan et al. 2021; Yuan et al. 2021; Zhai et al. 2022). Similar to the interventions above, baicalein promotes SLC7A11/GPX4 expression, but its neuroprotective effects after HIBI are also associated with reduced lipid peroxidation through ACSL4 suppression and increased levels of the negative

regulator of ferroptosis ACSL3 (Li et al. 2022). Rosiglitazone, liproxstatin-1, and ferrostatin-1 also decrease lipid peroxidation by inhibition of ACSL4, resulting in reduced lipid peroxidation and infarct volume, while promoting white matter integrity and cognitive function after HIBI (Chen et al., 2021; Tuo et al. 2017; Sayan-Ozacmak et al. 2012; Han et al. 2015).

Although concentrations of CoQ10 may not be reduced in the brain after HIBI (Tsukahara et al. 1999), exogenous CoQ10 administration has been shown to attenuate functional deficits and cerebral infarction in an animal model of adult stroke (Nasoohi et al. 2019). Similarly, the combination of CoQ10 and lipid-lowering statins produced enhanced neuroprotection above that of either intervention alone (Abd El-Aal et al. 2017). Another antioxidant, UBIAD1, is involved in the production of CoQ10 in Golgi membranes and regulates ischemic ferroptosis by repairing mitochondrial and other organelle functions in damaged brain tissue (Huang et al. 2022). N-acetylcysteine (NAC), vitamin D, and melatonin are all used clinically for other indications but are currently being evaluated in ferroptosis due to their free radical scavenging abilities. Each of them has shown therapeutic potential for brain ischemia, especially in neonates (Alonso-Alconada et al. 2013; Stessman and Peeples 2018; Adams et al. 2021). Neuroprotective effects of exogenous melatonin have been observed after HIBI in rats, with the protective effects being attributed to AKT/GPX4 pathways (Gou et al. 2020) and/or the suppression of ferritin (Rui et al. 2021; Gao et al. 2023).

To reduce the accumulation of ferroptosis-inducing free iron, certain iron chelators have been tested in vitro and in vivo to alleviate HIBI-related tissue injury. In this regard, intranasal administration of deferoxamine, a bacterial siderophore that chelates iron, protected against stroke damage following MCAO in rats (Hanson et al. 2009). Carthamin yellow, a flavonoid, has also shown anti-ferroptotic activity in MCAO rats by lowering iron and reactive oxygen species accumulation and reversing ACSL4 expression levels in the brain (Guo et al. 2021). Lastly, N-omega-nitro-L-arginine (NLA) administration after HIBI reduces plasma concentrations of free iron, resulting in suppressed nitric oxide formation and lower brain malondialdehyde concentrations (Dorrepaal et al. 1997).

14.5 Conclusions

"Ferroptosis" is a relatively new term coined to describe the deteriorating cell functions primarily characterized by imbalance in metabolic pathways mediated by triggers like free iron accumulation and disruption of antioxidant GSH system and resulting in lipid peroxidation. Since its inception in the last decade, researchers have demonstrated the involvement of ferroptosis in many different neurodegenerative diseases and specifically in both neonatal and adult HIBI. Despite the growing preclinical data outlined in this chapter, there are minimal human data in this field to date. That said, therapeutic targeting of ferroptosis pathways has shown some early promise in providing neuroprotective benefits.

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Involvement of Ferroptosis in Lupus Nephritis

15

Keiko Hosohata

Abstract

Lupus nephritis (LN) is one of the most common severe organ manifestations of systemic lupus erythematosus (SLE). LN is often associated with a poor prognosis, a higher risk of progression to end-stage renal disease, the need for replacement therapy, and mortality. LN is considered to start in the glomeruli with the massive deposition of immune complexes and then locally produced inflammatory mediators recruit leukocytes. In LN, glomerular injury results in increased leakage of transferrin-bound and non-transferrin-bound iron, leading to tubular iron accumulation that triggers ferroptosis, a recently recognized form of nonapoptotic cell death. At the same time, loss of glomerular permeability causes tubulointerstitial injury, which is sustained by an intrinsic tubular cell inflammatory phenotype and infiltrating immune cells, mediated by reactive oxygen species (ROS). Ferroptosis is also triggered by ROS due to iron overload, in addition to lipid peroxidation accumulation, or inhibition of phospholipid hydroperoxidase glutathione peroxidase 4 (GPX4). The kidney in particular has higher rates of O_2 consumption in its mitochondria than other organs; therefore, it is susceptible to imbalances between ROS and antioxidants. In this chapter, we summarize the role of ferroptosis in the etiopathology of LN.

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15.1 Introduction

Lupus nephritis (LN), glomerulonephritis with severe proteinuria, is a serious complication of systemic lupus erythematosus (SLE) (Jaryal and Vikrant 2017). Approximately 200 of every 100,000 people develop SLE in Europe (Barber et al. 2021), and it is more common in women; however, men who develop SLE may have a more severe form (Barber et al. 2021). A meta-analysis in the USA showed a higher incidence and prevalence of SLE in minority populations and women across all registries (Izmirly et al. 2021). A population-based registry of individuals with SLE residing in San Francisco County showed a higher age-standardized prevalence of SLE in women among ethnic minorities: in Blacks (458.1 per 100,000 persons), in Hispanics (177.9 per 100,000 persons), in Asians (149.7 per 100,000 persons), and in Whites (109.8 per 100,000 persons) (Dall'Era et al. 2017). In the UK, people of Black Caribbean ethnicity had the highest incidence and prevalence of SLE, although the incidence of SLE has been declining, the prevalence has been increasing in the UK in recent years (Rees et al. 2016). It has been suggested that 40–60% of SLE patients are at risk of developing LN during their lifetime, causing irreversible nephron loss (Murphy and Isenberg 2019).

LN is almost twice as prevalent in children than in adults and is associated with long-term morbidity (Huang et al. 2022). The clinical features of LN in childhood are similar across regions, whereas the prevalence of LN, disease severity, comorbidities, and outcomes might vary in developing and developed countries (Pereira et al. 2011). A large population-based registry from North America, including 184 children with SLE, showed that LN was more prevalent in African Americans, Asian/Pacific Islanders, and Hispanic patients, that African Americans had increased neurological manifestations, and that both African Americans and Asian/Pacific Islanders had increased hematological manifestations compared with Whites (Maningding et al. 2020).

In 5–30% of patients with LN, end-stage kidney disease (ESKD) still develops within 10 years of diagnosis. The gold standard for the diagnosis and classification of LN is the percutaneous kidney biopsy. The treatment response in LN is defined clinically and generally stratified into complete (CR), partial (PR), and no response. There is no consensus definition of CR in LN across guidelines, and proteinuria is the most important clinical variable used for definition of response. Generally, a reduction in protein excretion to <0.5 g/d based on a 24-hour urine collection with normal serum creatinine or serum creatinine level within 15% of previous baseline values is considered a CR (Parikh et al. 2020).

15.2 Glomerular and Tubular Injury in LN

15.2.1 Glomerular Injury in LN

LN is considered to start in the glomeruli with the massive deposition of immune complexes, composed of antinuclear, anti-C1q, and cross-reactive anti-glomerular autoantibodies (Trouw et al. 2004). Macrophages are abundant in glomerular lesions in LN [21], and their numbers are associated with clinical and histopathological parameters related to disease (Bos et al. 2022). Several studies showed that the number of CD68+ macrophages in tissue is associated with LN disease parameters (increased anti-dsDNA levels, complement depletion, high SLEDAI, high proteinuria) and is also predictive of kidney outcome (Hill et al. 2001). After immune complex deposition along the microvascular structures of the kidney locally produced inflammatory mediators recruit leukocytes. The antigen-presenting cells (APCs) present self-antigens from various sources to T lymphocytes, generating autoreactive T cells with a low activation threshold. These CD4 T lymphocytes in turn instruct B cells to produce autoantibodies of different specificities. In situ-generated or circulating immune complex deposits in the glomeruli are the most plausible culprits. This leads to progressive glomerular pathology and secretion of chemokines, cytokines, and matrix proteins, resulting in immune cell infiltration and glomerular damage. Loss of glomerular permeability causes tubulointerstitial injury that is sustained by an intrinsic tubular cell inflammatory phenotype and infiltrating immune cells. The renal tubules play an important role in the maintenance of body fluid homeostasis and defense of the body against toxic reactions via the absorption and secretion of various xenobiotics and endogenous compounds. Then, tubulointerstitial injury eventually leads to kidney injury (Fig. 15.1).

15.2.2 Tubular Injury in LN

Renal tubulointerstitial injury in LN is considered to be of great value to evaluate the degree of renal damage and to guide management and determine prognosis (Parikh et al. 2014). Previous studies have reported that patients with LN with tubulointerstitial inflammation had the greatest risk for progression to renal failure, whereas there was no relationship between the probability of remission and tubulointerstitial inflammation (Chang et al. 2011; Alsuwaida 2013). A constant feature of LN is the concomitant presence of tubulointerstitial inflammation (Theilig 2010). Importantly, tubulointerstitial inflammation, fibrosis, and tubular atrophy are strongly correlated with poor renal outcomes independent of the extent of glomerular damage (Broder et al. 2018). The enhanced glomerular permeability along with glomerular injury leads to overabsorption of many proteins via proximal tubular epithelial cells, which trigger tubulointerstitial inflammation, scarring, and renal function deterioration (Abbate and Remuzzi 1999). Immune complex deposits are observed in the tubular basement membrane in many patients with LN, and the quantity of immune complex deposition correlates with the severity of

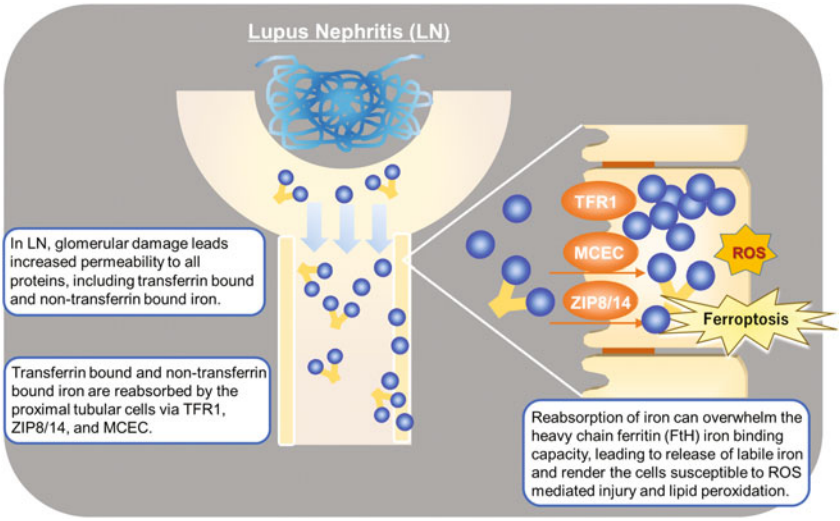

Fig. 15.1 In lupus nephritis (LN), glomerular damage leads increased permeability to all proteins, including transferrin-bound and non-transferrin-bound iron. Transferrin-bound and non-transferrin-bound iron are reabsorbed by the proximal tubular cells via TFR1, ZIP8/14, and MCEC. This can overwhelm the heavy chain ferritin (FtH) iron binding capacity, leading to release of labile iron and render the PTEC susceptible to ROS-mediated injury and lipid peroxidation

tubulointerstitial inflammation (Chang et al. 2011). Tubulointerstitial inflammation may be less responsive to current immunosuppressive treatment than glomerular proliferative changes (Yung et al. 2005). Proximal tubular cells are susceptible to injury by autoantibodies, including anti-dsDNA antibodies. When proximal renal tubular cells are exposed to an identical anti-dsDNA IgG concentration, cellular hydrogen peroxide (H_2O_2) is increased, activating the reactive oxidative species (ROS)-sensitive transcription factors NF-kB, ERK, MAPK, and the downstream JNK signaling pathway (Yung et al. 2005). This has been shown to result in IL-6, CXCL8, CCL2, and soluble fibronectin secretion and a downstream increase in profibrotic TGF-β1 and collagen synthesis (Yung et al. 2017).

15.2.3 Involvement of Oxidative Stress in Tubular Injury in LN

Proximal renal tubular cells are metabolically active compared to other renal cells. They reabsorb approximately 80% of the glomerular filtrate, including glucose, ions, and nutrients, and they contain more mitochondria than any other renal cells to provide sufficient energy to enable them to remove waste from the blood, reabsorb nutrients, regulate the balance of electrolytes and fluid, maintain acid–base homeostasis, and regulate blood pressure (Bhargava and Schnellmann 2017). β-oxidation is the most efficient mechanism to produce ATP in renal proximal tubules, so high consumption of oxygen is observed in proximal tubules, and they are thus more

susceptible than other cell types to changes in oxygen levels (Forbes 2016). A decrease in oxygen levels can lead to impaired β-oxidation and a reduction in ATP production.

A redox imbalance is observed in patients with active LN, and it is considered to be involved in lipid peroxidation of the glomerular basal membrane, which impairs renal tubular function (Bona et al. 2020). High ROS levels were observed in the serum from patients with active LN (Moroni et al. 2010). Oxidative stress is a phenomenon caused by an imbalance between production and removal of ROS in cells and tissues (Pizzino et al. 2017). ROS are induced by exogenous and endogenous sources. The sensitive balance between the production and elimination of ROS dictates the normal function of cells. However, when cells are unable to keep redox homeostasis via the detoxification of these reactive species and/or repair the damage, oxidative stress appears. Accumulation of cellular ROS can affect cellular contents such as lipids, proteins, and DNA. Commonly, ROS are defined as superoxide anion radical ($O_2^{\bullet -}$), hydroxyl radical ($HO^{\bullet}$), H_2O_2, and singlet oxygen ($^1O_2$), which have highly reactive properties; they are generated as metabolic by-products by biological systems (Navarro-Yepes et al. 2014). ROS are involved in the maintenance of redox homeostasis and various cellular signaling pathways. ROS are generated from diverse sources including the mitochondrial respiratory chain, enzymatic activation of cytochrome p450, and NADPH oxidases, further suggesting their involvement in a complex array of cellular processes (Bae et al. 2011). Excessive generation of ROS is harmful to cells, because they cause the oxidation of lipids, proteins, and DNA. In particular, $O_2^{\bullet -}$, $HO^{\bullet}$, and H_2O_2 are detrimental to tissues. Mitochondria possess various cellular functions and produce ATP, thereby supplying the energy source for basal cell functions (Bhargava and Schnellmann 2017). As a result, mitochondria are the main source of ROS by production of highly reactive and toxic hydroxyl radicals (Fleury et al. 2002).

Nitric oxide (NO) and its derivate reactive nitrogen species (RNS) are also produced in peroxisomes. Peroxisomes can regulate ROS and NO/RNS levels in their role as signaling molecules (Sandalio et al. 2023). There is a close relationship between ROS and RNS. Major RNS include nitric oxide ($^{\bullet}NO$), dinitrogen trioxide (N_2O_3), peroxynitrite ($ONOO^-$), nitrogen dioxide ($^{\bullet}NO_2$), and other oxides of nitrogen. $O_2^{\bullet -}$ can also react with nitric oxide ($NO^{\bullet}$) to produce peroxynitrite ($ONOO^-$). The most reactive and damaging are $HO^{\bullet}$ and $ONOO^-$. In particular, these reactants are abundantly produced in mitochondria where molecular oxygen (O_2) is reduced to $O_2^{\bullet -}$ by electrons that escape from the respiratory chain, mainly at mitochondrial complexes I and III. The degradation of $ONOO^-$ yields highly oxidizing intermediates, such as nitrogen dioxide ($NO_2^{\bullet}$) and the hydroxyl radical ($OH^{\bullet}$); finally, stable nitrite (NO_3^-) is generated. In the kidney, NO synthase (NOS) is expressed at various sites (Bachmann et al. 1995), and especially, higher $^{\bullet}NO$ levels are observed in the medulla (Kim et al. 2016). Generally, NO behaves as a vasodilator and contributes to lowering vascular tone in the kidney (Navar et al. 1996). In contrast, $^{\bullet}NO$ is produced at the macula densa and is involved in renin secretion and tubuloglomerular feedback via vasoconstriction of afferent arteries (Castrop et al. 2004) (Fig. 15.2).

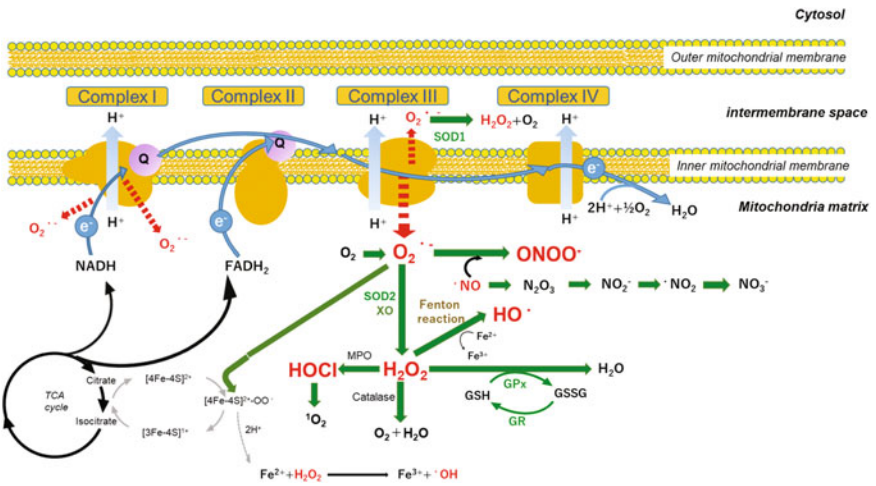

Fig. 15.2 Summary of reactive oxygen species (ROS) and reactive nitrogen species (RNS) interactions. Hydroxyl radical ($HO^{\bullet}$) and peroxynitrite ($ONOO^{-}$) are especially abundantly produced in mitochondria where molecular oxygen (O_2) is reduced to superoxide anion radical ($O_2^{\bullet -}$) by electrons that escape from the respiratory chain. By the cytosolic and mitochondrial superoxide dismutases (SODs), $O_2^{\bullet -}$ is dismutated to hydrogen peroxide (H_2O_2), which is in turn converted into innocuous products by glutathione peroxidase (GPx). Under the presence of myeloperoxidase (MPO), H_2O_2 is converted to HOCl and H_2O. GR, glutathione reductase; GSH, glutathione; GSSG, glutathione disulfide; H_2O_2, hydrogen peroxide; NOX, NADPH oxidase; NO, nitric oxide; $ONOO^{-}$, peroxynitrite; O_2, oxygen; $O_2^{\bullet -}$, superoxide anion radical; XO, xanthine oxidase

Reactive carbonyl species (RCS) are generated predominantly through the oxidative degradation of lipid peroxides (LOOH), most of which are formed by the oxidation of polyunsaturated fatty acids (PUFAs) by ROS, such as·OH in particular, or in the reaction mediated by lipoxygenases (Sandalio et al. 2023).

15.3 Role of Iron in LN

15.3.1 Iron in the Physiological Condition

Iron is an essential element for diverse biological processes, from enzyme activity regulation, immune function, and oxygen transport to mitochondrial function and DNA synthesis and repair. In humans, most iron is used for heme and hemoglobin synthesis (Camaschella 2019), which act as the carriers of oxygen to tissues throughout the human body. Iron has two states, the reduced form (Fe (II) or "ferrous") and the oxidized form (Fe (III) or "ferric"), which are present and altered by redox reactions. Therefore, iron is an essential cofactor in reactions involving ROS and handling oxidative stress. Dietary iron is not readily absorbed because it is insoluble at intestinal pH and requires promotion of compounds found in food, and there are no specific iron excretion mechanisms in the body. Duodenal enterocytes

are the major site of dietary iron uptake (Choi et al. 2012). Iron is mainly regulated by the liver, reticuloendothelial system, and several mediators. Several organs also have unique local iron regulation (van Swelm et al. 2020). In particular, overload of iron is detrimental and affects several parenchymal organs (Cabantchik 2014). In this condition, commonly affected organs are the endocrine glands, heart, and liver. The kidney can also be damaged by iron.

Under physiological conditions, there is little transferrin-bound and non-transferrin-bound iron, which is filtered by the glomerular assembly and then reabsorbed and cycled by the proximal tubular cells. At a cellular level, under physiological conditions, iron entry occurs through the cell membrane by the transferrin receptor. After iron enters the cell, excess iron is usually stored in ferritin. Ferritin is an essentially intracellular protein that serves to store iron safely. It is also present at very low concentrations in serum, likely via secretion by macrophages (Cohen et al. 2010). Normal values usually range from 30 to 200 μg/L or 300 μg/L in females and males, respectively. The function of secreted extracellular ferritin remains largely unknown. Excess iron is also carried to the mitochondria for heme synthesis and formation of proteins containing iron–sulfur (Fe-S) clusters (Maio and Rouault 2020). In these clusters, both iron and sulfur can donate or accept electrons and form a crucial component of many enzymes in the body. Abnormal distribution and excess content of iron in cells promotes the Fenton reaction which produces the hydroxyl radical ($HO^{\bullet}$) and other ROS, leading to cell and tissue damage (Lai et al. 2021). Thus, there is a close association between iron overload and ROS, indicating that tissue-iron accumulation causes ROS damage and its related toxicities.

15.3.2 Iron in Pathological Condition

In LN, glomerular injury results in increased leakage of transferrin-bound and non-transferrin-bound iron, which can be reabsorbed by the proximal renal tubular cells via TfR1, ZIP8/14, and MCEC. Iron has been shown to accumulate within lysosomes in the proximal tubules in patients with chronic kidney disease (CKD) (Nankivell et al. 1992). In renal biopsies of primary glomerulopathies such as focal segmental glomerulosclerosis, anti-glomerular basement membrane disease, and IgA nephropathy, as well as secondary ones such as LN, iron was deposited in a granular pattern in both proximal and distal tubules (van Raaij et al. 2018). Tubular iron accumulation is also a feature in animal models of LN (Theut et al. 2020).

15.4 Role of Ferroptosis in LN

15.4.1 Ferroptosis

Under physiological or pathological conditions, cell death is an unavoidable and important link in development, growth, aging, and regeneration. Traditionally, cell death has been classified into apoptosis and necrosis. Recently, in addition to

necrosis and apoptosis, there are also other new programmed cell death forms, such as autophagy, necrosis, and necrotic apoptosis, which have unique biological processes and pathophysiological characteristics. In 2012, a new concept of cell death, "ferroptosis," was developed. Ferroptosis is a new type of cell death that is usually accompanied by a large amount of iron accumulation and lipid peroxidation during the cell death process (Dixon et al. 2012). Ferroptosis occurs iron-dependently and shows a non-apoptotic form, and its molecular features have been recognized as distinct (Dixon et al. 2012). Ferroptosis differs from apoptosis, autophagy, and necroptosis, and its morphological characteristics are largely observed in mitochondria. Morphologically, in ferroptotic cells, shrinking mitochondria are observed, which leads to increased density of the mitochondrial membrane, rupture or vanishing of mitochondrial cristae, and a ruptured outer membrane, whereas the cell membrane remains intact, the nucleus is normal in size, and there is no concentration of chromatin (Xie et al. 2016).

15.4.2 Involvement of Iron in Ferroptosis

During development or normal homeostatic tissue turnover, ferroptosis could be triggered by the accumulation of 1) iron (Li et al. 2021; Zhang et al. 2020; Wlazlo et al. 2021; Liu et al. 2021), 2) PUFAs (Yang and Stockwell 2016; Yang et al. 2016; Kagan et al. 2017; Dubin et al. 2017), or by 3) depletion of the antioxidant glutathione (GSH) (Tang et al. 2022), decreased function of glutathione peroxidase 4 (GPX4), which mediates the reduction of lipid peroxides(Maiorino et al. 2018), or activation of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (Wen et al. 2021). Lipid peroxidation is an important biological reaction. In particular, PUFAs are easily peroxidized by free radicals and enzymes. PUFAs are subject to lipid peroxidation, and the peroxidated PUFAs drive ferroptosis. Of the PUFA-related phospholipids, phosphatidylethanolamines (PEs) with arachidonoyl (AA) or its derivative adrenaline adrenoyl (AdA) moieties are important substrates of oxidation in ferroptosis (Kagan et al. 2017). Inside the cells, superoxide dismutase (SOD)1 is the most abundant among antioxidant enzymes and can transform $O_2^{\bullet -}$ to H_2O (Fridovich 1986). On the other hand, the effect of exogenous SOD1 is limited due to its low cell membrane permeability. Further decomposition of H_2O_2 to H_2O and O_2 is catalyzed by other antioxidative enzymes within mitochondria, such as glutathione peroxidase (GPx) and peroxiredoxin (PRx)/thioredoxin (TRx) (Mailloux 2015). GPX4 is the only enzyme that can decrease lipid hydroperoxides within biological membranes (Brigelius-Flohe and Maiorino 2013). In addition, GPX4 consumes glutathione as an essential cofactor for its enzymatic activity (Brigelius-Flohe and Maiorino 2013). GPX4 decreases hydroperoxides of PUFAs (PUFA-OOH) and phospholipids (PL-OOH) (Imai and Nakagawa 2003). Esterification of PUFAs into phospholipids requires acyl-CoA synthase-catalyzed formation of PUFA-CoA. The depletion of GSH reduces GPX4 activity, leading to the production of excess lipid ROS (Xie et al. 2016).

15.4.3 Involvement of ROS in Ferroptosis

The rates of O_2 consumption are higher in kidney mitochondria than in those of other organs (Cancherini et al. 2003), and H_2O_2 release accounts for 0.1–0.2% of the total consumed oxygen (Tahara et al. 2009). Oxidative stress is an imbalance between ROS production and its removal due to overproduction of ROS and/or a decrease in antioxidant defense activity (Pizzino et al. 2017). ROS are induced by exogenous and endogenous sources. Accumulation of cellular ROS can affect cellular contents such as lipids, proteins, and DNA. Major ROS are superoxide anion radical ($O_2^{\bullet -}$), $HO^{\bullet}$, H_2O_2, and singlet oxygen ($^1O_2$), which have highly reactive properties. It has been demonstrated that excessive generation of ROS is harmful to cells, because they cause the oxidation of lipids, proteins, and DNA (Bae et al. 2011). In particular, $O_2^{\bullet -}$, $HO^{\bullet}$, and H_2O_2 are detrimental to tissues. Decreased antioxidant capacity and chronic inflammation commonly occur in patients with chronic kidney disease. ROS can react with PUFAs of lipid membranes (Yang and Stockwell 2016; Yang et al. 2016; Kagan et al. 2017; Dubin et al. 2017), PUFAs are subject to lipid peroxidation, and the peroxidated PUFAs drive ferroptosis (Yang et al. 2016). Of the PUFA-related phospholipids, phosphatidylethanolamines (PEs) with arachidonoyl (AA) or its derivative adrenaline adrenoyl (AdA) moieties are important substrates of oxidation in ferroptosis (Kagan et al. 2017).

Among the ROS, the superoxide anion radical (O_2^{-}) is a key redox signaling molecule prominently generated by the NADPH oxidase (Nox) enzyme family and by the mitochondrial electron transport chain (Pecchillo Cimmino et al. 2023; Xie et al. 2017; Chen et al. 2022). Nox is present in the renal cortex, medulla, and blood vessels (Gill and Wilcox 2006). Among the members of the Nox family, the Nox2 isoform of NAD(P)H primarily generates O_2^{-} (Araujo and Wilcox 2014; Huang et al. 2017). On the other hand, the Nox4 isoform of NAD(P)H oxidase primarily produces H_2O_2 (Brown et al. 2014; Huang et al. 2017). ROS have been shown to trigger an inflammatory response through the activation of the tumor necrosis factor-α (TNF-α) pathway (Gloire et al. 2006). TNF-α binds to its cellular TNFR1 receptor, which triggers signaling cascades that activate nuclear factor kappa B (NF-κB) and AP-1 transcription factors. TNF-α promotes monocyte chemotaxis toward mesangial cells by regulating the expression of monocyte chemoattractant protein-1 (MCP-1) via a Rho-kinase/p38 mitogen-activated protein kinase (MAPK)-dependent pathway (Matoba et al. 2010). In addition, ROS activates p38MAPK and nuclear factor kappa B (NF-κB), leading to proinflammatory cytokine release and the accumulation of inflammatory cells in the kidney (Bao et al. 2007). Thus, sustained inflammatory responses may contribute to the progression of renal injury.

15.5 Biomarkers in LN

15.5.1 Current Biomarkers in LN

Many candidate LN biomarkers have been evaluated in several cohorts (Hosohata 2021). Most of these potential LN biomarkers have been assessed in cross-sectional studies, with few reports of markers prospectively evaluated. In kidney biopsies of LN patients, increased expression of monocyte chemoattractant protein 1 (MCP-1), which belongs to the C-C chemokine family, on epithelial or endothelial cells and infiltrating mononuclear leukocytes in the tubulointerstitial regions has been reported (Abozaid et al. 2020). In addition, TNF-like weaker inducer of apoptosis (TWEAK), neutrophil gelatinase-associated lipocalin (NGAL), and uric acid exhibited good ability to differentiate LN patients from non-renal SLE patients. TWEAK is another TNF-superfamily member with clinical potential. NGAL is a low-molecular-weight protein that is produced and secreted by neutrophil leukocytes (Mishra et al. 2003). Because lipid peroxidation is aberrantly activated in LN, the activation of ferroptosis is suggested, and LTF, CYBB, CCL5, G0S2, and AKR1C1, especially CYBB, might be good biomarkers of ferroptosis in LN (Wang et al. 2022).

NGAL is produced by the proximal tubules and is secreted into the urine in a variety of renal diseases, including acute kidney injury and CKD. However, NGAL is also synthesized by various types of tissues, such as the kidney, lung, stomach, and colon (Mishra et al. 2003). Therefore, its levels increase not only with kidney injury, but also with bacterial infection and chronic and systemic diseases without bacterial infection (Tornblom et al. 2020).

15.5.2 Future Biomarkers in LN

Several studies have shown the association between oxidative stress and renal injury involved in glomerular damage. However, tubular injury also affects glomerular filtration function via tubuloglomerular feedback (Wang et al. 2018). Thus, it is possible that biomarkers for renal tubular injury such as KIM-1 and vanin-1 could reflect renal damage in LN patients.

KIM-1 was isolated from the rat kidney and T cells as a protein with different functions (Ichimura et al. 1998). Functionally, KIM-1 regulates the immune response by modulating cytokine production, and, thus, it is also called T-cell immunoglobulin and mucin domain-containing molecule-1 (TIM-1). KIM-1 and TIM-1 have the same sequence of amino acids. KIM-1 is also known as hepatitis A virus cellular receptor-1 (HAVCR1) (Ichimura et al. 1998). The HAVCR-1 polypeptide is an attachment receptor for HAV, which suggests that it is also a functional receptor that mediates HAV infection (Kaplan et al. 1996). KIM-1 is a type 1 membrane protein containing a unique Ig-like domain and a mucin domain in its extracellular portion (Ichimura et al. 1998). KIM-1 is not detected in the normal kidney; however, it is strongly induced by damage to tubular epithelial cells (Kaplan

et al. 1996). The extracellular domain of KIM-1 is divided in a protease-dependent manner and secreted into the urine (Bailly et al. 2002). The biological function of KIM-1 currently remains unclear; however, it was identified as a phosphatidylserine receptor that is responsible for the phagocytosis and processing of apoptotic cells (Brooks et al. 2015). KIM-1 has the ability to specifically recognize the phosphatidylserine of apoptotic cell surface-specific epitopes and oxidized lipoproteins expressed by apoptotic tubular epithelial cells (Ichimura et al. 2008). Thus, KIM-1-expressing kidney epithelial cells actively phagocytose apoptotic and necrotic cells.

Vanin-1 was originally characterized as a membrane molecule expressed by a subset of thymic stromal cells and is considered to be involved in the homing of bone marrow precursor cells into the thymus in rats (Aurrand-Lions et al. 1996). Vanin-1 is a prototypic member of a family containing at least three genes in humans (i.e., VNN1, VNN2, and VNN3), two in mice (vanin-1 and vanin-3), and one *Drosophila* homologue (Galland et al. 1998; Granjeaud et al. 1999). Vanin-1 is anchored at the surface of epithelial cells by a glycosylphosphatidyl inositol moiety, with an ectoenzyme as a pantetheinase that catalyzes the hydrolysis of pantetheine to produce pantothenic acid (vitamin B5) and cysteamine (Pitari et al. 2000). In turn, cysteamine, which is the decarboxylated derivative of the amino acid cysteine, can be oxidized to cystamine. Cysteamine and cystamine are important sensors of oxidative stress, maintaining the thiol-disulfide equilibrium through protein disulfide exchange (Ferreira et al. 2015). Thus, vanin-1 promotes inflammation, similar to several other mediators of oxidative stress. Vanin-1 mRNA is strongly expressed in the kidney; however, its expression has a broad tissue distribution (Pitari et al. 2000). In the early phase of a variety of renal diseases, the urinary excretion of vanin-1 was elevated before traditional biomarkers in rats with nephrotoxicant- and drug-induced renal tubular injury (Hosohata et al. 2011; Hosohata et al. 2012, 2014; Hosohata et al. 2016b; Washino et al. 2018; Hosohata et al. 2018, 2019; Hosohata et al. 2021a), as well as in patients with AKI and CKD (Hosohata et al. 2016a; Washino et al. 2019; Hosohata et al. 2020; Hosohata et al. 2021b). AKI is often detected with urinary tract obstruction, which inhibits urinary flow and increases intratubular pressure. A strong correlation has been reported between vanin-1 and the severity of urinary tract obstruction.

15.6 Perspective in LN

Renal iron accumulation is associated with increased damage and inflammation in LN. Ferroptosis is considered to be involved in LN as an integral component in the vicious cycle of immune dysfunction, inflammation, and tissue damage (Hosohata et al. 2022). Renal tubular ferroptosis is a pathological feature in both human and murine LN, thus identifying a novel medication target to treat a disease still managed mainly by immunosuppression. The mechanism by which iron mediates and perpetuates tubulointerstitial inflammation following glomerular injury is important in terms of development of LN biomarkers and/or treatment of LN. This chapter

shows that ferroptosis has important prospects in the progression of LN. However, further studies are needed to fill the knowledge gaps of the relationship between ferroptosis and LN, shed more light on the pathogenesis of LN, and provide a new perspective on ferroptosis-based immunotherapy for LN.

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Ferroptosis and Infectious Diseases

16

Yang Zhang and Jen-Tsan Chi

Abstract

Ferroptosis is a newly recognized form of regulated cell death characterized by oxidative stress, iron dependency, and lipid peroxidation. The importance of ferroptosis has been appreciated in various pathological conditions, such as cancer, neurodegeneration, and ischemia injury. Interestingly, there is emerging evidence that ferroptosis may play an important and multifaceted role in the infection of various pathogens, including bacteria, viruses, and parasites. Here, we wish to summarize the several distinct ways that ferroptosis may play in host–pathogen interactions. First, the infected host cells may undergo ferroptosis, which may trigger damage-associated molecular pattern (DAMP) molecules to be recognized by immune cells for clearance and immune activation. Second, certain pathogens may modulate ferroptosis response by interacting with ferroptosis machinery to promote their propagation. Third, ferroptosis may also contribute to organ injuries during uncontrolled infection and sepsis. Importantly, since multiple compounds are available to enhance or inhibit ferroptosis, it is possible to modulate ferroptosis for therapeutic gain. Therefore, a detailed understanding will allow us to modulate ferroptosis to eliminate pathogens and ameliorate the acute and chronic adverse outcomes of infectious diseases.

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16.1 Introduction

The fate of life vs. death is one of the most fundamental decisions for all organisms. Cell death is part of the process involved in organogenesis and regeneration. Dysregulation of such life vs. death fate is strongly associated with various pathologic processes, such as cancer, heart disease, stroke, and infectious diseases. Cell death was initially thought to be a non-programmed, spontaneous event in response to injury or extreme stress, but many studies have revealed that this is a highly regulated process via different pathways (Nössing and Ryan 2022; Galluzzi et al. 2014; Christgen et al. 2022; Tang et al. 2019). Apoptosis and necrosis were thought to be the main forms of cell death. Apoptosis during development or homeostatic cell turnover is a "quiet" form of cell death as it avoids activation of inflammatory immune reactions. In contrast, necrosis and other cell deaths are immunologically active and associated with the release of inflammatory stimuli into the surrounding environment to recruit various immune cells and inflammatory reactions. Recently, several additional programmed cell death (PCD) have been described, including pyroptosis, necroptosis, NETosis, alkaliptosis (Song et al. 2018), and ferroptosis. For example, pyroptosis of host cells is triggered by membrane pores formed by gasdermins during pathogenic infection. Pyroptosis is characterized by inflammasome formation, caspase activation, and proinflammatory cytokine release (Christgen et al. 2020; Swanson et al. 2019). Necroptosis involves the activation of receptor-interacting protein (RIP) RIP1 and RIPK3, and subsequent phosphorylation of the pseudokinase mixed lineage kinase domain-like (MLKL) that polymerizes to form a membrane pore (Sun et al. 2012). NETosis mainly occurs in neutrophils that are associated with the formation of neutrophil extracellular traps (NETs) comprising modified chromatin decorated with various bactericidal proteins from neutrophils' granules and cytoplasm. NETosis is triggered by an excessive amount of reactive oxygen species (ROS) produced by NADPH oxidase (Vorobjeva and Chernyak 2020). In contrast, alkaliptosis is a novel cell death resulting from intracellular alkalinization after the downregulation of carbonic anhydrase IX (CA9) and loss of the ability to maintain pH homeostasis (Kuang et al. 2020).

16.2 The Hallmarks of Ferroptosis

Ferroptosis is a recently recognized form of cell death first described in 2012 (Dixon et al. 2012). Although the term and concept of ferroptosis were recently proposed, similar oxidative stress-induced cell death has been described before (Eagle et al. 1961; Dolma et al. 2003; Yagoda et al. 2007; Yang and Stockwell 2008). During the past decade, the research of ferroptosis has been broadly expended to different systems and diseases. We will mainly review the characteristics and mechanisms of ferroptosis in infectious diseases. Ferroptosis has been demonstrated in the pathogenesis of several acute and chronic diseases such as acute kidney injury, hepatic fibrosis, diabetes, neurodegenerative diseases, and cancers (Jiang et al. 2021; Dixon and Stockwell 2019; Du et al. 2023). There is some evidence showing that

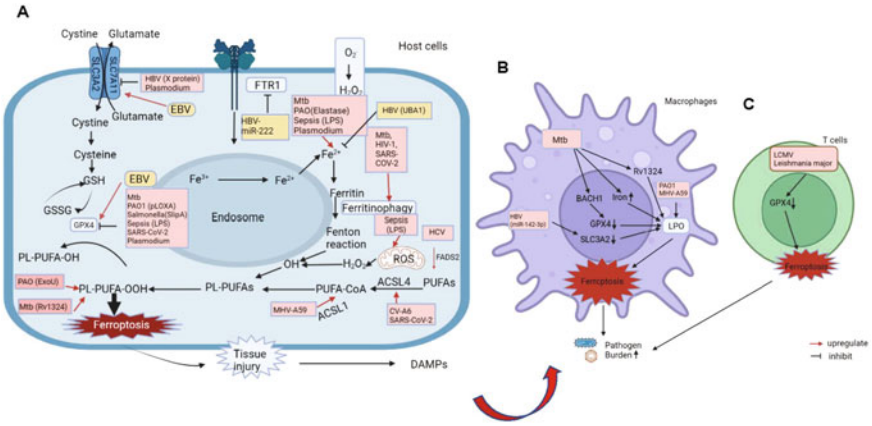

Fig. 16.1 Hallmarks of ferroptosis and crosstalk between ferroptosis regulators and pathogens. (**a**) Ferroptosis is an iron-dependent cell death that is characterized by the accumulation of ROS and lipid peroxidation. GPX4-dependent pathway serves as the most dominant antioxidant system. On the one hand, pathogens can suppress the expression level of system Xc- or GPX4 triggering ferroptosis which is further leading to tissue injury or pathogens dissemination. On the other hand, some of the pathogens can alter iron homeostasis and accumulation of lipid ROS which eventually regulate iron-dependent cell death in host cells. (**b**) Ferroptosis has also occurred in macrophages upon some pathogen infections, such as Mtb and PAO. (**c**) Under pathogen infection, especially some viruses, T cells can undergo ferroptosis as well

targeting ferroptosis may be a potential efficiency therapeutic strategy, especially in cancers. However, much remains unknown about the role of ferroptosis in other diseases. Here, we focus on infectious diseases and reviews on ferroptosis on these themes.

Ferroptosis is characterized by iron-dependent oxidative damage, accumulation of ROS, and lipid peroxidation (Fig. 16.1a). This would further lead to plasma membrane ruptures (Chen et al. 2020b; Ursini and Maiorino 2020). Morphologically, ferroptotic cells manifest as reduced mitochondrial volume, increased bilayer membrane density, and reduced mitochondrial cristae (Xie et al. 2016). Mechanically, ferroptosis can be mediated by the glutathione peroxidase 4 (GPX4)-dependent pathway and GPX4-independent pathway. In addition, iron oxidizes lipids in a Fenton-like manner that will result in the accumulation of ROS promoting ferroptosis (Yang and Stockwell 2008; Friedmann Angeli et al. 2014). Recently, zinc and zinc transporter have been shown to promote ferroptosis independent of iron (Chen and Chi 2021; Chen et al. 2021a). Genetically, ferroptosis is regulated by multiple genes, including SLC7A11, GPXs, ferroptosis suppressor protein (FSP1), related regulator of those genes, such as tumor protein 53 (p53) and erythroid 2-related factor 2 (NRF2). Genetic changes in iron homeostasis and lipid peroxidation metabolism are reported to be involved in ferroptosis (Li et al. 2020). These distinct features of ferroptosis make it different from other types of cell deaths.

16.3 Induction and Inhibitions of Ferroptosis

Ferroptosis occurs when intracellular lipid ROS accumulation exceeds the antioxidation capacity (Gao et al. 2022). As a redox defense, there are three types of antioxidants against ferroptosis: the glutathione (GSH)/GPX4 antioxidant system, apoptosis-inducing factor mitochondria-associated 2 [AIFM2/FSP1]-mediated coenzyme Q pathway and GTP cyclohydrolase 1-regulated tetrahydrobiopterin (BH4, THB) synthesis pathway. GSH/GPX4 system is the most well-established and most dominant mechanism in ferroptosis.

System Xc- effects as cystine and glutamate transporter located in cell membrane which is composed of two subunits encoded by *solute carrier family 7 member 11* (*SLC7A11*) and *solute carrier family 3 member 2* (*SLC3A2*) (Fig. 16.1a). Cystine and glutamate are utilized to synthesize GSH. GSH reduces ROS and reactive nitrogen under the action of GPXs. Ferroptosis can be induced by system Xc- inhibition which resulted in reduced GSH/GPXs activities and further leads to lipid ROS accumulation. System Xc- can be regulated by different mechanisms through protein–protein interaction, gene expression, and protein stabilization (Chen et al. 2021c). For example, P53 was established to be another inducer of ferroptosis by regulating the expression of SLC7A11 (Jiang et al. 2015). One of the classic ferroptosis inducers, such as erastin, sorafenib, and sulfasalazine, can induce ferroptosis with reduced GSH levels by directly inhibiting system Xc- (Chen et al. 2021c). Similarly, ferroptosis of different cancer cells (Tang et al. 2016; Tang et al. 2017; Yang et al. 2019b) can be induced by cystine deprivation that also lead to the cysteine/glutathione deletion, compromised GPX4 activities, and ferroptosis.

Among GPXs, GPX4 serves as the key GSH-dependent regulator of ferroptosis by neutralizing lipid peroxidation. GPX4 converts GSH into oxidized glutathione (GSSG) and reduces cytotoxic lipid peroxides (L-OOH) to the L-OH. Inhibition of GPX4 is another inducer for lipid peroxides that drive to ferroptosis. Accordingly, RSL3, DPI7, and DPI10 are the examples of ferroptosis inducers which act as GPX4 inhibitors resulting in accumulation of lipid peroxides (Yang et al. 2014). In addition, selenocysteine is essentially needed for the synthesis of GPX4. The mevalonate (MVA) pathway can regulate the maturation of selenocysteine tRNA which results in altered GPX4 synthesis. Therefore, inhibition of MVA pathway can induce ferroptosis through GPX4 regulation as well (Kryukov et al. 2003).

Apart from those above, AIFM2/FSP1-mediated CoQ production and GTP cyclohydrolase 1-regulated tetrahydrobiopterin synthesis plays an alternative role in the regulation of ferroptosis (Bersuker et al. 2019; Kraft et al. 2019). FIN56 and FINO2 are another classic ferroptosis inducers which act through those alternative pathways. FIN56 can not only promote GPX4 degradation but also deplete endogenous antioxidant coenzyme Q10 (CoQ10) that results in lipid peroxidation and ferroptosis. FINO2 induces ferroptosis due to oxidation of labile iron and the inactivation of GPX4 (Liang et al. 2019). There are also other regulators which contribute to ferroptosis. Our group identified that MESH1 can deplete NADPH and sensitize cells to ferroptosis in cancer cells (Ding et al. 2020). Further study showed that an increased cell density, adhesion, and connection can rescue the cancer cells

from ferroptosis through Hippo pathway or the activation of the integrin pathway (Brown et al. 2017; Wu et al. 2019; Yang et al. 2019a).

16.4 Iron Metabolism

Iron has been extremely precious for life during evolution, especially for organism to utilize the increased oxygen in the atmosphere. As the most abundant heavy metals, the adult human body contains as much as 2.5–4 g of iron (Ganz 2019). In mammals, iron can be uptake by DMT1 (SLC11A2), an iron transporter, expressed in duodenal mucosal cells (Yanatori and Kishi 2019). Iron solubility is altered with different pH. At neutral pH, Fe(II) is much more soluble than Fe(III), which makes Fe (II) easier to exchange through cell membrane via transporters. Fe(II) can initiate Fenton reaction and produce lipid ROS promoting lipid peroxidase and ferroptosis.

It has been well known that iron is a major player in oxidative stress resulting in cell death, especially ferroptosis. Iron-mediated ROS by Fenton reaction are responsible for lipid peroxidation in ferroptosis. Moreover, iron chelators have been revealed to be efficient ferroptosis inhibitor by deferent groups in vitro and in vivo (Vlahakos et al. 2012; Dixon et al. 2012).

Iron can be transported into cells after binding to transferrin and stored in ferritin as Fe(III). Given the importance of iron in ferroptosis, it is not surprising that several regulators of iron metabolism may impact the sensitivity of ferroptosis. Researchers have found that ferroptosis can be regulated by multiple mechanisms, such as transcriptional regulation (e.g., HIF-1), ATM kinase, posttranscriptional (e.g., IRP-1/2), ubiquitin-proteasomal (e.g., FBXL-5), and hormonal (e.g., hepcidin) (Hirota 2019; Moroishi et al. 2011; Iwai 2019; Ganz and Nemeth 2012; Chen et al. 2020a).

16.5 Lipid Peroxides and Lipid Peroxidation

Ferroptosis inhibitors such as iron chelators, ferrostatin-1 (Fer-1), liproxstatin-1, and vitamin E can rescue cell death by inhibiting the formation of lipid peroxide. Lipid peroxidation is identified by the formation of L-OOH and their decomposition leading to a series of products including reactive electrophiles. Rapid production of free radicals has occurred in minutes from L-OOH. This process is composed of three phases: 1) initiation phase—formation of the first lipid radical initiating the peroxidative chain reaction; 2) propagation phase—a peroxidative chain reaction with continued free radicals production; and 3) termination—arrest of the chain reaction by radical–radical interaction (Yin et al. 2011; Ursini and Maiorino 2020). Electrophiles and other toxic products are produced during lipid peroxidation, which can serve as biomarkers of ferroptosis. Among them, malondialdehyde (MDA) and 4-hydroxynonenal (4-HNE) are the toxic lipids to induce cell death (Ayala et al. 2014; Chen et al. 2022a) and often used as markers for ferroptosis (Dixon and Stockwell 2019).

16.6 Ferroptosis and Infectious Diseases

Given that the infection of host cells by pathogens is known to affect various biological processes regulating ferroptosis, it is not surprising that the ferroptosis plays a role during different stages of the pathogens. Similarly, pathogens may also affect the ferroptosis of host cells to enhance their survival and dissemination. Understanding such complicated and multifaceted role of ferroptosis in the host–pathogen interaction may identify novel means to control the acute dissemination and tissue injuries and prevent/treat the long-term consequences of infection, including malignant transformation and resulting tumors.

16.7 Bacterial Infections

16.7.1 *Mycobacterial tuberculosis* (*Mtb*)

Mycobacterial tuberculosis (*Mtb*) infection resulted in tuberculosis which remains a major global public health problem. Macrophages are the first-line responders against Mtb infections. Previous studies have shown that Mtb growth within macrophages is limited when macrophages undergo programmed cell death such as apoptosis and autophagy (Molloy et al. 1994; Oddo et al. 1998; Riendeau and Kornfeld 2003). The dead macrophages can then undergo efferocytosis by uninfected macrophages (Martin et al. 2012). On the other hand, non-programmed death of Mtb-infected macrophages results in bacterial release into the extracellular environment and enhances the spread of Mtb. Therefore, targeting such death mechanism may suppress the spread of Mtb as a potential therapeutic approach (Pan et al. 2005; Kiran et al. 2016).

Cell deaths including necroptosis, pyroptosis, ferroptosis, ETosis, parthanatos, and PANoptosis are reported to be involved in Mtb infection (Nisa et al. 2022). For example, Mtb necrotizing toxin (TNT) is the major cytotoxicity factor in macrophages that depletes the host nicotinamide adenine dinucleotide (NAD+) and activates RIPK3 and MLKL leading to necroptosis of Mtb-infected macrophages (Pajuelo et al. 2018). Pyroptosis also serves as an efficient antimicrobial defense pathway as the IL-1b-, IL-18-, and IL-1R-deficient mice exacerbated pathological stages upon Mtb infection (Labbe and Saleh 2008; Rastogi and Briken 2022).

Recently several studies implicated ferroptosis as a major death mechanism in Mtb infection and as a potential target for host-directed therapy of tuberculosis (Amaral et al. 2019; Shi et al. 2023) (Yaqoob et al. 2022; Luo et al. 2022). First, Eduardo and their colleagues found Mtb-infected macrophages exhibited several hallmarks of ferroptosis, including reduced glutathione and Gpx4, increased free iron, mitochondrial superoxide, and lipids peroxidation (Amaral and Namasivayam 2021). Importance of such an observation is shown that ferroptosis inhibitor ferrostatin-1 (Fer-1) can limit the death in Mtb-infected macrophages and bacterial burden in mice (Amaral and Namasivayam 2021). Another group pointed out that

the transcription factor BACH-1 downregulates the expression of GPX4 and enhances Mtb-induced ferroptosis in macrophages (Nishizawa et al. 2020; Amaral et al. 2020) (Fig. 16.1a, b).

Second, Mtb infection is associated with dysregulated iron metabolism. TB infection leads to iron overload in microphages that contribute to an excessive inflammatory response during Mtb infection (Boelaert et al. 2007; Reddy et al. 2018). The iron overload associated with ferritin heavy chain (FtH) deficiency leads to increased bacterial burden and increased mortality rate in Mtb-infected mice. Importantly, increased iron levels are also noted in human TB lung tissue (Reddy et al. 2018) (Fig. 16.1a, b). Other groups also revealed that ferritin degradation through the ferritinophagy triggers ferroptosis as well (Mancias et al. 2014). More recently, another group also revealed that Hmox1a, a paralog of mammalian iron regulator heme oxygenase 1 (HMOX1) in Zebrafish, protects host against *Mycobacterium marinum* infection by reducing infection-induced iron accumulation and reduced ferroptosis (Luo et al. 2022).

Third, Rv1324, a specific virulence factor secreted by Mtb, was reported as an antioxidant to enhance bacterial persistence in macrophages and further induce ferroptosis with reduced GPX4/GSH and increased lipid peroxidation in mouse lung tissue under M. Smegmatis infection (Shi et al. 2023) (Fig. 16.1a, b). Together, these findings highpoint the role of ferroptosis in Mtb infection-induced cell death. The molecular process by which Mtb promotes macrophages' ferroptosis and the crosstalk of different cell death pathways would need to be further explored. This will contribute to our effort to eradicate human tuberculosis by modulating cell death pathways.

16.7.2 *Pseudomonas aeruginosa*

P. aeruginosa is an opportunistic pathogen in immunosuppressed proinflammatory host and environments. Reported studies have shown that *P. aeruginosa* can release some virulence factors that can induce ferroptosis in host cells. For example, 15-lipoxygenase (pLoxA), a toxin derived from *P. aeruginosa* strain PAO1, can selectively oxidize the host's phospholipids and generate 15-hyddroperoxy-arachidonoyl-PE (15-HpETE-PE) which further induce ferroptosis in host cells (Dar et al. 2019). Such increased ferroptosis associated with GSH/GPX4 reduction dramatically boosts the injury caused by total body irradiation within the gut in mice (Fig. 16.1a). Moreover, pharmacological inhibition of the pLoxA by baicalein significantly reduced the ferroptosis of intestinal epithelial cells and mouse mortality (Dar et al. 2022).

ExoU, another lytic phospholipase toxin released from *P. aeruginosa* can trigger ferroptotic-like death associated with increased phospholipid peroxidation in macrophages and in non-immune cells as well (Fig. 16.1a, b). Importantly, one of the ferroptosis inhibitor, ferrostatin-1, can delay such ExoU-induced cell death and improve bacterial clearance in mice, suggesting blocking ferroptosis would be a

potential therapeutic target in the pathogenesis of *P. aeruginosa* infection (Bagayoko et al. 2021).

Other than lipase-like toxin, *P. aeruginosa* also expresses elastase, similar to neutrophil elastase, which can modify transferrin forming catalytic iron complexes and generating hydroxyl radicals (Britigan and Edeker 1991) and contribute to ferroptosis of host cells.

Apart from the virulence factors from *P. aeruginosa,* another study states that PAO degrades GPX4 though autophagy that further induce ferroptosis in epithelial cells. In contrast, host macrophages-produced NO• can prevent the epithelial cells from PAO-induced ferroptosis. Inhibition of NO• by pharmacological targeting of iNOS attenuated its anti-ferroptosis function though reduced lipid peroxidation(Dar et al. 2021). This finding shows that evidence of a potential new target against *P. aeruginosa*-induced ferroptosis in epithelial cells.

16.7.3 *Salmonella*

Several studies indicate ferroptosis may be involved in the pathologic process of Salmonella infections. Schauser et al. reported a non-caspase-dependent cell death in intestinal epithelial cell upon *S. typhimurium* infection (Schauser et al. 2005), which is suggestive of ferroptosis process may be involved. Further study revealed that the GPX4 level was used by the function of SipA, a factor released from *S. typhimurium* (Agbor et al. 2014), implying a potential to sensitize the host cells to ferroptosis (Fig. 16.1a).

Further evidence of the involvement of ferroptosis is from reported treatment with antioxidant in *S. typhimurium*-infected rats. Researchers reported that dichloromethane, a gradient from plants, can improve the infection-associated mortality, and these benefits may mediate through ferroptosis inhibition since they are associated with increased levels of glutathione and enhanced superoxide dismutase and catalase (Fankem et al. 2019; Jain et al. 2009).

16.7.4 *Chlamydia trachomatis*

A recent study reported the late stage of *Chlamydia trachomatis* infection cycle triggers several hallmarks of ferroptosis including the reduced level of SLC7A11, glutathione, and increased lipid peroxidation (Chen et al. 2021b). In addition, ferrostatin-1 and liproxstatin-1 can abolish *C. Trachomatis*-induced cell death and block bacteria dissemination (Chen et al. 2021b), implying the importance of ferroptosis in the dissemination of *Chlamydia*. Another group also found that hydroperoxide lipids are induced by *Chlamydia trachomatis* in multiple cell lines (Azenabor and Mahony 2000). Further investigation is expected to explore the importance of ferroptosis in the pathogenesis of *Chlamydia trachomatis* infection.

16.7.5 Polymicrobial-Induced Sepsis

Sepsis is a potentially life-threatening condition that occurs when the host's defense against an infection damages its own homeostasis. The activated immune response during sepsis can lead to tissue damage and multiple organ failure. Moreover, during COVID-19 pandemic, sepsis is a major contributor to the poor prognosis (Lei et al. 2022). Similar to other forms of cell death, ferroptosis can be a double-edged sword in sepsis. First, it helps immune cells to eliminate pathogens and infected host cells. On the other hand, the ferroptosis of immune cells and non-immune cells can promote bacterial invasion and damage in the infected organs affected by sepsis.

In the multiple organ injuries induced by sepsis, ferroptosis was noted in cardiomyocytes and pharmacological inhibition of ferroptosis significantly attenuated cardiac injury in cell culture and murine models of LPS-induced sepsis (Dai et al. 2020a; Dai et al. 2020b; Xiao et al. 2021). Similarly, ferroptosis is also found to be relevant in the sepsis-induced acute lung injury and ferrostatin-1 can rescue lung damage during sepsis (Liu et al. 2020; Xu et al. 2021; Zhang et al. 2022c; He et al. 2022; Li et al. 2022). Furthermore, sepsis-associated acute renal injury is reported to be induced by ferroptosis via iron overload and mitochondrial-derived ROS (Liang et al. 2022b; Yao et al. 2022) (Fig. 16.1a). Inhibition of mitochondrial-derived antioxidant NADPH oxidase (NOX) can protect renal cells from sepsis-induced ferroptosis. Apart from the above tissue injuries, ferroptosis is being identified as a role in other organ damages that happened during sepsis such as neuron, liver, and immune system (Tauber et al. 2021; Xie et al. 2022; Wei et al. 2020).

Although the pathologic process of sepsis requires multiple mediators, several ferroptosis inhibitors, such as vitamin E, deferoxamine, N-acetyl-L-cysteine (NAC) can rescue the mice from lethal sepsis that is revealed by different groups (Valnier Steckert et al. 2014; Vlahakos et al. 2012; Ritter et al. 2004). These studies indicate that the role of ferroptosis and its regulatory effectors such as GPX4-dependent and GPX4-independent molecules would be potential target for tissue damage during sepsis.

16.8 Viral Infections

16.8.1 Lymphocytic Choriomeningitis

The role of ferroptosis during viral infection has been also explored. Previously, researchers tried to uncover the relevance of GPX4 in T-cell development by using T-cell-specific *Gpx4*-deficient mice. During the process of lymphocytic choriomeningitis virus infection, both antigen-specific GPX4 null CD8+ and CD4+ T cells failed to expand and underwent to ferroptosis with accumulated membrane lipid peroxides. In addition, vitamin E rescued the phenotype induced by LCMV. These results indicate the important role of GPX4 and ferroptosis protection in the T-cell expansion during LCMV infection (Matsushita et al.

2015). Ferroptosis protection can also promote the longevity of virus-specific memory CD4+ T cells via mTORC2-AKT-GSK3β pathway (Wang et al. 2022). During LCMV infection, follicular helper T cells (Tfh cells) also show features of ferroptosis such as higher lipid ROS and lipid peroxidation regulated by GPX4 activity (Yao et al. 2021) (Fig. 16.1c).

16.8.2 Epstein-Barr Virus

Epstein-Barr virus (EBV) can infect B cells and drive the transformation of infected B cells to become Burkitt lymphoma and posttransplant lymphoproliferative (PTLD). Such transformation progress was found to generate lipid ROS that may sensitize ferroptosis in the Burkitt-like phase of B cells (Burton et al. 2022). Therefore, the induction of ferroptosis may have therapeutic value in preventing or treating EBV-mediated transformation (Burton et al. 2022). In nasopharyngeal carcinoma cells (NPC), acute EBV infection conferred ferroptosis resistance, which may play a part in the EBV-mediated transformation of NPC cells. Such resistance is mediated by activating NRF2 via p62-mediated KEAP1 degradation and upregulation of SLC7A11 and GPX4 expression (Yuan et al. 2022). These results indicate the importance of the ferroptosis in the EBV infection and transformation (Fig. 16.1a).

16.9 Enterovirus and Coronaviruses

Coxsackie virus is one of human enteroviruses which can cause common diseases such as hand, foot, and mouth disease (HFMD) and herpangina. During the process of virus replication, assembly, and release, membrane rearrangement is required. Acyl-coenzyme A synthetase long-chain family member (ACSL4) is involved in membrane synthesis and also a common host factor in RNA virus replication. During the infection of coxsackie A6, ACSL4 was found to be essential in viral replication organelle formation. ASCL4-deficient cells exhibited reduced ferroptosis accompanied by reduced virus titers upon Coxsackie virus A6 and SARS-CoV-2 infection (Fig. 16.1a). Two FDA-approved ACSL4 inhibitors, rosiglitazone and pioglitazoneare, can reduce the viral load of human enteroviruses and coronaviruses suggesting ACSL4 as a promising therapeutic target via ferroptosis (Kung et al. 2022). CVB3, another common type of coxsackie virus in myocarditis, can cause ferroptosis with an accumulation of lipid peroxides via Sp1/FRC/Fe axis (Yi et al. 2022).

Ferroptosis is reported to be involved in the pathogenesis of SARS-CoV-2 infection as well. First, SARS-CoV-2-infected cells express high level of ROS and reduced level of GPX4 which are the hallmarks of ferroptosis. Ferroptosis inhibitors rescued the GPX4 expression and mitigated the increased ROS during viral infection. Second, the infection increased free iron and hyperferritinemia contributes to the ROS-induced oxidative damage, inflammation, and ferroptosis (Cavezzi et al.

2020) (Fig. 16.1a). Study has shown that ferritin serum levels are correlated to disease severity in COVID-19 patients (Colafrancesco et al. 2020). Another piece of evidence is iron chelator that can reduce tissue injuries in COVID-19 patients which indicate ferroptosis may be involved in such pathological process (Habib et al. 2021). Third, the envelope (E) protein of SARS-CoV-2 can induce ferroptosis in host cells by inhibiting an epigenetic reader, bromodomain-containing protein 4 (BRD4) through regulating ferroptosis-related genes such as system Xct (Sui et al. 2019; Tang et al. 2022; Du et al. 2023). Last, ferroptosis also contributes to the pathologic processes and tissue injuries of liver and brain during SARS-CoV-2 infection (Chen et al. 2022c; Zhang et al. 2022b).

16.9.1 Human Immunodeficiency Virus

Ferroptosis is also relevant in HIV infection. HIV-infected individuals exhibit hallmarks of ferroptosis, such as decreased GSH and increased ROS. In addition, NAC treatment inhibits HIV replication in infected T cells and monocytic cells (Kalebic et al. 1991). Moreover, antiretroviral therapy can trigger ferroptosis in both CD4+ immune cells upon HIV-1 infection and cancer cells (Li et al. 2021; Xiao et al. 2022). Ferroptosis may be also involved in the HIV-associated neurodegeneration as the disease processes is characterized by enhanced lysosomal ferritinophagy that releases excessive amounts of iron, Ca2+, and cathepsin B (Sfera et al. 2022). In addition, HIV-1 gp120 protein can also induce ferroptosis via activating iron-responsive element-binding protein 2 (IRP-2) and affecting labile iron pools (Shimizu et al. 2007) (Fig. 16.1a).

16.9.2 Hepatitis Virus

Ferroptosis is also important in various hepatitis virus. Hepatitis B virus (HBV) X protein can sensitive hepatocytes to ferroptosis by suppression of *SLC7A11* (Liu et al. 2021). In HBV-related hepatocellular carcinoma, ubiquitin-like modifier activating enzyme 1 (UBA1) was overexpressed and associated with poor outcomes. The underlying mechanism for such association may be through the ability of UBA1 to regulate ferroptosis by affecting Fe2+ and malondialdehyde levels via Nrf2 (Shan et al. 2020). This finding indicates that ferroptosis may exert tumor suppressive capacity of HBV-related hepatocellular carcinoma cells (Fig. 16.1a). In addition, HBV-infected hepatocytes and M1-type macrophages may also undergo ferroptosis (Fig. 16.1b). The hepatocyte-enriched miR-222 promotes liver fibrosis through inhibiting ferroptosis (Zhang et al. 2022a). In contrast, miR-142-3p promoted ferroptosis of macrophages through suppressing *SLC3A2* (Hu et al. 2022).

Apart from HBV, hepatitis A virus 3C protease (3Cpro)-induced cell death exhibits features of ferroptosis that can be blocked by ferroptosis inhibitors (Komissarov et al. 2021). In hepatitis C, increased lipid peroxidation is shown to suppress HCV replicase activity in human hepatoma cells and primary hepatocytes.

The reduced HCV replicase activity then contributes to the viral persistence in damaged liver tissue. Further study indicated that ferroptosis-like lipid peroxidation of host cells suppresses HCV replication and the iron chelator deferoxamine abolished lipid peroxidation and promoted HCV replication (Yamane et al. 2022). Moreover, low dose of erastin can disrupt HCV replicase function and enhancing the efficiency of oral HCV antivirals to promote viral clearance. Mechanically, fatty acid desaturase 2 (FADS2) is a key determinant factor to sensitize the HCV-infected hepatoma cells to ferroptosis (Yamane et al. 2022) (Fig. 16.1a). Murine hepatitis virus strain A59 (MHV-A59) can upregulate ACSL1 and promote ferroptosis via NF-kB activation in macrophages (Xia et al. 2021) (Fig. 16.1a, b). Therefore, the ferroptosis inhibition can be a therapeutic strategy to protect hosts from MHV-A59 infection.

16.9.3 Other Viruses

The infection of HSV-1 and Zika virus also exhibit features of ferroptosis. For example, in the brain of HSV-1 infected brain, ferroptosis features were observed in the astrocytes and microglia cells via KEAP1-mediated NRF2 modification (Xu et al. 2022). Therefore, both ferroptosis inhibitor and a proteasome inhibitor suppressing Nrf2 degradation alleviated HSV-1 encephalitis (Xu et al. 2022), indicating the importance of ferroptosis in such disease processes. Zika virus is a neurotropic flavivirus and can induce brain damage. RNA-seq of the brain from a more recent study indicated the activation of ferroptosis pathways in Zika virus-induced mice (Yan et al. 2023).

16.10 Parasite Infections

16.10.1 Plasmodium

Iron is an essential nutrient for not only humans but also malaria parasites of different Plasmodium species (Drakesmith and Prentice 2012; Zhang et al. 2018). Therefore, it is not surprising that the ferroptosis plays an important role in the Plasmodium infection. During the blood stage of the malaria parasite life cycle, the cleavage of hemoglobin leads to increased oxidative stress due to the release of free heme. Consistently, Plasmodium-infected RBCs exhibit an accumulation of lipid peroxidation accompanied by reduced antioxidants GSH (Das and Nanda 1999; Jonscher et al. 2019). The oxidation of Fe2-heme causes the production of ROS via Fenton reaction which further results in lipid peroxidation and ferroptosis in RBCs (Xie et al. 2020; Vaid et al. 2010). Apart from this, researchers found that non-synonymous SNP rs1800371 of p53 alters the regulation of ferroptosis-related genes, such as GLS2 and SLC7A11 (Singh et al. 2020). In liver stage, a recent work showed that *P. yoelii*-infected hepatocytes underwent lipid peroxidation and ferroptosis by blocking SLC7A11 and GPX4 (Kain et al. 2020). Ferroptosis is also

involved in the cerebral malaria-associated neuronal damage with increased MDA and iron levels (Liang et al. 2022a). Therefore, there seems to be a strong role in the ferroptosis during Plasmodium infection (Fig. 16.1a).

16.10.2 Leishmania Major

Another ferroptosis-associated parasite is *Leishmania major* responsible for leishmaniasis. Proper regulation of lipid peroxidation in T cells is found to be essential to against *L. major* in the adaptive immune response. GPX4 deficiency in T cells triggered ferroptosis of both antigen-specific CD8(+) and CD4(+) T cells that abolish the expression of these T cells and reduced protection against *L. major* parasite infections, which were rescued with treatment of vitamin E that protect ferroptosis (Matsushita et al. 2015) (Fig. 16.1c). Therefore, the ferroptosis of the T cells may impair the immune response against *L. major*.

16.10.3 Fungi

Ferroptosis is also relevant during fungal infection. Both *Candida albicans* and *Aspergillus fumigatus* increase the levels of lipid peroxidation (Gross et al. 2000), implying a relevant role of ferroptosis in fungal infections. A recent study showed that novel polysaccharide (AUM-1) from marine *Aureobasidium melanogenum* acts as a ferroptosis-related immunomodulator by affecting the expression of GSH/GPX4 in RAW 264.7 cells (Lin et al. 2022). In plants, ferroptosis may play a role in the protection of rice against *Magnaporthe oryzae* (Caseys 2019; Shen et al. 2020; Dangol et al. 2019). *M. oryzae* infection modulates the expression of genes encoding proteins in the maintenance of iron homeostasis (Sánchez-Sanuy et al. 2022). OsNLP2 has been revealed as a negative regulator of ferroptotic cell death and defense responses in rice-infected *M. oryzae* (Chen et al. 2022b).

16.11 Conclusion and Perspectives

As summarized before, ferroptosis is an iron-dependent non-apoptotic programmed cell death involved in the host defense against different pathogens. As described above, multiple functions of ferroptosis in infectious diseases effects as a double-edged sword. Ferroptosis can diminish the pathogen within the immune cells. At meantime, DAMPs from ferroptotic cells can active immune response and facilitate the host control of infections. On the other hand, ferroptosis of host cell death may play a role in the pathogen dissemination by releasing pathogens and other pro-pathogen factors. Furthermore, the ferroptosis is a tumor suppression mechanism that can be of therapeutic approach to prevent or treat virus-induced oncogenesis. Reciprocally, during the continuous arm race between pathogens and hosts, pathogens may also manipulate the machinery involved in the ferroptosis to enhance

their survival and dissemination. Understanding the molecular basis of such host-pathogens may allow us to induce or inhibit ferroptosis to control infection and mitigate the organ injuries and oncogenesis from the infections as novel therapeutic approaches.

However, comparing to other cell deaths such as pyroptosis, study of ferroptosis is still needed to be fully expanded. For example, the specific markers or specific DAMPs generated by ferroptosis remain to be studied. Most cell death form a protein pore leading the membrane damage, there is still unclear whether ferroptotic cells have a pore or not. Regarding to infectious disease, most of the studies have been shown pyroptosis that plays a vital role in the host defense against pathogen infection. Ferroptosis also shares some similarities with other types of cell death, the crosstalk between them needed fully explored. The function of ferroptosis within immune systems is still not clear. Undoubtedly, there are several questions to be solved in the further studies. We believe that with the increasing understanding of the mechanisms between ferroptosis and infectious diseases, the regulation of ferroptosis would serve as potential therapeutic targets in balancing between host defense and inflammation during pathogen infections, which would further benefit the control of the threatening infectious diseases.

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Selenium Metabolic Pathway in Ferroptotic Cell Death

17

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Abstract

Selenoproteins play a crucial role in various complex biological processes, including cell growth, differentiation, apoptosis, and ferroptosis. In response to increased oxidative damage and altered nutrient metabolism known as the Warburg switch, cancer cells adapt via selenoprotein synthesis that are essential for their survival. Especially, selenoprotein thioredoxin reductase 1 (TrxR1) and glutathione peroxidase-4 (GPX4) have significant roles in controlling hydroperoxides and resisting ferroptosis. These selenoproteins offer protection to cancer cells from ferroptosis and oxidative damage by utilizing selenium-related mechanisms to counteract the toxic effects of chemotherapy and radiotherapy. The bioavailability of selenium is often overlooked as a determinant of oxidative defense status and has emerged as a critical factor in cancer cells. Hence, the impact of dietary selenium depletion in the context of anticancer treatment highlighted the need for further investigation into the potential therapeutic applications of selenium or selenoproteins in cancer treatment. This

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chapter explores the potential application of selenoproteins as therapeutic targets in cancer treatment, with a specific focus on ferroptosis.

17.1 Introduction

Ferroptosis, a form of regulated cell death, is controlled by a selenium-dependent antioxidant glutathione peroxidase 4 (GPX4) in the presence of iron and selenium (Seibt et al. 2019). A reduction in GPX4 activity due to decreased selenium levels can lead to oxidative stress and ferroptosis in cancer (Ingold et al. 2018). GPX4 and other 25 selenoproteins in humans are notably ferroptosis sensitive. Despite their potential role in preventing the progression of cancer, the precise role of other selenoproteins and the thioredoxin system in the ferroptosis pathway is poorly understood (Cai et al. 2020; Kalimuthu et al. 2022). However, due to the antioxidant properties of selenoproteins, they may play a significant role in the regulation of ferroptosis. A pan-cancer study on selenoprotein expression found that the expression of genes belonging to the GPX and TrxR families can vary between different cancer types and even within individual cancer cases (Wu et al. 2021). Few studies in mice models have shown that administering selenomethionine (SeMet) before irradiation can delay tumor formation. On the other hand, continued use of SeMet can lead to uncontrolled growth of tumors (Jeter et al. 2019). The complexity of selenoproteins classified is based on the type and stage of cancer. Hence, further investigation is needed to fully understand their role in carcinogenesis. In addition, selenoproteins play a role in various molecular signaling pathways and can influence the expression of transcription factors (Ghelichkhani et al. 2022).

Ferroptosis may serve as a therapeutic target cancer cells with enhanced sensitivity, as evidenced by the altered regulation of selenoproteins in the cancer cells. Apolipoprotein E receptor 2 LRP8 (also known as ApoER2) can promote resistance to ferroptosis in cancer cells by regulating selenium and selenoprotein levels. In response to limited selenium availability, the levels of GPX4 rapidly decrease in cancer cells leading to ferroptosis (Li et al. 2022). Eagle and group highlight the potential of targeting selenophosphate synthetase 2 (SEPHS2) and selenium detoxification as a therapeutic strategy for acute myeloid leukemia (AML). The authors also observed other selenoproteins that help to suppress programmed cell death caused by iron-dependent lipid peroxidation, i.e., ferroptosis. Few other studies also suggested that selenium depletion may be used as a part of anticancer therapies (Eagle et al. 2022; Jankowski and Rabinowitz 2022). This book chapter points out the possibility to use selenium or selenoprotein levels as a therapeutic strategy against cancer and for regulating the ferroptosis pathway.

17.2 Ferroptosis

Before the term "ferroptosis" was coined, Dolma and group discovered inducers of this form of cell death through a high-throughput screening method in which they evaluated 23,550 compounds for their cell-killing ability on tumorigenic cells induced by multiple oncogenes, including RAS (which is mutated in approximately 25% of cancers, as described by Dixon et al (Dixon et al. 2012)). Human fibroblast cells were transformed into tumor cells through the introduction of human telomerase reverse transcriptase (hTERT), the catalytic component of telomerase, and the large T (LT) and small T (ST) oncoproteins of SV40 and onco-RAS protein. The researchers found nine compounds that were four times more effective on these tumorigenic cells, with erastin being effective among the identified compounds and exhibiting specificity toward these cells (as an eradicator of RAS and ST cells). The morphological studies suggested that erastin did not result in any changes in nuclear morphology and neither caused DNA fragmentation nor activated caspases, indicating that the compound induces a type of non-apoptotic cell death (Dolma et al. 2003). In 2007, Yagoda and coworkers reported that this form of cell death is reliant on the RAS-RAF-MEK signaling pathway and can be suppressed by antioxidants, suggesting that reactive oxygen species (ROS), particularly lipids, might play a role in inducing cell death (Yagoda et al. 2007).

The researchers discovered that erastin binds to voltage-dependent anion channels (VDACs) and changes the permeability of the outer membrane of the mitochondria. Out of 47,725 compounds tested, two compounds, RSL3 and RSL5, were found to exhibit anticancer activity through a mode of action like erastin, which involves the activation of the RAS-RAF-MEK pathway, binding to VDACs, and the involvement of lipid-derived ROS (Yang and Stockwell 2008). The authors also observed that two iron chelators, including deferoxamine (DFX), can block the effects of RSLs and erastin, indicating a role for intracellular iron in this new form of cell death (Yang and Stockwell 2008). They characterized ferroptosis as being distinct from apoptosis, necrosis, and autophagy in terms of biochemistry, morphology, and genetics (Dixon et al. 2012).

17.3 Characteristics of Ferroptosis

Ferroptosis can be distinguished from other forms of cell death as it does not result in chromatin condensation as seen in apoptosis or cytoplasmic swelling and plasma membrane rupture as seen in necrosis. Additionally, ferroptosis does not involve the formation of double-membrane vacuoles, which is a hallmark of autophagy (Dixon et al. 2012). Biochemically, ferroptosis can be differentiated from apoptosis as it does not involve the activation of caspases, cytochrome c release, or cleavage of PARP-1 (Yagoda et al. 2007). Furthermore, ferroptosis does not result in ATP depletion, a key feature of necrosis (Dixon et al. 2012). To identify the unique genetic network underlying ferroptosis, Dixon and researchers used an shRNA library targeting 1087 genes involved in mitochondrial functions. The results of

this study showed no correlation between shRNAs that protected cells from erastin-induced ferroptosis and staurosporine-induced apoptosis, indicating that the genetic networks involved in these two forms of cell death are distinct (Dixon et al. 2012). They also reported six genes that are essential for ferroptosis: RPL8 (ribosomal protein L8), IREB2 (iron response element-binding protein 2), ATP5G3 (ATP synthase F0 complex subunit C3), CS (citrate synthase), TTC35 (tetratricopeptide repeat domain 35), and ACSF2 (acyl-CoA synthetase family member 2). The knockout (KO) of these genes was found to protect cells against ferroptosis, but not against cell death induced by other agents such as staurosporine, rotenone, or rapamycin (Dixon et al. 2012). These findings support the notion that ferroptosis is regulated by a specific genetic network.

17.4 Role of Selenium in Ferroptosis Pathway

The critical role of selenium in the ferroptosis pathway can be understood from the results of various biochemical assays such as western blot and promoter assay. However, the previous studies on ferroptosis suggest that these assays were often not reproducible even when the same cell lines were used in different laboratories. Why do the results differ? The findings of Karlenius and coworkers from gene promoter reporter assay indicate that the results were altered when the laboratory switched to a different vendor of FBS supplier. To investigate this further, they determined the serum selenium concentrations of FBS from various country vendors and their corresponding activity for gene promoter, cell proliferation, and antioxidant enzyme assays. The chemical composition of different batches of sera used for cell culture may vary including selenium concentration, which is not routinely tested before shipping. The significance of redox control systems in regulating a range of biological processes such as gene expression and cell signaling is now widely accepted. Different growth mediums may lead to the activation of different key redox systems in the cells grown which may have various downstream effects. For example, many transcription factors are redox-regulated which in turn regulates the activity of other gene promoters (Shahdadfar et al. 2005; Turanov et al. 2010). The variation in the serum-containing cell culture medium is a key factor in the output of conflicting results for many tests such as gene reporter assays, mRNA quantification, and western blot assay. A recent report suggests that the metabolic environment of tumors in vivo cannot be recreated using currently available cell culture media (Karlenius 2011). To test this, the effect of a new physiological medium called Plasmax was compared with commercial media for animal cell culture. It was found that cancer cells undergo metabolic disturbances because of the unbalanced nutrient composition of commercial media (Gardner et al. 2022; Kalimuthu et al. 2022). The high levels of pyruvate and arginine in this context have specific effects on the regulation of gene expression and the urea cycle. Pyruvate stabilizes HIF-1α in normal oxygen conditions, leading to a false hypoxia response. Arginine interferes with the normal functioning of the urea cycle enzyme argininosuccinate lyase; however, this effect can be prevented in vitro by Plasmax. Ferroptosis influenced

by the varying levels of selenium in serum inhibited the ability of cancer cells to form colonies on commercial media, whereas predetermined selenium in Plasmax was able to restore this ability (Voorde Vande et al. 2019). Recent study on the effects of selenium on cancer stem cells is consistent with the previous reports which indicate that selenium offer resistance to cancer drug (Eagle et al. 2022). Hence, we propose that optimal levels of selenium in the medium could serve as a supplement and can be employed in drug discovery experiments for drug-resistant cancer.

17.5 Ferroptosis-Induced ER Stress

Endoplasmic reticulum (ER) stress is one of the attractive biological phenomena that ensue as part of ferroptosis (Lee et al. 2020). The unfolded protein response (UPR) pathway is triggered by three transmembrane proteins, IRE1 (inositol-requiring enzyme 1), ATF6 (activated transcription factor 6), and PERK (protein kinase RNA (PKR)-like endoplasmic reticulum kinase) (Moncan et al. 2021). In addition, the presence of seven selenoproteins in the endoplasmic reticulum membrane demonstrates the diversity within this subgroup and their impact on cancer processes such as proliferation, survival, and apoptosis. Recent studies have highlighted the role of selenoproteins in endoplasmic reticulum stress in promoting tumor growth and metastasis in various solid tumors. Although most selenoproteins, including SELENO F, SELENO K, SELENO M, SELENO N, SELENO S, and SELENO T are involved in protein degradation and calcium homeostasis (Fig. 17.1), the exact mechanism through which they protect cancer cells from apoptosis and ferroptosis cell death is still unclear (Varlamova et al. 2022). There is mounting evidence suggesting that the endoplasmic reticulum membrane represents a crucial location where lipid peroxidation occurs, leading to various cellular dysfunctions and pathologies (Varlamova et al. 2022; von Krusenstiern et al. 2023). In fact, GPX4 act as a protector against lipid peroxidation in ER and other subcellular membranes.

The AAA+ ATPase p97 (also known as VCP in metazoans and Cdc48 in lower eukaryotes) regulates the entire autophagy process, from initiation to maturation and autophagy-mediated degradation. This highly abundant protein constitutes about 1% of total cellular protein and is essential for endoplasmic reticulum-associated protein degradation (ERAD). During the ERAD process, the recruitment of VCP/p97 to the ER membrane is necessary for substrate degradation and is facilitated by the selenoproteins SELENO S and SELENO K. The binding of SELENO K to p97 is essential for the recruitment of SELENO S. The binding between SELENO K and SELENO S is not influenced by the redox state of SELENO S and does not involve the Cys173 and Sec188 residues of SELENO S. (Lee et al. 2015). Notably, both SELENO S and SELENO K bind to derlins that are involved in the ERAD complex with an association of p97 (VCP). It is well known that selenoprotein S functions in cell survival by adaptive regulation of ER stress. Selenoprotein K is also one of the ER stress-regulated proteins and plays an important role in resistance against ER stress-mediated apoptosis. This extensive list corresponds with the reports that at

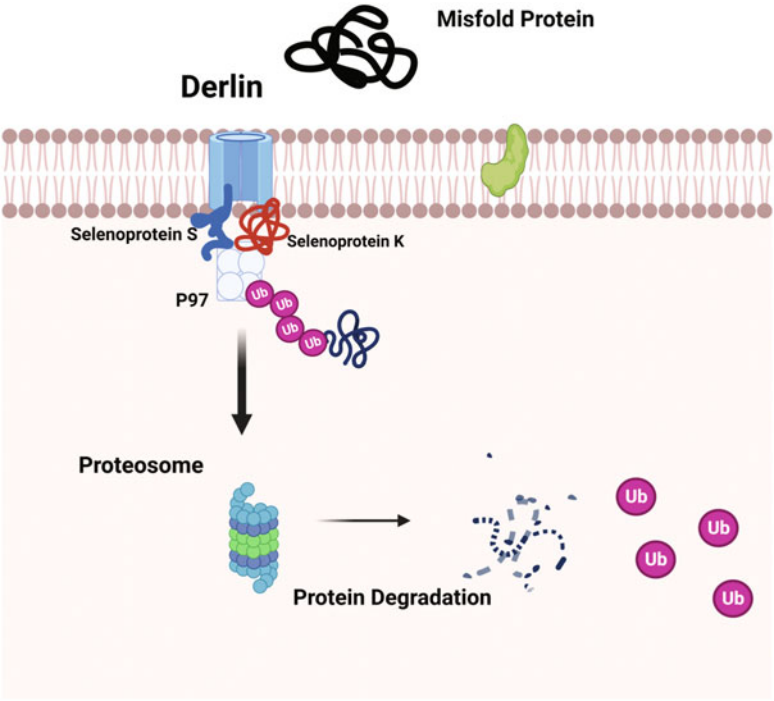

Fig. 17.1 Role of selenoprotein in the proteasomal degradation of misfolded proteins

least some of SELENO S and SELENO K cellular roles are tied to the protein degradation process and lipid storage (Shchedrina et al. 2011).

According to some reports, SELENO K promotes melanoma tumor progression and metastasis. It binds to the DHHC6 (each letter represents amino acids Asp-His-His-Cys) acyltransferase enzyme to catalyze the palmitoylation of the IP3R which is required for ER membrane stability. Although, it has been seen that SELENO K disturbs the IP3R palmitoylation and Ca^{2+} in the ER membrane, however, increasing SELENO K expression may not be in proportionality for IP3R palmitoylation as well as the Ca^{2+} in the ER membrane (Marciel and Hoffmann 2019). Genetic manipulation of the SELENO K gene results in significantly lower levels of store-operated calcium (Ca^{2+}) entry protein in melanoma cancer cells in vitro and in vivo. According to recent studies, selenium deficiency may be used in cancer treatment (Eagle et al. 2022; Jankowski and Rabinowitz 2022). Recently, few studies have revealed that targeting SELENO K can effectively reduce tumor size by modulating ferroptosis, thereby contributing a favorable establishment for the development of potential therapies for cervical cancer (Abdurahman et al. 2023). Previously, it was believed that proteins that trigger ferroptosis such as RSL3 and ML162 were inhibited by GPX4, TNXRD1 and SELENO K. Recent discovery suggests that GPX4 does not directly inhibit RSL3 and ML162 (Cheff et al. 2023). However, SELENO K and TNXRD1 may directly inhibit those ferroptosis triggerers.

17.6 p62/Keap1/NRF2 Axis Protects against Ferroptosis

Brigelius-Flohe proposed that selenium metabolites like selenols and selenenic acids can modify protein thiols to form selenylsulfides (Se-S), selenotrisulfides (S-Se-S), selenocysteine (SeSec), and selenomethionine (SeMet) (Brigelius-Flohé 2008). Recently, some reports revealed that selenium-containing natural compounds have opened new avenues for selenometabolomics in eukaryotic cells (Hou and Xu 2022; Kayrouz et al. 2022; Abdalla and Mühling 2023). The changes in the levels of these selenometabolites may play an important role in the survival of cancer cells. Selenium metabolites mainly regulate the nuclear factor erythroid 2-related factor 2 (NRF2) pathway (Fig. 17.2). First, NRF2 protects cells against oncogenic attacks such as ROS and electrophilic carcinogenic species. Conversely, once malignant transformation occurs within a cell, the NRF2-KEAP1 signaling pathway can protect the tumor from oxidative stress and chemo- or radiotherapy-induced cytotoxicity through the cooperation of various cells populations and functions.

The p62-KEAP1-NRF2 pathway is involved in the protection of cancer cells against ferroptosis through the up-regulation of several genes such as quinone oxidoreductase 1 [NQO1], heme oxygenase-1 [HO1], glutathione peroxidase (GPx), thioredoxin reductase (Trx), and ferritin heavy chain 1 [FTH1] that are intricately involved in iron metabolism (Sun et al. 2016). Upon treatment with an electrophilic precursor of methylselenol, methylseleninic acid first activates the

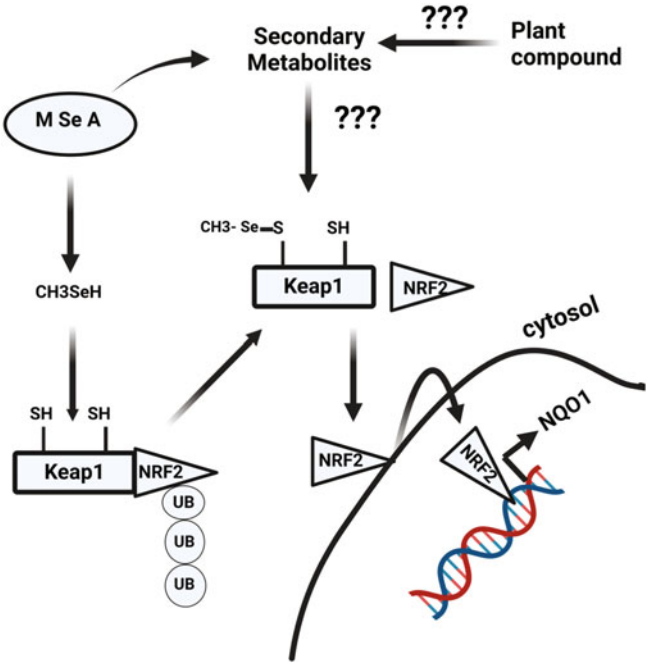

Fig. 17.2 Regulation of the NRF2/Keap1 axis by the action of phytochemicals

transcription factor NRF2, leading to the induction of the gene encoding NQO-1 through direct modification of selenium-KEAP1 (Park et al. 2018). These findings provide a novel mechanism by which methylseleninic acid exhibits a significant ability to induce oxidative/stress-responsive gene expression associated with selenium metabolites. There is documented evidence that inhibition of NRF2 significantly enhances the anticancer effects of ferroptosis inducers in several cancers (Lei et al. 2019). Selenium is an essential micronutrient that is required for the proper functioning of various cellular pathways, including the antioxidant defense system. Recently, the researcher has discovered an unexpected outcome associated with the plant-derived compound known as artesunate. While the mechanism of how artesunate alters KEAP1 protein expression is not yet fully understood, it has been suggested that it may be related to changes in selenium metabolism. However, further research is needed to fully elucidate the relationship between artesunate, KEAP1, and selenium metabolism (Hill et al. 2021). Lee et al. 2003 demonstrated that selenium can maintain the growth of specific human hepatocellular carcinoma cell lines in the serum-free medium containing 0.1% of serum (Lee et al. 2003). We propose for the first time that selenium-dependent cell growth and proliferation can be defined as selenium-dependent selenoplasia which is one of several types of metalloplasia. It is in line with other metalloplasia such as cuproplasia which is copper-dependent cell proliferation resulting from the modulation of signaling pathways (Ge et al. 2022). However, the precise signaling mechanism of selenoplasia that underlies cancer formation has not yet been identified and it is the survival, particularly via acquiring resistance to ferroptosis.

17.7 BRF2 Selenoprotein: Cancer Cell

The UGA stop codon is responsible for encoding selenocysteines in the protein molecule. During translation, cellular processes depend on specific machinery to ensure the correct insertion of specific residues. Selenocysteine insertion sequence-binding protein 2 (SBP2) can recognize a specific stem-loop in the mRNA known as the selenocysteine insertion sequence (SECIS) element which allows the recruitment of eEFsec (selenocysteine-specific elongation factor. In addition, eEFsec is responsible for binding SeCystRNA which is an essential step in the process of ensuring that selenoproteins contain SeCys (Howard et al. 2007). It is important to note that the reduced level of GPx4 is the result of the depletion of SBP2 or eEFSec. Some reports demonstrate that SBP2 knockout increases ROS production and leads to ferroptosis and apoptosis (Arimbasseri and Maraia 2016; Jehan et al. 2022). Ultimately, the researcher suggests that the SeCystRNA transcription is a response to control redox reaction. More specifically, RNA polymerase III is a core transcription factor composed of three subunits (TATA-binding protein) TBP, Bdp1, and Brf2). Researchers highlighted a direct link between Brf2 and the overexpression of many cancers including breast and lung cancer. SeCystRNA contributes to the regulation of the selenoprotein level which is the main transcription factor of Brf2 (Jenkins and Gouge 2021). The researchers believe that Brf2 plays the role of a master regulator in

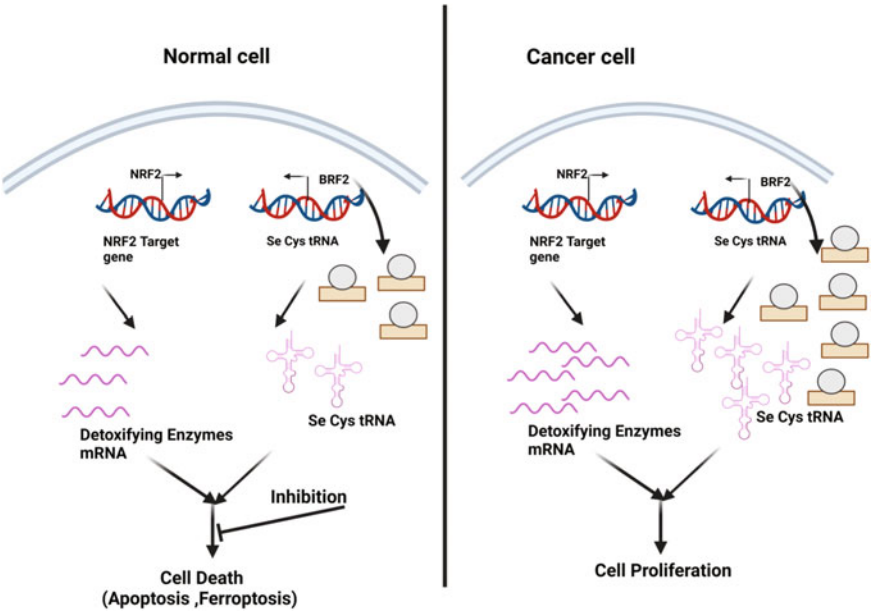

Fig. 17.3 Differential role of selenoproteins in normal cells and cancerous cells and its implication in cell proliferation

the oxidative stress response which is accomplished by regulating SeCystRNA levels in a redox-dependent manner (Fig. 17.3). Cancer cells suppress this regulation and prevent apoptosis by overexpressing Brf2. Notably, this phenomenon occurs even in the presence of activated Nrf2 (Gouge and Vannini 2018; Rashidieh et al. 2021).

17.8 Protein Synthesis

Ferroptosis is related to deregulated redox metabolism. Lee and coworkers were initially interested in examining the potential role of glucose starvation in promoting ferroptosis since it reduces ATP generation and increases the production of reactive oxygen species (ROS). However, they made an unexpected discovery that glucose starvation along with other energy stress-inducing conditions prevents cells from ferroptosis (Lee et al. 2020). AMP-activated protein kinase (AMPK) response to energy stress depletes ATP and temporarily slows protein synthesis. Also, AMPK along with one of its major downstream targets, rapamycin (mTOR) regulates the rate of cell proliferation. mTOR is a serine/threonine protein kinase that regulates two distinct protein complexes called mTOR complex 1 (mTORC1) and 2 (mTORC2) by acting as a catalytic component. mTORC1 plays an important role in cancer cell growth and cell division. In addition, activation of p70S6 kinase (p70S6K) and 4E-binding protein (4E-BP) increases protein synthesis and

effectively involves de novo lipid synthesis. It is well known that human eukaryotic initiation factor 4G (eIF4G) is a binding site on eIF4E which is essential for translation initiation (Rehman et al. 2014). However, when 4E-BP is phosphorylated by mTOR, it can no longer bind with eIF4E. Multiple types of stress inhibit mTORC1 activity resulting in phosphorylated 4E-BPs that block the translation initiation factor eIF4E. In contrast, mTORC1 inhibition results in only a 60% decrease in the total translation of proteins. The remaining 40% of protein translation may not necessarily be occurring via the cap-independent mechanism. Over the past decade, researchers have believed that cap-dependent translation is normally mediated by the protein eIF4F. In recent years, researchers have postulated that cap-dependent translation is typically mediated by the eIF4F protein. Surprisingly, the discovery of eIF3d as a new player in cap-dependent mRNA translation has expanded our understanding of the regulation of protein synthesis. The requirement of conserved stem-loop structures in the 5' UTR of mRNAs for eIF3d-mediated translation raises questions about the mechanism of recognition and how these structures block canonical eIF4F binding. The specific role of eIF3d in glucose deprivation-mediated translation raises questions about the presence of other types of switches for stimulus-specific translation reprogramming (Lamper et al. 2020; Jia and Qian 2021). These questions highlight the need for further research to fully understand the complex regulation of protein synthesis and the role of eIF3d in this process. Similarly, the interactions of eIF3 with selenoprotein mRNAs may provide a means to steer mRNAs into specific translation pathways. Further studies will be necessary to determine if eIF3 regulates the translation of selenoprotein mRNAs and how it contributes to the establishment of the selenoprotein expression hierarchy in stressful conditions (Hayek et al. 2022). If potent and selective AMPK inhibitors are developed and used in clinical settings, combining them with other specific inhibitory eIF3d chemotherapy agents may help to kill cancer through the ferroptosis pathway.

The production of ribosomes is a complex process that involves the assembly of ribosomal subunits, the transcription of ribosomal RNA, and the folding and modification of ribosomal proteins. These events are tightly regulated to ensure proper ribosome biogenesis and optimal protein synthesis, making ribosomes a critical component of cell biology (Harish and Caetano-Anollés 2012). Malinouski et al. suggest that deficiency in levels of certain ribosomal proteins may affect the ribosome structure and result in an increased insertion of Selenocysteine(SeSec). This could also happen if the knockdown of these proteins decreases the rate of protein synthesis, which supports the slow SeSec insertion process. The control of selenoprotein expression appears to be regulated globally through ribosome structure and function, but more research is needed to understand the specific mechanism. The same group suggests that the knockdown of ribosomal structural protein ribosomal protein L14 (RPL14) has been shown to increase selenium levels and increase the expression of the genes TrxR1 and selenoprotein S (Seleno S). In addition to that some other proteins were deficient in the expression of a potassium voltage-gated channel subfamily A member 1 (KCNA1). KCNA1 deficiency has been found to also decrease the expression of several selenoproteins, with molecular weights under

25 kilodaltons (kDa). This study found that several genes have an impact on selenium levels in HeLa cells by altering selenoprotein expression. These findings suggest that RPL14 may play a role in regulating selenium metabolism and the expression of related genes (Malinouski et al. 2014).

The recent study suggests that regulating the metabolism of selenoaminoacids in pancreatic cancer cell lines can be achieved through the interaction of diaphanous-related formin 3(DIAPH3) with key proteins involved in selenoaminoacid metabolism. It has been previously reported that ribosomal proteins, including RPL6, play a crucial role in maintaining selenium and selenoprotein levels, and a deficiency in these proteins can affect the ribosome structure, leading to increased Sec insertion and selenoprotein expression. The interaction between DIAPH3 and RPL6 may alter the ribosome structure, leading to increased expression of the selenoprotein TrxR1. It is also possible that this interaction decreases the rate of protein synthesis, indirectly supporting slow Sec insertion and increased TrxR1 expression. TrxR1 may be a potential target for inhibiting the progression of pancreatic cancer (Rong et al. 2021).

17.9 Selenoprotein's Regulation of Multiple Transcription Factors

Selenoproteins are involved in a variety of molecular signaling pathways and may be closely linked to several complex cellular network signaling pathways. The cellular role of selenoproteins in the regulation of many networks at the transcriptional level was to protect antioxidant pathways. Many transcription factors play a significant role in regulating selenoprotein mRNA transcription. According to the report by Stoytcheva and Berry, the common potential promoter of the selenoprotein family is 21 transcription factors (Stoytcheva and Berry 2009). Other transcription factor binding sites for selenoprotein gene subsets were discovered. Experimentally, most of the transcriptional factors are responsible for the different selenoprotein gene expressions. MITF is one of the most important transcriptional regulators of melanocytes and melanoma cancer. Recently, another group published research related to TrxR1 (selenoprotein) and its crucial regulatory role in the redox control and stability of the transcriptional factor MITF (Kline et al. 2022). Some recent studies suggest that the metabolism of seleno-aminoacids is through the interaction of ferroptosis. Indisputably, in the future, studies will reveal the important role of selenoprotein in the ferroptosis pathway as well as in drug-resistant cancer cells to adapt to oxidative stress conditions at the transcription level.

17.10 Conclusion

The incorporation of selenium into the body occurs through the selenocysteine (SeSec) biosynthesis pathway, which is crucial for the production of several selenoproteins, including glutathione peroxidases and thioredoxin reductases.

Selenoproteins regulate several intracellular biological processes including ferroptosis, drug resistance, protein quality control, and lipid production. In addition, selenium uptake during protein synthesis also guides many epigenetic processes. Selenoproteins act as a strategic moderator of cancer redox homeostasis in ferroptosis. Cancer cells are highly susceptible to chemodrugs and another therapeutic regimen which induce the production of free radicals. Interestingly, this susceptibility of cancer cells to free radicals is associated with selenoproteins. This suggests that deficiency of selenium through diet would aid in improving the efficacy of various anticancer therapies.

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Epigenetic and Posttranslational Regulation of Ferroptosis

18

Yilei Zhang, Jingwen Xiong, and Minglin Wang

Abstract

Ferroptosis is tightly controlled by pro-ferroptotic pathways, which involve iron and lipid metabolism, as well as anti-ferroptotic pathways, such as SLC7A11/GSH/GPX4 and FSP1/CoQ, as mentioned in the relevant chapters. The key molecules involved in these regulatory circuits determine the extent to which ferroptotic cell death ultimately occurs. Therefore, there has been significant attention focused on the detailed regulation of these ferroptosis-related genes at the transcriptional, posttranscriptional, and posttranslational levels in recent years. In this chapter, we will discuss and summarize the epigenetic and post-translational regulation of genes and proteins involved in ferroptosis. These mechanisms represent major complementary mechanisms in the regulation of ferroptosis and response to therapy. The chapter will cover histone modifications, DNA modifications, and mRNA modifications, which control gene expression levels at the transcriptional and posttranscriptional levels, as part of the epigenetic regulation of ferroptosis. Additionally, extensive review will be provided on posttranslational modifications, which affect the activity, stability, and localization of ferroptosis-modulating proteins. The rewiring of the epigenome or PTMs through pharmacological interventions can offer therapeutic advantages for various diseases involving ferroptotic cell death.

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18.1 Introduction

Along with the emerging modulators that have been identified to control ferroptosis, increasing studies focus on the upstream regulation of these key factors, including histone modifications, DNA or mRNA modifications, and posttranslational modifications, which indirectly determine the expression level of molecules contributing to ferroptosis regulation. As such, knowing the epigenetic regulation and posttranslational modification of ferroptosis-related modulators is important for finding new drug targets and exploring potential therapeutic strategies.

The concept of "epigenetics" was firstly proposed by Conrad Waddington to describe heritable changes in gene expression profiles in cells that are not related to changes in DNA sequence (Gonzalez-Recio et al. 2015). Epigenetic regulation includes chromatin remodeling, dynamic changes in DNA modification and histone modifications, as well as noncoding RNA-mediated regulation (Lan et al. 2023). Epigenetic regulation is an essential regulatory mechanism in embryonic development, cell differentiation, and cell characterization maintenance. Epigenetic regulation enriches the manners of regulating gene expression and enhances the ability of the body to adapt to the environment. It has been demonstrated that the reprogramming of gene expression induced by epigenetic regulation contributes to tumorigenesis and cancer progression. Accumulating evidence suggests that epigenetics regulates cell sensitivity to ferroptosis through modulating the expression of ferroptosis-associated genes, including histone modifications like ubiquitination and methylation, DNA methylation, and m6A modifications of RNA (Tang et al. 2021) (Table 18.1 and Fig. 18.1).

Posttranslational modifications (PTMs) refer to the modification of protein after translation. It can change the structure, function, stability, localization, and interaction of proteins, so as to affect intracellular signal transduction, metabolism, immunity, and other life activities. The PTMs of key proteins involved in ferroptosis regulation like phosphorylation and ubiquitination could affect their expression or activity, eventually leading to the changes in ferroptosis sensitivity.

18.2 Epigenetic Regulation of Ferroptosis Modulators

18.2.1 Chromatin Remodeling

Chromatin, the carrier of genetic information in eukaryotes, undergoes structural changes that regulate gene expression. Chromatin remodeling encompasses a variety of processes that alter chromatin from a condensed state to a transcriptionally accessible state. These remodeling outcomes include nucleosome disassembly, nucleosome assembly, histone exchange, and nucleosome sliding. The maintenance of chromatin homeostasis relies on the activity of chromatin-remodeling factors, such as the switching defective/sucrose nonfermenting (SWI/SNF) family remodeling factors, the imitation switch (ISWI) family remodeling factors, the chromodomain, helicase, DNA-binding (CDH) family remodeling factors, and the

Table 18.1 Epigenetic and posttranslational regulation of ferroptosis

Modification regulation			Ferroptosis regulation		
Group	Types	Regulators	Targets	Effects	Functional impacts
Epigenetic regulation and modification	Chromatin remodeling	ARID1A	SLC7A11	Directly promote the recruitment of SWI/SNF to SLC7A11 transcriptional start site and lead to chromatin remodeling.	Activate SLC7A11 transcription induced by NRF2 and RNAPII, enhance cystine uptake and GSH synthesis.
	H2A monoubiquitination	BAP1	SLC7A11	Directly inhibit the histone H2AK119ub on the SLC7A11 promoter.	Downregulate SLC7A11 mRNA level, promote ferroptosis.
		PRC1	SLC7A11	Directly promote the histone H2AK119ub on the SLC7A11 promoter.	Downregulate SLC7A11 mRNA level, promote ferroptosis.
	H2B monoubiquitination	USP7	SLC7A11	Directly inhibit the H2BK120ub on the SLC7A11 promoter.	Downregulate SLC7A11 mRNA level, promote ferroptosis.
	Histone methylation	KDM3B	SLC7A11	Promote the elevation of SLC7A11 promoter activities activated by ATF4.	Upregulate SLC7A11 mRNA level, inhibit ferroptosis.
		KDM4A	SLC7A11	Directly decrease the H3K9me3 levels on the promoter region of SLC7A11.	Upregulate SLC7A11 mRNA level, inhibit osteosarcoma cells ferroptosis
		SUV39H1	DPP4	SUV39H1 deficiency directly decrease the H3K9me3 levels at the DPP4 promoter.	SUV39H1 deficiency upregulates DDP4 mRNA level, induces ferroptosis during ccRCC cell growth.
		LSH	SCD1, FASD2	Regulate Histone methylation levels.	Upregulate SCD1 and FASD2 mRNA level, inhibit ferroptosis.
	Histone hydroxybutyrylation	NA	GPX4	β-hydroxybutyrate indirectly promotes H3K9bhb enrichment in promotor regions of GPX4.	Upregulate GPX4 mRNA level, inhibit ferroptosis.
	DNA methylation	DNMT3a	GPX4	Promote GPX4 promoter methylation	Downregulate GPX4 mRNA level, induce ferroptosis.

(continued)

Table 18.1 (continued)

Modification regulation			Ferroptosis regulation		
Group	Types	Regulators	Targets	Effects	Functional impacts
	m6A modification	IGF2BP1	SLC7A11	Suppress SLC7A11 mRNA deadenylation in an m6A dependent manner	Upregulate SLC7A11 expression level and promote ferroptosis.
		YTHDF2	SLC7A11	Promote SLC7A11 mRNA degradation in an m6A dependent manner	Downregulate SLC7A11 expression level and promote ferroptosis.
		YTHDC2	SLC7A11	Promote SLC7A11 decay in an m6A dependent manner	Downregulate SLC7A11 expression level and promote ferroptosis.
		FTO	SLC7A11	Remove m6A modification	Downregulate SLC7A11 expression level and promote ferroptosis.
		ALKBH5	SLC7A11	Remove m6A modification	Downregulate SLC7A11 expression level and promote ferroptosis.
		NKAP	SLC7A11	Promote SLC7A11 mRNA splicing and maturation in an m6A dependent manner	Upregulate SLC7A11 expression level and promote ferroptosis.
		YTHDF1	SLC7A11	Destabilized SLC7A11 mRNA in an m6A-dependent manner	Upregulate free iron levels, induce ferroptosis
		MIR-4443	FSP1	Decrease the m6A enrichment of FSP1 and elevate the FSP1 mRNA levels	Upregulate FSP1 levels, inhibit ferroptosis
	N4-acetylcytidine modification	NAT10	FSP1	Improve the stability of FSP1 mRNA through N4- acetylation of the FSP1 mRNA and enhance the FSP1 expression	Upregulate FSP1 protein levels, inhibit ferroptosis
Post-translational modification	Phosphorylation	mTORC2	SLC7A11	Phosphorylate SLC7A11 at serine 26	Inhibit the transporter activity induce ferroptosis
		AKT			
		PKCIIβ	ACSL4	Phosphorylate ACSL4 at Thr-328	Increase ACSL4 activity and enhanced incorporation of PUFA into membrane phospholipids then promotes ferroptosis

	UFMylation	NA	SLC7A11	Covalent attachment UFM1 to SLC7A11	Stabilize the protein level, inhibit ferroptosis
	Ubiquitination	TRIM26		Ubiquitinate SLC7A11	Promote SLC7A11 protein degradation, induce ferroptosis
		SOCS2		Transfer the attached ubiquitin to SLC7A11 and promoted K48-linked polyubiquitination	
		OTUB1		Deubiquitinate SLC7A11	Stabilize the protein level and increase the SLC7A11 protein half-life, inhibit ferroptosis
		NA	GPX4	Ubiquitinate GPX4	Promote the GPX4 degradation, induce ferroptosis
		NA	ACSL4	Phosphorylate ACSL4 at Thr-328	Increase ACSL4 activity and enhanced incorporation of PUFA into membrane phospholipids then promotes ferroptosis
	Alkylation	NA	GPX4	Alkylate the catalytic selenocysteine residue of GPX4	Lead to inhibition of its activity and induction of ferroptosis
	Succination	NA		Catalaze succination of GPX4 at C93	Decrease GPX4 activity and increased susceptibility to ferroptosis
	Myristoylation	NMT	FSP1	Transfer the myristoyl group from myristoyl-CoA to the N-terminal glycine residue of FSP1	Localize to the mitochondria and interacts with CoQ10 to scavenge lipid peroxyl radicals and prevent lipid peroxidation, thereby inhibiting ferroptosis.

Abbreviations: *ARID1A* AT-rich interactive domain-containing protein 1A, *BAP1* BRCA1-associated protein 1, *PRC1* Polycomb repressive complex 1, *USP7* Ubiquitin-specific protease 7, *KDM3B* Lysine-specific demethylase 3B, *KDM4A* Lysine-specific demethylase 4A, *SUV39H1* Suppressor of variegation 3-9 homolog 1, *LSH* Lymphoid-specific helicase, *DNMT3a* DNA methyltransferase 3A, *IGF2BP1* Insulin-like growth factor 2, mRNA-binding protein 1, *YTHDF2* YTH N6-methyladenosine RNA-binding protein 2, *YTHDC2* YTH domain-containing protein 2, *FTO* Fat mass and obesity-associated protein, *ALKBH5* AlkB homolog 5, *NKAP* NF-κB activating protein, *YTHDF1* YTH N6-methyladenosine RNA-binding protein 1, *NAT10* N-acetyltransferase 10, *mTORC2* Mechanistic target of rapamycin complex 2, *AKT* Protein kinase B, *PKCIIβ* Protein kinase C II beta, *UFM1* Ubiquitin-fold modifier 1, *TRIM26* Tripartite motif-containing protein 26, *SOCS2* Suppressor of cytokine signaling 2, *OTUB1* Ubiquitin thioesterase OTUB1, *NMT* N-myristoyltransferase, *SLC7A11* Solute carrier family 7 member 11, *DPP4* Dipeptidyl peptidase 4, *SCD1* Stearoyl-CoA desaturase-1, *FASD2* Fatty acid synthase dehydratase 2, *GPX4* Glutathione peroxidase 4, *FSP1* Ferroptosis suppressor protein 1, *ACSL4* Long-chain-fatty-acid-CoA ligase 4, *NA* not available

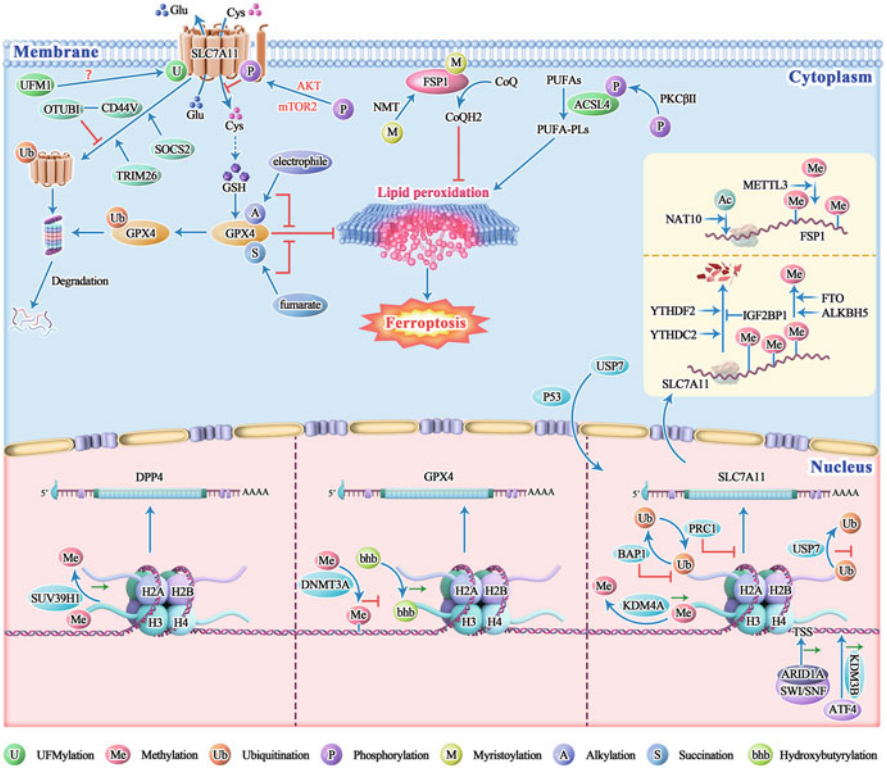

Fig. 18.1 Mechanisms and regulations of ferroptosis

inositol requiring 80 (INO80) family remodeling factors (Clapier and Cairns 2009). Chromatin remodeling not only governs gene expression but also plays a critical role in DNA replication, repair, and other cellular processes.

AT-rich interaction domain 1A (ARID1A), a frequently mutated gene in various cancer types, encodes a subunit of the SWI/SNF chromatin-remodeling complex (Wu et al. 2009). Ogiwara et al. reported that ARID1A-deficient cancer cells exhibited sensitivity to glutathione (GSH) inhibition compared to ARID1A-proficient cells. They found that the mRNA expression of SLC7A11, a key component of the cystine/glutamate antiporter system xc-, was downregulated in ARID1A-deficient cells. Further investigation revealed that ARID1A localized to the transcription start site (TSS) of SLC7A11, and the catalytic subunit of the SWI/SNF complex, BRG1, also occupied the SLC7A11 TSS in an ARID1A-dependent manner. Moreover, the recruitment of RNA polymerase II (RNAPII) and the transcription factor NRF2 to the SLC7A11 TSS were significantly impaired in ARID1A knockout cells. These findings suggest that the recruitment of the SWI/SNF complex to the SLC7A11 TSS leads to chromatin remodeling, facilitating SLC7A11 transcription through RNAPII and NRF2. Given that upregulation of

SLC7A11 enhances cystine uptake, promotes glutathione synthesis, and confers resistance to GSH inhibition in ARID1A wild-type cells, it is tempting to speculate that ARID1A-mediated SLC7A11 expression may contribute to ferroptosis resistance. However, further investigation is warranted to elucidate the precise role of ARID1A-mediated SLC7A11 expression in ferroptosis (Ogiwara et al. 2019).

18.2.2 Histone Modification

The basic unit of chromatin is the nucleosome, which consists of an octamer comprising four core histones (H3, H4, H2A, H2B). This octamer includes an H3–H4 tetramer and two H2A–H2B dimers, surrounded by 147 pairs of DNA base pairs. The core histones form a compact spherical core particle, while their N-terminal tails extend outward from the core. Histone modifications primarily occur on these N-terminal tails. Over 10 different covalent modifications have been identified on specific amino acid residues of the core histones, including methylation, acetylation, ubiquitination, phosphorylation, sumoylation, carbonylation, and glycosylation (Chan and Maze 2020). These histone modifications significantly impact chromatin structure, compaction, and accessibility, ultimately influencing gene transcriptional activity. In the context of ferroptosis, we will delve into the histone modifications that regulate ferroptosis-related genes and explore their impact on the occurrence of ferroptotic cell death.

18.2.2.1 Histone Ubiquitination

Histone ubiquitination plays a crucial role in the regulation of the transcriptional state of the genome. It involves the addition of mono-ubiquitin moieties to histone proteins, particularly H2A and H2B. Notably, ubiquitination can occur at lysine 119 (K119ub) in H2A or lysine 120 (K120ub) in H2B, leading to significant effects on nucleosome dynamics and influencing the activation or repression of gene transcription. Moreover, histone ubiquitination can also modulate gene expression by promoting histone methylation. The process of histone ubiquitination is primarily mediated by ubiquitin-activating enzymes (E1), ubiquitin-conjugating enzymes (E2), and ubiquitin ligases (E3). Conversely, the removal of histone ubiquitination is facilitated by deubiquitinating enzymes, such as the polycomb repressive deubiquitinase (PR-DUB) complexes and ubiquitin-specific peptidases (USPs), which are responsible for erasing mono-ubiquitination marks from histones.

In a study by Zhang et al., it was revealed that BRCA1-associated protein 1 (BAP1), a key deubiquitinase within the polycomb repressive deubiquitinase (PR-DUB) complex, plays a role in epigenetically reducing the level of H2A mono-ubiquitination (H2Aub) on the SLC7A11 gene. This reduction in H2Aub leads to the suppression of SLC7A11 expression, ultimately promoting increased sensitivity to ferroptosis in kidney cancer cells (Zhang et al. 2018). Additionally, the polycomb repressive complex 1 (PRC1) has been identified as the responsible entity for mono-ubiquitinating H2A at Lys-119 on the SLC7A11 promoter. Interestingly, PRC1-mediated H2Aub also contributes to the repression of SLC7A11 expression,

enhancing ferroptosis susceptibility. These findings suggest a potential synergistic repression of SLC7A11 expression by BAP1 and PRC1 through dynamic regulation of H2Aub levels on the SLC7A11 promoter (Zhang et al. 2019). At the genomic level, H2Aub is typically associated with transcriptional repression. However, the exact mechanism by which H2Aub regulates SLC7A11 expression requires further elucidation, including the identification of specific factors involved in H2Aub regulation and SLC7A11 transcription.

On the contrary, mono-ubiquitination of histone H2B on lysine 120 (H2Bub1) is generally associated with transcriptional activation and leads to an open chromatin state that facilitates gene transcription. Wang et al. discovered that the deubiquitinase USP7 plays a crucial role in regulating ferroptosis by mediating the deubiquitination of H2B on the SLC7A11 gene. Mechanistically, p53 promotes the nuclear translocation of USP7, which in turn reduces the levels of H2Bub and suppresses SLC7A11 expression. The study also demonstrated that erastin treatment results in the removal of H2Bub1 and repression of SLC7A11 expression by enhancing the interaction between p53 and USP7, ultimately sensitizing cells to erastin-induced ferroptosis (Wang et al. 2019).

18.2.2.2 Histone Methylation

Histone methylation modifications primarily occur on lysine and arginine residues of histones H3 and H4. These modifications are regulated by methyltransferases and demethylases. Lysine can undergo mono-, di-, and trimethylation, and the effects of lysine methylation vary depending on the specific methylation site, while arginine methylation is generally associated with transcriptional activation. Methylated histones can be recognized by readers of histone methylation, leading to downstream signaling and functional alterations. It has been established that histone methylation plays an epigenetic role in regulating ferroptosis-related genes involved in cystine metabolism and lipid metabolism.

KDM3B belongs to the JMJD family of proteins and contains the JmjC domain. It exhibits specific demethylation activity on monomethyl and dimethyl histone H3 lysine 9 (H3K9) residues and possesses arginine demethylase activity on histone H4 arginine 3 symmetric dimethylarginine (H4R3me2s) residues (Li et al. 2018). Studies have shown that KDM3B can protect cells from erastin-induced ferroptosis. Overexpression of KDM3B in HT-1080 cells leads to upregulation of SLC7A11, while knockdown of KDM3B attenuates the ATF4-induced elevation of SLC7A11, suggesting that KDM3B may function as a coregulator of ATF4 in the regulation of SLC7A11 (Wang et al. 2020). Similarly, KDM4A, another demethylase from the JMJD family, removes di- and trimethyl marks from H3K9 and H3K36 residues and regulates SLC7A11 gene expression by targeting H3K9 demethylation in the promoter region of SLC7A11 (Dixon et al. 2012). Knockdown of KDM4A in osteosarcoma cells leads to downregulation of SLC7A11 expression and reduced glutathione (GSH) levels, resulting in increased sensitivity to ferroptosis (Chen et al. 2021). These findings indicate that histone methylation can suppress SLC7A11 expression and promote ferroptosis, while reducing the levels of methylated histones through demethylases such as KDM3B and KDM4A significantly upregulates SLC7A11 and

confers resistance to ferroptosis. Targeting histone demethylases could be a potential strategy to induce ferroptosis in disease therapy, including anticancer treatments.

Suppressor of variegation 3–9 homolog 1 (SUV39H1) encodes a histone 3 lysine 9 methyltransferase. In clear cell renal cell carcinoma (ccRCC), Wang et al. demonstrated that SUV39H1 deficiency leads to increased levels of intracellular lipid reactive oxygen species (ROS), mitochondrial superoxide, and iron. Further investigation revealed that SUV39H1 deficiency promotes the expression of dipeptidyl-peptidase-4 (DPP4), a serine exopeptidase primarily located at the plasma membrane, which cleaves X-proline dipeptides from the N-terminus of polypeptides. DPP4 forms a complex with NOX1, known as the DPP4-NOX1 complex, which enhances lipid peroxidation and accelerates lipid peroxidation-induced ferroptosis. Consequently, depletion of SUV39H1 upregulates DPP4 expression and promotes ferroptosis by reducing H3K9me3 levels at the DPP4 promoter (Wang et al. 2021).

Lymphoid-specific helicase (LSH), a member of the sucrose nonfermenting 2 (SNF2) family of chromatin-remodeling ATPases, is known for its role in establishing proper DNA methylation levels and patterns. However, according to Jiang et al., LSH only minimally affects the levels of H3K4Me3, H3K27Me3, and H3K9Me3 modifications. Interestingly, in cells overexpressing LSH, these subtle changes in histone modifications lead to the recruitment of WDR76 to the promoters of genes involved in lipid metabolism, such as stearoyl-CoA desaturase 1 (SCD1) and fatty acid desaturase 2 (FADS2). This recruitment results in the upregulation of SCD1 and FADS2 expression. The increased expression of SCD1 and FADS2 leads to a reduction in intracellular lipid reactive oxygen species (ROS) and iron accumulation, ultimately conferring resistance to ferroptosis in LSH-overexpressed cells (Jiang et al. 2017a, b).

18.2.2.3 Histone β-hydroxybutyrylation

Histone β-hydroxybutyrylation (Kbhb) has emerged as a novel epigenetic mark that links metabolism and gene expression in response to stress conditions such as fasting and starvation. β-hydroxybutyrate (β-HB), a ketone body produced by the liver, plays a role as an alternative energy source in extrahepatic tissues and functions as a chromatin regulator through the β-hydroxybutyrylation of lysine residues on histones H3 and H4, thereby modulating gene expression at the transcriptional level (Xie et al. 2016). In the context of ferroptosis, Zheng et al. demonstrated that treatment with β-HB in BxPC-3 cells leads to significant enrichment of H3K9bhb marks in the promoter regions of ferroptosis suppressor genes, such as GPX4, GCLC, ACSL3, and CTH. This enrichment of H3K9bhb marks results in the upregulation of these genes, both in the presence and absence of the ferroptosis inducer homocysteic acid, a glutamate analog. Mechanistically, β-HB maintains H3K9bhb marks within the regulatory regions of ferroptosis suppressor genes, leading to an open chromatin state that promotes gene expression (Zheng et al. 2022b). Consequently, β-HB functions as a ferroptosis inhibitor and suppresses ferroptosis during pancreatic injury following acute liver failure. These findings highlight the significance of β-HB as a signaling metabolite that systemically and

epigenetically promotes the expression of ferroptosis suppressor genes through H3K9bhb-mediated chromatin remodeling. They also suggest that maintaining an appropriate physiological concentration of β-HB is crucial in defending against ferroptosis in vivo.

18.2.3 DNA Methylation

As previously mentioned, the SLC7A11/GPX4 axis is a central pathway in protecting cells from ferroptosis. Recent research has demonstrated the significance of modulating GPX4 promoter methylation in the regulation of ferroptosis in various diseases (Ling et al. 2022; Zhang et al. 2020). DNA methylation, a well-known epigenetic modification, involves the addition of methyl groups to the DNA molecule by DNA methyltransferases (DNMTs), typically resulting in gene repression (Greenberg and Bourc'his 2019). In the context of rheumatoid arthritis, glycine treatment has been shown to decrease GPX4 expression and induce ferroptosis in RA fibroblast-like synoviocytes through S-adenosylmethionine (SAM)-mediated GPX4 promoter methylation (Ling et al. 2022). Similarly, in nucleus pulposus cells, homocysteine-induced oxidative stress and ferroptosis are associated with enhanced GPX4 promoter methylation, which is mediated by increased levels of DNMT3a, a key DNA methyltransferase involved in DNA methylation (Zhang et al. 2020). These findings highlight the critical role of GPX4 promoter methylation as a regulatory mechanism in ferroptosis and provide insights into the development of novel therapeutics for diseases characterized by ferroptosis induction. They emphasize the importance of key methyl donor SAM and methyltransferases such as DNMT3a in the regulation of GPX4 promoter methylation and ferroptosis.

18.3 mRNA Modification

18.3.1 N6-methyladenosine

First identified in the 1970s (Desrosiers et al. 1974), N6-methyladenosine (m6A) is the most abundant epitranscriptomic modification among the more than 100 identified in eukaryotic RNAs (Shi et al. 2019; Roundtree et al. 2017). m6A modifications are typically incorporated into the conserved DRACH sequence (D = A, G, U; R = A, G; H = A, C, U) and are frequently located near stop codons (Shi et al. 2019). The process of m6A modification is facilitated by a methyltransferase complex, known as the "writer," while a demethylase, or "eraser," removes m6A marks (Jia et al. 2011; Mauer et al. 2017). "Reader" proteins recognize m6A-modified RNA and initiate downstream activities. The expression of genes encoding the writer, eraser, and reader proteins modulates m6A levels, thereby controlling RNA stability, splicing, and translation (Pan et al. 2018).

Recent investigations have linked m6A modification to genes involved in the regulation of ferroptosis. One mechanism by which m6A modification regulates

ferroptosis is through the modulation of mRNA stability of key genes involved in cellular redox homeostasis. For instance, in hepatoblastoma cells, the methyltransferase protein METTL3 mediates m6A modification of SLC7A11 mRNA, which is recognized by IGF2 mRNA-binding protein 1 (IGF2BP1), enhancing SLC7A11 mRNA stability and increasing its expression. Mechanistically, IGF2BP1 competitively binds to poly(A)-binding protein 1 (PABPC1), thereby blocking the recruitment of the BTG2/CCR4-NOT complex and suppressing mRNA deadenylation.

However, the effects of m6A modification on SLC7A11 mRNA appear to be inconsistent and dependent on distinct reader proteins. In septic myocardial injury, METTL3 installs m6A modifications on SLC7A11 mRNA, and the reader protein YTHDF2 (YTH N6-methyladenosine RNA-binding protein F2) accelerates its degradation, resulting in reduced SLC7A11 protein levels and the induction of ferroptosis in LPS-induced H9C2 cells (Shen et al. 2023). Similarly, in lung adenocarcinoma (LUAD) cells, the reader protein YTHDF1 destabilizes SLC7A11 mRNA in an m6A-dependent manner. YTHDF1 preferentially binds to m6A-modified SLC7A11 mRNA and promotes its decay, rendering the cells more sensitive to ferroptosis (Xu et al. 2022). In thyroid cancer cells, the eraser protein FTO catalyzes the demethylation of m6A on SLC7A11, leading to decreased expression of SLC7A11 and increased sensitivity to ferroptosis (Ji et al. 2022). Similarly, ALKBH5, another eraser protein, reduces the mRNA stability of SLC7A11 by erasing m6A modifications on SLC7A11 mRNA, thereby promoting ferroptosis in colorectal cancer cells (Luo et al. 2023).

Importantly, m6A modification can also impact the splicing and maturation of SLC7A11 mRNA. The m6A modification reader, NF-κB activating protein (NKAP), can bind to m6A-modified SLC7A11 mRNA and recruit the splicing factor proline and glutamine-rich (SFPQ) to recognize the splice site, leading to splicing events on the SLC7A11 transcript and retention of the last exon in glioblastoma cells (Sun et al. 2022). Consequently, NKAP promotes SLC7A11 expression and acts as a suppressor of ferroptosis in glioblastoma cells.

Additionally, m6A modification has been implicated in the regulation of ferroptosis through the modulation of autophagy and exosomal miRNA signaling pathways. In hepatic stellate cells (HSC), the reader protein YTHDF1 promotes the stability of BECN1 mRNA by recognizing the m6A binding site, thereby activating autophagy and facilitating the degradation of ferritin through autophagy, ultimately leading to ferroptotic cell death (Shen et al. 2021). In non-small cell lung carcinoma (NSCLC) cells, exosomal miR-4443 has been shown to promote cell resistance to cisplatin through m6A-mediated regulation of FSP1 and ferroptosis (Song et al. 2021). The study revealed that tumor-derived exosomal miR-4443 downregulates METTL3, reduces m6A enrichment of FSP1, and increases FSP1 mRNA levels, resulting in resistance to cisplatin-induced ferroptosis in NSCLC.

In summary, these studies highlight the significant role of m6A modification in the regulation of ferroptosis and its therapeutic potential for the treatment of

ferroptosis-related diseases such as cancer and neurodegeneration. The modulation of m6A modification can influence various aspects of ferroptosis, including mRNA stability, splicing, autophagy, and exosomal miRNA signaling pathways.

18.3.2 N4-acetylcytidine

N-acetyltransferase 10 (NAT10) is a lysine acetyltransferase that acetylates RNA and is responsible for mediating the N4-acetylcytidine (ac4C) modification of mRNA, which plays a crucial role in mRNA stability and translation efficiency (Dalhat et al. 2021; Balmus et al. 2018). The ac4C modification is considered an ancient and conserved form of mRNA modification (Yang et al. 2021; Montgomery et al. 2016). A recent study has shown that NAT10 enhances the stability of FSP1 mRNA through N4-acetylation of the FSP1 mRNA, resulting in increased FSP1 expression in colon cancer cells. The FSP1/CoQ system, which operates independently of the SLC7A11/GSH/GPX4 system, acts as a defense mechanism against ferroptosis. Consequently, the high expression of NAT10 promotes cancer progression by inhibiting ferroptosis in a FSP1-dependent manner (Zheng et al. 2022a). However, the impact of ac4C modification on other ferroptosis-related genes and its broader regulation of ferroptosis in different pathophysiological conditions remain unclear. Further research is needed to elucidate the full extent of ac4C modification's influence on ferroptosis and its implications in various disease contexts.

18.4 Posttranslational Regulation of Ferroptosis Regulators

18.4.1 Phosphorylation

Phosphorylation is a fundamental and highly conserved PTM that involves the covalent addition of a phosphate group to specific amino acid residues, predominantly serine, threonine, or tyrosine, in eukaryotic proteins. This reversible modification plays a pivotal role in regulating various cellular processes, including signal transduction, gene expression, cell division, and cell death. Several studies have highlighted the importance of protein phosphorylation in the regulation of ferroptosis.

In the context of ferroptosis, SLC7A11, a well-established target, is regulated not only at the epigenetic, transcriptional, and translational levels but also through various PTMs. Phosphorylation is the first reported PTM of SLC7A11, and its regulation has been shown to impact the activity and stability of the transporter. For instance, the mechanistic target of rapamycin complex 2 (mTORC2), a serine/threonine kinase and a core component of altered growth factor receptor signaling in certain cancer types, was identified as the enzyme responsible for phosphorylating serine 26, located in the cytoplasmic region of SLC7A11. This discovery was made using immunoprecipitation combined with mass spectrometry in glioblastoma (GBM) cells (Gu et al. 2017). Inhibition of mTORC2 or the use of a SLC7A11

mutant lacking the serine 26 residue ($SLC7A11^{S26A}$) led to decreased phosphorylation of SLC7A11 (Gu et al. 2017). Interestingly, AKT, a major substrate of mTORC2 and also known as protein kinase B (PKB), was reported to directly phosphorylate SLC7A11 at the same site, as demonstrated in human breast epithelial cell lines (MCF10A) using a similar method. Consistently, inhibition of AKT or the use of $SLC7A11^{S26A}$ resulted in impaired transporter activity of SLC7A11 and increased sensitivity to ferroptosis-inducing agents (Lien et al. 2017). These findings provide crucial evidence supporting the notion that phosphorylation of SLC7A11 at the Ser26 residue inhibits cystine uptake mediated by SLC7A11, consequently reducing glutathione (GSH) levels and increasing sensitivity to ferroptosis. This highlights an alternative approach to modulate ferroptosis through the manipulation of SLC7A11 phosphorylation.

Acyl-CoA synthetase long-chain family member 4 (ACSL4) plays a critical role in ferroptosis by facilitating the incorporation of long-chain polyunsaturated fatty acids (PUFAs) into membrane phospholipids, thereby increasing cellular susceptibility to oxidative damage (Doll et al. 2017). Recent research has revealed that the phosphorylation of ACSL4 by protein kinase C βII (PKCβII) represents an important regulatory mechanism in ferroptosis induction (Zhang et al. 2022b). In this study, PKCβII was found to phosphorylate ACSL4 at serine 659, which enhanced its enzymatic activity, leading to increased incorporation of PUFAs into membrane phospholipids and subsequent PUFA accumulation (Zhang et al. 2022b). This process, in turn, amplified lipid peroxidation and triggered ferroptotic cell death. Furthermore, the authors demonstrated that inhibition of the PKCβII–ACSL4 pathway reduced the effectiveness of immunotherapy by suppressing ferroptosis. These findings suggest that PKCβII enhances the efficacy of immunotherapy by promoting ferroptosis and provides a potential therapeutic strategy for cancer treatment.

18.4.2 Ubiquitination

Ubiquitination is a reversible process that involves the addition of ubiquitin molecules to lysine residues on substrate proteins by E1, E2, and E3 enzymes. Polyubiquitination, in particular, leads to the formation of ubiquitin chains on cytoplasmic proteins, marking them for degradation by the proteasome and thereby affecting their intracellular levels (Popovic et al. 2014). On the other hand, deubiquitinases (DUBs) remove ubiquitin chains from proteins, thereby enhancing protein stability (Ronau et al. 2016). Ubiquitin-mediated regulation of protein homeostasis plays a critical role in various biological processes, including cell differentiation and development, cell cycle and division, immune response, DNA transcription and repair, as well as cell death pathways such as apoptosis and ferroptosis.

The regulation of SLC7A11 protein stability has been investigated in previous studies. It has been observed that SLC7A11 interacts with CD44 variant (CD44v), a membrane-localized protein. High levels of CD44v have been shown to enhance SLC7A11 expression and the synthesis of glutathione (GSH), potentially by

promoting SLC7A11 stability (Ishimoto et al. 2011). One key regulator of SLC7A11 stability is OTUB1, a deubiquitinase belonging to the ovarian tumor (OTU) family. OTUB1 stabilizes the protein level of SLC7A11 and increases its half-life. Further research has demonstrated that OTUB1, CD44v, and SLC7A11 form a trimeric complex, with CD44v promoting SLC7A11 stability by facilitating the interaction between OTUB1 and SLC7A11 (Liu et al. 2019; Gan 2019). Additionally, the E3 ubiquitin ligase tripartite motif-containing protein 26 (TRIM26) has been identified as an inducer of ferroptosis that suppresses the activation of hepatic stellate cells and decreases liver fibrosis through SLC7A11 ubiquitination (Zhu et al. 2021b). Another study has shown that suppressor of cytokine signaling 2 (SOCS2) mediates the ubiquitination of SLC7A11 in hepatocellular carcinoma. Mechanistically, SOCS2 acts as a bridge to transfer attached ubiquitin to SLC7A11, promoting K48-linked polyubiquitination and subsequent protein degradation, although the specific E3 ubiquitin ligase involved in this process is not yet clear (Chen et al. 2023). Furthermore, ubiquitinated SLC7A11 has been observed in carbon tetrachloride (CCl_4)-induced acute liver injury (ALI) and long noncoding RNA (LncRNA) HEPFAL-induced ferroptosis in hepatocellular carcinoma (Zhang et al. 2022a; Lin et al. 2022). These studies collectively demonstrate that ubiquitination of SLC7A11 leads to its degradation in a proteasome-dependent manner, ultimately inducing ferroptosis. A comprehensive understanding of SLC7A11 stability regulation could provide valuable insights into potential targets for modulating ferroptosis.

In addition to SLC7A11, independent studies have demonstrated that the protein stability of GPX4 can also be regulated through ubiquitination-mediated degradation. In non-small cell lung cancer cells, treatment with the deubiquitinase (DUB) inhibitor pyridinium sulfur palladium complex (PdPT) leads to the ubiquitination and degradation of GPX4 (Yang et al. 2020). Another study provided further evidence of proteasome-mediated degradation of GPX4. A drug derived from parthenolide called DMOCPTL directly binds to the active site of GPX4, resulting in GPX4 ubiquitination in triple-negative breast cancer cells (Ding et al. 2021). However, the specific E3 enzymes and DUBs involved in the ubiquitination system that regulate GPX4 stability, as well as the detailed molecular mechanism, remain unclear. Further investigation into the molecular mechanisms underlying ubiquitination and its impact on ferroptosis-related modulators may provide novel insights into the regulation of ferroptosis and identify potential therapeutic targets for diseases associated with this form of cell death.

18.4.3 UFMylation

UFMylation is a posttranslational modification (PTM) that involves the covalent attachment of a ubiquitin-like protein called UFM1 (ubiquitin-fold modifier 1) to target proteins (Komatsu et al. 2004). Similar to ubiquitination, this process is mediated by a three-step enzymatic cascade involving the E1 activating enzyme (UBA5), the E2 conjugating enzyme (UFC1), and E3 ligases (such as UFL1)

(Tatsumi et al. 2010). UFMylation has been implicated in various cellular processes, including protein stability, ER stress response, and autophagy.

Recent studies have demonstrated that UFMylation plays a role in modulating the stability of SLC7A11 and its involvement in ferroptosis (Zhu et al. 2021a). These studies have shown that UFM1 targets and modifies SLC7A11. This modification stabilizes the SLC7A11 protein, enhancing its function and promoting cellular resistance to ferroptosis. In contrast, inhibition or knockout of UFM1 leads to downregulation of SLC7A11 protein stability, increasing susceptibility to ferroptosis. UFMylation is a newly identified posttranslational modification that affects the stability of SLC7A11, which plays a crucial role in cellular redox homeostasis and ferroptosis regulation. However, the precise E3 ligase responsible for SLC7A11 UFMylation remains to be fully elucidated, and the existence of enzymes that can reverse UFMylation, similar to DUBs in ubiquitination, is still uncertain. Identification of these enzymes would provide insights into the dynamic nature of UFMylation regulation and its role in regulating ferroptosis. Furthermore, exploration of UFMylation inhibitors and activators for SLC7A11 and investigation of whether other ferroptosis-related proteins undergo similar modifications are areas that require further exploration.

18.4.4 Alkylation

Alkylation is a chemical modification process that involves the transfer of an alkyl group to a target molecule, including proteins. This posttranslational modification can induce conformational changes in proteins, potentially affecting their function, stability, and interactions with other molecules.

Recent studies have demonstrated that GPX4 can be targeted by alkylation, specifically by electrophiles under class II FINs treatment (Eaton et al. 2020; Hassannia et al. 2018; Vučković et al. 2020). These electrophiles can alkylate the catalytic selenocysteine residue of GPX4, leading to the inhibition of its peroxidase activity. Additionally, a study revealed that alkylation of GPX4 resulted in ferroptosis in high-risk neuroblastoma (Hassannia et al. 2018). Therefore, it is possible to modulate cellular susceptibility to ferroptosis by inducing GPX4 alkylation. Further research into the mechanisms of GPX4 alkylation and its biological consequences may uncover novel ferroptosis inducers that target ferroptotic cell death in various pathological contexts associated with ferroptosis resistance.

18.4.5 Succination

Succination is a posttranslational modification that involves the covalent attachment of a succinyl group to a protein, resulting in changes to protein function, stability, and localization. Succination occurs in response to cellular stress, such as elevated levels of fumarate, an intermediate of the Krebs cycle in mitochondria. In the

absence of the enzyme, fumarate can bind to the sulfhydryl group of cysteine residues (Blatnik et al. 2008; Manuel and Frizzell 2013).

A recent study has shown that succination of GPX4 can occur in hereditary leiomyomatosis and renal cell cancer (HLRCC) (Kerins et al. 2018). This modification leads to a decrease in GPX4 activity and an increased susceptibility to ferroptosis. When GPX4 undergoes succination, its ability to neutralize lipid peroxides is compromised, resulting in the accumulation of lipid peroxides, increased lipid peroxidation, and the induction of ferroptosis. Further investigation is necessary to uncover the precise mechanism underlying the regulation of succination on GPX4, as well as to explore other potential candidates involved in the regulation of ferroptosis.

18.4.6 Myristoylation

Myristoylation is a posttranslational modification that involves the covalent attachment of a 14-carbon saturated fatty acid, known as myristic acid, to the N-terminal glycine residue of a protein. This modification occurs during protein translation and plays a crucial role in modulating the function, localization, and protein–protein interactions of the modified protein (Wright et al. 2010; Adam et al. 2007; Wang et al. 2016).

Ferroptosis suppressor protein 1 (FSP1), also called AIFM2, is a recently discovered glutathione-independent ferroptosis suppressor that works alongside GPX4 to inhibit ferroptosis. FSP1 functions by reducing coenzyme Q10 (CoQ10) to ubiquinol, which scavenges lipid peroxyl radicals and prevents lipid peroxidation, a critical event in ferroptosis (Doll et al. 2019; Bersuker et al. 2019). Studies have demonstrated that FSP1 undergoes myristoylation, which is essential for its inhibitory function against ferroptosis. The myristoylation of FSP1 is mediated by the enzyme *N*-myristoyltransferase (NMT), which transfers the myristoyl group from myristoyl-CoA to the N-terminal glycine residue of FSP1. Inhibition of NMT activity or mutation of the myristoylation site in FSP1 (G2A) abolishes its ferroptosis-inhibitory function. Myristoylation facilitates the proper localization of FSP1 to the plasma membrane, enabling it to efficiently reduce CoQ10 and suppress lipid peroxidation. The myristoylation-mediated plasma membrane localization is crucial for FSP1's ability to inhibit ferroptosis (Bersuker et al. 2019).

18.5 Conclusion

Understanding the impact of posttranslational modifications, including phosphorylation, ubiquitination, UFMylation, alkylation, succination, and myristoylation, on the stability and activity of proteins involved in ferroptosis can provide valuable insights for modulating ferroptosis and developing targeted therapies for various pathological conditions associated with ferroptotic cell death. These modifications can influence protein function, localization, and interactions, thereby affecting the

regulation of ferroptosis-related pathways. Exploring the dynamic nature of these posttranslational modifications and their specific roles in ferroptosis regulation may unveil new therapeutic targets and strategies for intervening in ferroptosis-related diseases.

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Phospholipid Peroxidation in Health and Disease

19

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Abstract

Phospholipid peroxidation plays a crucial role in modulating the fluidity, integrity, and stability of cellular and organelle membranes. This process has significant implications in the initiation of ferroptosis, a form of cell death, and has been associated with various pathological processes in diverse diseases. Targeting phospholipid peroxidation represents a promising approach for the development of novel therapeutic interventions. This chapter provides a comprehensive overview of the oxidation, reduction, and remodeling processes of phospholipids, along with the methods for detecting phospholipid peroxidation. Furthermore, it examines the impact of phospholipid peroxidation on signal transduction and its involvement in the onset and progression of various diseases. The knowledge presented here offers a solid theoretical foundation for the development of targeted interventions aimed at preventing diseases by modulating phospholipid peroxidation.

19.1 Introduction

Polyunsaturated fatty acid-containing phospholipids (PUFA-PLs) are essential constituents of cellular membranes, playing a critical role in maintaining membrane fluidity necessary for proper cellular functions. However, due to the presence of multiple double bonds within their fatty acyl chains, PUFA-PLs are highly reactive and prone to be modified by reactive oxygen species (ROS) via a nonenzyme

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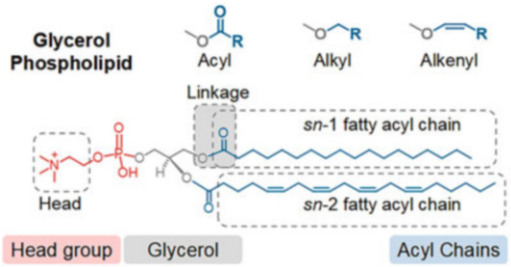
pathway or oxidized by enzymes to form oxidized phospholipids (ox-PLs) (O'Donnell et al. 2019). The relationship between phospholipid peroxidation and cell death has been a topic of investigation since the 1960s (Stockwell and Jiang 2020). In 2012, Dr. Brent R. Stockwell and his research team discovered a novel form of cell death, ferroptosis, which is induced by the accumulation of iron-dependent lipid peroxidation (Dixon et al. 2012). Among its key features is the loss of cell membrane integrity, which results from phospholipid peroxidation of the cell membrane (Doll et al. 2019; Yang et al. 2014). The discovery of ferroptosis has bridged the gap between the accumulation of phospholipid peroxidation products and the organ dysfunction phenotypes observed in human pathology (Yang et al. 2014). Presently, ferroptosis has been implicated in the pathological and physiological processes of several human diseases, including tumors, neurodegenerative disorders, acute kidney injury, drug-induced liver injury, and liver/heart ischemia-reperfusion injury (Jiang et al. 2021; Yan et al. 2021).

Over the past decade, with the rapid increase of research in ferroptosis, phospholipid peroxidation, the key process involved in driving ferroptosis, has led to a surge of interest in the regulation of various diseases. It is now well established that ox-PLs produced by phospholipid peroxidation potentially possess bioactive effects, participating in various biological processes (Bochkov et al. 2010). The oxidation of PUFA-PLs may dramatically alter the biological activity of phospholipids, such as membrane polarity, membrane permeability, phospholipid phase transition and phase separation, and the alteration of these properties (Jiang et al. 2021). Besides destabilizing the phospholipid bilayer structure (Wong-Ekkabut et al. 2007), ox-PLs may also act as cellular signal transduction messengers and thus shaping cell fate or affect membrane-dependent events such as phagocytosis (Bohdanowicz and Grinstein 2013; Lemke 2019). All that highlights the significance of comprehending the intricate interplay between phospholipid peroxidation and its roles in cellular signaling and regulation. In this chapter, we summarize the oxidation, reduction, and remodeling processes of phospholipids, the detection methods that monitor phospholipid peroxidation, the effect of phospholipid peroxidation in the signal transduction, as well as the roles of phospholipid peroxidation in the occurrence and progression of diverse diseases, to provide a theoretical basis for targeted intervention of phospholipid peroxidation as a potential strategy to prevent diseases.

19.2 Oxidation Process of Phospholipids

Phospholipids are typically composed of a hydrophilic head group made up of a phosphate group and a hydrophobic tail composed of fatty acid chains connected through a glycerol backbone, giving them amphipathic properties. The hydrophilic ends of the phospholipids can interact with each other, while the hydrophobic tails also interact with each other, forming a phospholipid bilayer together with proteins, glycolipids, and cholesterol, which is a basic structure of biological membranes (Dowhan 2017). The phosphate group in phospholipids can be modified by organic small molecules such as glycerol, choline, ethanolamine, and serine. Common



PL	Head Group
PA	-
PC	Choline
PE	Ethanolamine
PG	Glycerol
PI	Inositol
PS	Serine
CL	Phosphatidylglycerol

Fig. 19.1 Composition of phospholipids. PA phosphatidic acid, PC phosphatidylcholine, PE phosphatidylethanolamine, PG phosphatidylglycerol, PI phosphatidylinositol, PS phosphatidylserine, CL cardiolipin

phospholipids found in biological membranes include phosphatidic acid (PA), phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylserine (PS), phosphatidylinositol (PI), phosphatidylglycerol (PG), and cardiolipin (CL) (Fig. 19.1). Fatty acid chains are attached to the glycerol backbone through ester or ether bonds at the first and second carbon atoms, which are represented as *sn*-1 and *sn*-2 acylated fatty chains (Harayama and Riezman 2018).

PUFA-PLs are crucial components of cellular membranes due to their multiple double bonds within the fatty acyl chains, which provide membrane fluidity necessary for proper cellular functions (Stillwell and Wassall 2003). Additionally, these double bonds render PUFA-PLs more reactive, making them prone to oxidation (Ayala et al. 2014). The degree of unsaturation directly influences the reactivity of these lipids, as the presence of more double bonds correlates with increased susceptibility to oxidation. Oxidized PUFA-PLs serve as signaling molecules that participate in various biological processes (Bochkov et al. 2010).

The oxidation process of phospholipids can be classified into three phases: initiation, propagation, and termination (Yin et al. 2011). During the initiation phase, PUFA-PLs are susceptible to attack by free radicals (nonenzymatic) or oxidation-related enzymes (enzymatic) to abstract a hydrogen atom from diallyl methylene, forming phospholipid radicals (PL·). In the propagation phase, PL· reacts quickly with oxygen molecules, leading to the formation of primary oxidation products, such as phospholipid peroxyl radicals (PLOO·). PLOO· reacts with other PUFA-PL, converting PLOO· into non-radical phospholipid hydroperoxides (PLOOH), generating new carbon-centered radicals that initiate a new peroxidation cycle (Bochkov et al. 2010). During the termination phase, two molecules of PLOO· react to terminate and form non-radical products. As the propagation of phospholipid peroxidation is a stochastic process independent of enzymes (Ashraf and Srivastava 2012), only the initiation phase of phospholipid oxidation is typically discussed when distinguishing between nonenzymatic and enzymatic oxidation (Fig. 19.2).

It is widely accepted that nonenzymatic phospholipid peroxidation arises from the assault on PUFA-PL by hydroxyl radicals (HO·) generated via the Fenton reaction (Lai and Piette 1978). The mitochondrial electron transport chain also can give rise

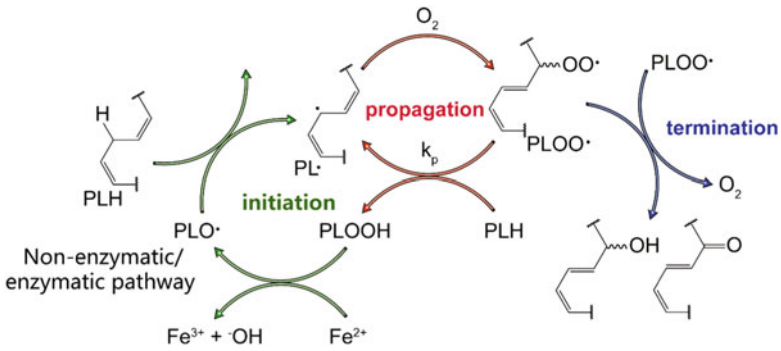


Fig. 19.2 Three phases of phospholipid peroxidation. PLOO· phospholipid peroxyl radical, PLOOH phospholipid hydroperoxide, PLO· phospholipid alkoxyl radical, PL· phospholipid radical

to various forms of ROS, which subsequently react with phospholipids, producing phospholipid peroxides (Zorov et al. 2014). As opposed to the randomness of the nonenzymatic oxidation pathway (O'Donnell et al. 2019), the enzymatic oxidation of phospholipids is a highly regulated process that is catalyzed by enzymes such as 15-lipoxygenase (15-LOX) (Anthonymuthu et al. 2018) and cytochrome P450 oxidoreductase (POR) (Ai et al. 2021; Zou et al. 2020). There are two distinct mechanisms of enzymatic oxidation of phospholipids: direct or indirect pathway (O'Donnell et al. 2019). Direct enzymatic oxidation of phospholipids predominantly takes place at the *sn*-2 position of the acylated fatty chain, exhibiting high regioselectivity and stereoselectivity (Bochkov et al. 2010; Kühn et al. 1994). PUFA-PLs, particularly PC and PE-containing arachidonic acid (AA), can be directly catalyzed by 15-LOX to generate free phospholipid-15-hydroperoxyeicosatetraenoic acid (15-HpETE-PL) (Singh and Rao 2019). Indirect enzymatic oxidation of phospholipids is achieved through the coupling of deacylation and reacylation via the eicosanoid and prostaglandin pathways (O'Donnell et al. 2019). This process encompasses: (1) phospholipids being cleaved by phospholipase A_2 (PLA_2) to remove the fatty acyl chain at the *sn*-2 position, generating free fatty acids and lysophospholipids (Lyso-PL) (Frasch and Bratton 2012); (2) free fatty acids being oxidized by lipoxygenase (LOX) or cyclooxygenase (COX) to generating lipid peroxides; (3) fatty acid oxidation product-coenzyme A (CoA) forming ox-PLs through the esterification of lysophosphatidylcholine acyltransferase 3 (LPCAT3) (Murphy and Folco 2019; O'Donnell et al. 2019) (Fig. 19.3).

Primary phospholipid peroxidation products formed by the oxidation process can undergo further oxidation, yielding secondary oxidation products through three distinct pathways. The first pathway involves PLOO· and PLOOH incorporating additional oxygen atoms to generate a series of ox-PLs with various functional groups, such as hydroperoxy products (hydroperoxy-PL), hydroxy products (hydroxy-PL), ketone-based products (keto-PL), and epoxy products (epoxy-PL). The second pathway entails the cyclization of PLOO·, leading to the formation of

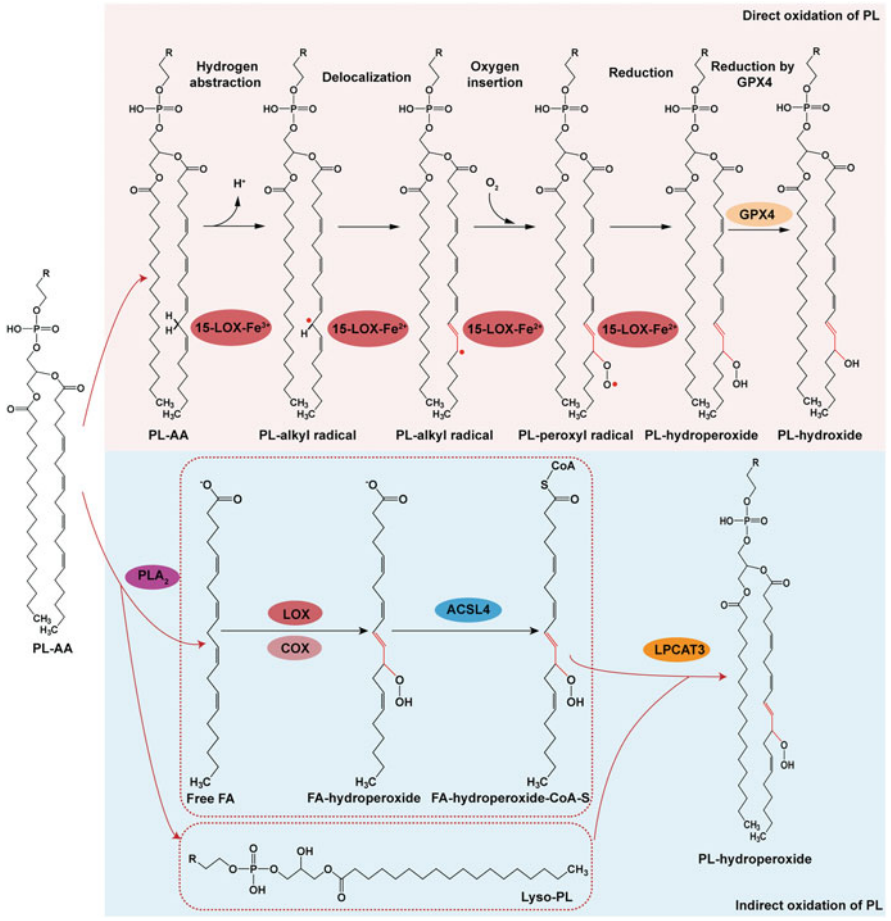


Fig. 19.3 Direct oxidation of phospholipids by 15-LOX and indirect oxidation of phospholipids

cyclic peroxides, which subsequently undergo internal cyclization and rearrangement to generate bicyclic endoperoxides, including isoprostanes, isothromboxanes (isoTx), and isolevuglandins (isoLG). Alternatively, these cyclic peroxides can oxidatively incorporate additional (non)-cyclic peroxide group molecules, undergoing further oxidation to yield isofurans. The third pathway involves the oxidation, cleavage, or polymerization of PLOOH, resulting in the production of γ-hydroxyl-α,β-unsaturated ox-PLs with terminal aldehyde groups, unsaturated ox-PLs with terminal furan groups, and saturated ox-PLs with terminal carbonyl groups. Oxidation products generated by the first two pathways are non-fragmented ox-PLs, while those produced by the third pathway are fragmented ox-PLs (Bochkov et al. 2010).

19.3 Reduction and Remodeling Process of Phospholipids

To respond to the produced ox-PLs, cells have evolved multiple mechanisms to reduce phospholipid peroxidation and prevent damage caused by the accumulation of phospholipid peroxides. These mechanisms primarily include glutathione peroxidase 4(GPX4)/GSH, ferroptosis suppressor protein 1 (FSP1)/CoQ_{10}, and GCH1/BH4 (Stockwell et al. 2020). The GPX4/GSH system functions by reducing phospholipid hydroperoxides to phospholipid alcohols (Seibt et al. 2019). FSP1/CoQ_{10} and GCH1/BH4 systems use reduced CoQ_{10} and BH4 as free radical scavengers to eliminate PLOO·, thereby preventing ferroptosis (Stockwell et al. 2020; Kraft et al. 2020). Besides these three systems, the phospholipid remodeling system also contributes to limiting the process of phospholipid peroxidation. PLA_2 selectively cleaves oxidized fatty acid chains at the *sn*-2 position of the glycerol backbone, generating the Lyso-PL, which can further react with AA-CoA under the action of LPCAT3 to synthesize new phospholipids, completing the process of phospholipid remodeling (Wang and Tontonoz 2019). Recently, calcium-independent phospholipase $A_2\beta$ (i$PLA_2\beta$) has been reported to play a crucial role in the process of remodeling ox-PLs. Specifically, i$PLA_2\beta$ exhibits a high selectivity toward the ferroptosis signal molecule PE-AA-OOH (Sun et al. 2021). Moreover, recent research has highlighted the role of dihydroorotate dehydrogenase (DHODH), an enzyme localized on the outer face of the mitochondrial inner membrane, in preventing cells from ferroptosis by suppressing mitochondrial lipid peroxidation (Mao et al. 2021). In addition, various endogenous or exogenous free radical scavenging antioxidants, such as Vitamin E and ferrostatin-1, also can eliminate lipid peroxides, thereby inhibiting ferroptosis (Magtanong et al. 2016).

19.4 Detection Methods of Phospholipid Peroxidation

Given the important role of phospholipid peroxidation in ferroptosis, different types of assays have been developed to monitor the process of phospholipid peroxidation, including fluorescent probe assay, chemiluminescence assay, immunoassay, and oxidative lipidomic analysis based on high-resolution liquid chromatography-mass spectrometry (LC-MS).

19.4.1 Fluorescent Probes

C11-BODIPY$^{581/591}$ is a lipophilic fluorescent probe that has been extensively utilized for the detection of lipid oxidation levels in live cells, particularly to indicate the accumulation of lipid peroxides in ferroptotic cells (Pap et al. 1999). Under normal conditions, this probe exhibits bright red fluorescence. However, upon oxidation of its polyunsaturated butadiene moiety, the peak of its fluorophore fluorescence emission shifts from 590 nm to 510 nm, resulting in a change in fluorescence color from red to green (Drummen et al. 2002) (Fig. 19.4a). The

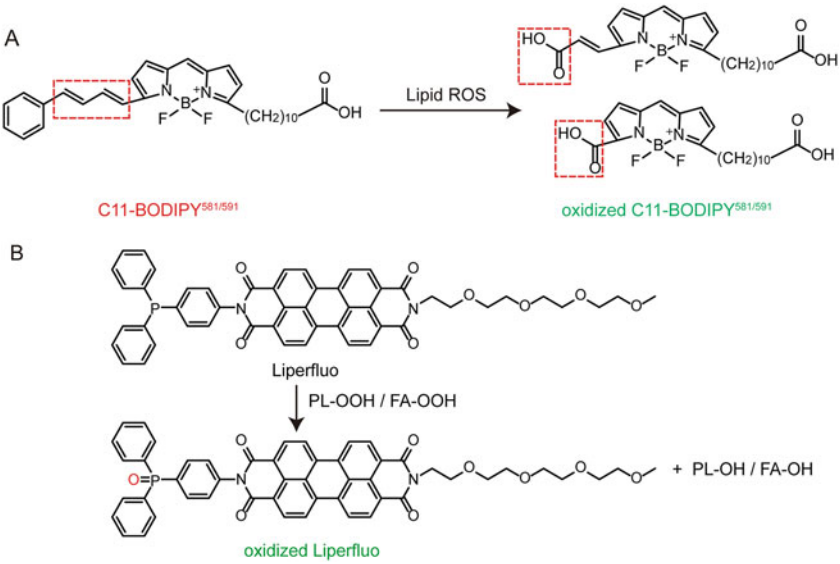


Fig. 19.4 Most commonly applied fluorescence probes for detecting phospholipid peroxidation. (**a**) C11-BODIPY$^{581/591}$; (**b**) Liperfluo

advantages of this probe include its insensitivity to O_2^-, nitric oxide (NO), ferric ions, and hydrogen peroxide (H_2O_2), its high stability and sensitivity, its low toxicity, and its relatively low cost. Nonetheless, C11-BODIPY$^{581/591}$ is sensitive to various ROS and peroxynitrite ($ONOO^-$) in lipid-soluble membranes, rendering it nonspecific in the detection of phospholipid peroxidation.

When compared to C11-BODIPY$^{581/591}$, Liperfluo, a probe optimized by Yamanaka (Yamanaka et al. 2012), is considered to be a more specific probe for the monitoring of phospholipid peroxidation. The reaction mechanism of Liperfluo is analogous to the reduction of LOOHs by GPX4. Upon interaction with LOOHs, it produces oxidized Liperfluo with green fluorescence. In an aqueous solution, the oxidized Liperfluo is almost non-fluorescent; however, it produces a strong green fluorescence in highly lipid-soluble sites, such as cell membranes (Fig. 19.4b). While this probe is widely utilized in the field of ferroptosis (Kagan et al. 2017), it also has some limitations. Specifically, it can only detect the total level of phospholipid peroxidation in cells and cannot differentiate between different categories of lipid peroxides, such as unsaturated fatty acid peroxides and phospholipid peroxides.

19.4.2 Chemiluminescence Assay and Immunoassay for Detecting Phospholipid Peroxidation Products

Phospholipid peroxidation generates a large amount of lipid peroxidation products, among which PLOOHs are the primary products that gradually decompose into a series of complex aldehyde compounds, including malondialdehyde (MDA), 4-hydroxynonenal (4-HNE), acetaldehyde, and hexanal, through the oxidative process. These reactive aldehyde compounds can attack biological macromolecules such as proteins, forming adducts and causing further damage to cells (Reis and Spickett 2012). The assessment of phospholipid peroxidation level can be achieved by detecting products generated at different stages of phospholipid peroxidation.

MDA is a highly produced product during lipid peroxidation, and the thiobarbituric acid reactive substances (TBARS) assay is frequently employed to measure MDA levels (Tsikas 2017). MDA readily reacts with thio-TBA to form a red adduct product, TBARS, whose absorbance (at 532 nm) can be measured to determine the amount of MDA. This method is straightforward and commonly employed in studying the lipid peroxidation level of biological samples. However, its drawback is the lack of specificity of TBA, which may cause false positives during the experimental procedure.

Due to their high reactivity, a significant proportion of MDA and 4-HNE can react with biomolecules such as proteins, DNA, or phospholipids to form large molecular adducts (Aldini et al. 2006). Currently, immunoassays are important analytical tools for detecting MDA and 4HNE protein adducts. Enzyme-linked immunosorbent assay (ELISA), protein immunoblotting (Western blot), and immunostaining, which utilize MDA and 4-HNE specific antibodies, can detect and quantify MDA and 4-HNE protein adducts based on the principle of antigen-antibody binding (Feng et al. 2020; Hao et al. 2021; Dierge et al. 2021). Despite their high specificity, these methods only provide information on the overall changes of protein adducts and cannot determine the type or precise location of the modified protein.

19.4.3 Oxidative Lipidomic Based on LC-MS

The investigation of biological processes involving ox-PLs presents a substantial challenge due to the intricate chemical structures and the highly variable levels of phospholipid peroxidation products (Bayır et al. 2020). Although various methods such as immunoassay, chemical or fluorescence photometry, and NMR have been used for the analysis of ox-PLs, their effectiveness is often limited in the qualitative and quantitative analysis of complex phospholipid peroxidation products in biological samples. In recent years, LC-ESI-MS/MS has emerged as a preferred method for the qualitative and quantitative analysis of ox-PLs in cells and tissues, providing a powerful tool for the study of phospholipid peroxidation and ferroptosis processes (Spickett and Pitt 2015). LC-MS-based ox-PLs analysis, akin to lipidomics, can be categorized into non-targeted and targeted modes, depending

on the technical process. Non-targeted analysis of ox-PLs typically employs multi-stage high-resolution mass spectrometry or ion trap mass spectrometry to collect samples and obtain abundant fragment ions containing information on oxidized phospholipid fatty chain species, oxidation types, and oxidation sites for structural identification. For example, Tyurin et al. introduced an analysis method for oxidized PS by utilizing normal-phase LC (NPLC) and high-resolution orbital ion trap (Orbitrap) tandem mass spectrometry, to identify ox-PSs as apoptotic "eat-me" signaling molecules (Tyurin et al. 2014). In a subsequent study, Kagan et al. established an oxidative lipidomic approach using NPLC-MS/MS to identify the oxidation products of PE-AA and PE-AdA, namely PE-AA-OOH and PE-AdA-OOH, which act as execution molecules of ferroptosis, among hundreds of ox-PLs (Kagan et al. 2017).

In addition, LC-MS technology could enrich the information on adducts formed by combining ox-PLs end products with proteins. Chen et al. developed a specific aniline probe m-APA and identified more than 400 carbonylated proteins in RSL3-induced ferroptosis cell models (Chen et al. 2018). In another study, Zhang et al. identified thousands of 4-HNE-modified proteins in cell samples by using aminoxyTMT labeling technology combined with LC-MS proteomics (Zhang et al. 2019).

Collectively, LC-MS-based technologies play crucial role in the discovery, identification, and quantification of novel ox-PLs and their derivatives related to ferroptosis.

19.5 The Role of Phospholipid Peroxidation in Cellular Signal Transduction

19.5.1 The Role of Phospholipid Peroxidation in Driving Ferroptosis

Ferroptosis is a novel type of iron-dependent oxidative cell death, characterized mainly by the inactivation of the intracellular antioxidant defense system, which induces phospholipid peroxidation to induce cell death (Dixon et al. 2012; Stockwell 2022). Phospholipid peroxidation is a hallmark of ferroptosis, and ox-PLs, such as ox-PEs, have emerged as important regulatory molecules involved in ferroptotic cell death signals (Fig. 19.5a). Through the application of LC-MS quantitative redox lipidomics combined with molecular biology studies, Kagan et al. have shown that phospholipid peroxidation during ferroptosis occurs in the endoplasmic reticulum, where PE is specifically oxidized by 15-LOX, particularly PE-containing *sn*-2 fatty acyl chains of AA (C20:4) and AdA (C22:4), generating PE-AA-OOH or PE-AdA-OOH to initiate ferroptosis (Kagan et al. 2017). In this process, phosphatidylethanolamine binding protein 1 (PEBP1) and 15-LOX form a protein complex that enhances the selectivity of 15-LOX for PE and specifically oxidizes the 15th carbon atom of AA-PE to yield the ferroptosis signaling molecule 15-HpETE-PE (Anthonymuthu et al. 2018; Wenzel et al. 2017).

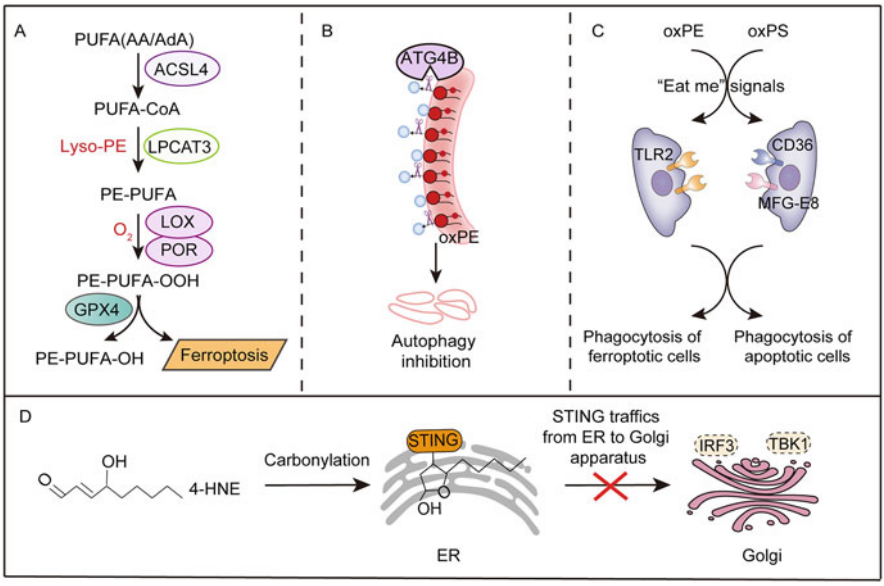


Fig. 19.5 Role of phospholipid peroxidation in cell signal transduction. (**a**) The role of phospholipid peroxidation in driving ferroptosis; (**b**) the role of phospholipid peroxidation in autophagy inhibition; (**c**) the role of phospholipid peroxidation in phagocytic signaling; and (**d**) the role of protein modification by phospholipid peroxidation products in signal transduction regulation

The induction of ferroptosis is mediated by various enzymes involved in the biosynthesis and remodeling of PUFA-PEs in the cell membrane, including acyl-CoA synthetase long-chain family member 4 (ACSL4) and LPCAT3 (Wang and Tontonoz 2019). Recently, Doll et al. conducted a genome-wide CRISPR-based genetic screen and microarray analysis of ferroptosis-resistant cell lines revealing that ACSL4 serves as a critical component for ferroptosis sensitivity by shaping cellular phospholipid composition and further confirming that ox-PEs are responsible for the execution of driving ferroptosis (Doll et al. 2017). Additionally, inhibiting LPCAT3, another critical enzyme involved in phospholipid remodeling, has been shown to protect cells from phospholipid peroxidation-induced ferroptotic cell death by counteracting changes in different classes of PUFA-PEs (Reed et al. 2022). Moreover, iPLA$_2\beta$ has been reported to preferentially hydrolyze peroxidized phospholipids and eliminate the ferroptotic 15-HpETE-PE death signal (Sun et al. 2021). Collectively, these findings suggest the crucial role of ox-PEs produced by phospholipid peroxidation in conducting the ferroptosis signal.

Although ox-PEs have been identified as the primary lipid signal to trigger ferroptosis, additional evidence suggests that oxidized phosphatidylcholines (ox-PCs) also act as the cellular ferroptosis mediator. It has been reported that myocardial ischemia-reperfusion (I/R) injury exacerbates the generation of ox-PC, which has been shown to disrupt mitochondrial bioenergetics and calcium transients,

leading to ferroptosis in cardiomyocytes. The neutralization of ox-PCs by monoclonal antibody EO6 that specifically binds to ox-PCs or the ferroptosis inhibitor ferrostatin-1 prevents cell death during reperfusion (Stamenkovic et al. 2021). By the oxidative lipidomics analysis of BMDMs during apoptosis, necroptosis, ferroptosis, and pyroptosis, Wiernicki et al. found that ox-PS and ox-PI, in addition to ox-PE, are the predominant phospholipid peroxidation products in ferroptotic BMDMs (Wiernicki et al. 2020), suggesting their potential roles in conducting the ferroptosis signal.

While a marked increase in ox-PLs is observed at the onset of ferroptosis, and phospholipid peroxidation is implicated in the propagation of cell swelling and ferroptotic cell death (Riegman et al. 2020), the precise mechanisms by which ox-PLs induce ferroptosis require further elucidation.

19.5.2 The Role of Phospholipid Peroxidation in the Autophagy Inhibition

Phospholipid peroxidation is involved in several vital metabolic processes in addition to its role as the lipid death signal in driving ferroptosis. One of these processes is autophagy, which is a highly conserved mechanism for the lysosomal degradation of dysfunctional or unnecessary cellular components. Autophagy is regulated by a set of core autophagy-associated proteins (ATGs), including LC3, which is essential in autophagosome formation. Initially, LC3 is processed by ATG4B in the cytoplasm to form LC3-I. Subsequently, LC3-I binds to PE to create the LC3-PE conjugate (LC3-II), which is recruited to the autophagosome membrane to induce autophagy (Tanida et al. 2008). Recent studies have uncovered evidence of the involvement of phospholipid peroxidation in the inhibition of autophagy. Through functional and oxidative lipidomics analysis, Li et al. showed that phospholipid peroxidation induced by GPX4 inhibition or ALOX15 overexpression inhibited autophagy via stimulating the ATG4B-mediated delipidation of oxidized LC3-PE (Li et al. 2022a) (Fig. 19.5b).

19.5.3 The Role of Phospholipid Peroxidation in Phagocytic Signaling

During apoptosis, the exposure of PS serves as an “eat-me” signal to macrophages for the clearance of apoptotic cells (Segawa and Nagata 2015; Fadok et al. 1992). Normally, PS is restricted to the cytoplasmic leaflets of the plasma membrane via a “flipping” mechanism, but apoptosis induces “scrambling,” causing rapid PS exposure on the cell surface, where it is recognized by macrophage receptors, such as CD36 and milk fat globule epidermal growth factor 8 (MFG-E8) (Tait and Smith 1999; Hanayama et al. 2002). Although natural PS is considered the primary ligand recognized by macrophages on apoptotic cells, it has been well-established that oxidized PS (ox-PS) may also serve as an “eat-me” signal during apoptosis (Kagan

et al. 2002; Matsura et al. 2005) (Fig. 19.5c). Recently, through ligand fishing, lipid blotting, and cellular heat transfer assay screening, Luo et al. have identified SAPE-OOH as a key "eat-me" signal on the surface of ferroptotic cell, which promotes its phagocytosis by macrophages through directly binding to TLR2 (Luo et al. 2021) (Fig. 19.5c). However, since ox-PE is a lipid signal that drives ferroptosis, it was also demonstrated in this study that ox-PE is a novel "eat-me" signal that promotes ferroptotic cell clearance by macrophages. Nevertheless, it remains to be further investigated whether ox-PE is involved in phagocytic signaling during late ferroptosis.

19.5.4 The Role of Protein Modification by Phospholipid Peroxidation Products in Signal Transduction Regulation

The process of phospholipid peroxidation generates various oxidation products, including MDA and 4-HNE, with the latter being the most toxic (Catalá 2009). 4-HNE has a preference for modifying certain protein amino acids, such as Cys, His, and Lys, through the formation of covalent adducts with protein nucleophilic side chains, ultimately impacting protein function and disease progression (Castro et al. 2017). The irreversible modification of proteins by reactive lipid aldehydes may be one of the main results of oxidative damage that accompanies neurodegenerative diseases, metabolic diseases, and aging (Castro et al. 2017; Li et al. 2022b). For instance, Tu et al. and Sun et al. observed varying degrees of accumulation of 4-HNE-modified proteins in mouse models of both amyotrophic lateral sclerosis and Parkinson's disease (Sun et al. 2021; Tu et al. 2023). Other researchers have reported strong modifications of histones by 4-HNE in adipose tissue of obese mice, both in vitro and in vivo (Hauck et al. 2020). Additionally, an age-associated decrease in proteasome activity has been shown to be positively associated with increased modification of proteins by the lipid aldehyde 4-HNE (Mihalas et al. 2018). The modification of proteins by 4-HNE leads to changes in protein function, which, in turn, results in alterations in cellular signaling. Jia et al. demonstrated that inactivation of GPX4 and the consequent increase in 4-HNE accumulation lead to the carbonylation of amino acids at C88 of the stimulator-of-interferon genes (STING), inhibiting its transport from the endoplasmic reticulum (ER) to the Golgi complex, thereby attenuating the STING DNA sensing pathway and impacting the innate immune process (Jia et al. 2020) (Fig. 19.5d). Similarly, Niu et al. reported that the phospholipid peroxidation end product 4-HNE forms adducts with the chondrogenic transcription factor SOX9, promoting its ubiquitin-dependent degradation, consequently impairing embryonic bone development (Niu et al. 2022).

19.6 Phospholipid Peroxidation and Diseases

Apart from the physiological functions of phospholipid peroxidation in cell signal transduction described above, phospholipid peroxidation has been implicated in the pathological processes of various diseases. Gaining an understanding of the role of phospholipid peroxidation in the progression of diseases could provide a foundation for developing prevention and treatment strategies, as well as identifying and developing targeted drugs.

19.6.1 Neurodegenerative Diseases

In recent years, studies have suggested that ferroptosis driven by membrane lipid peroxidation may be one of the mechanisms of progressive neuronal loss (Jiang et al. 2021; Masaldan et al. 2019). The main features of ferroptosis are iron load and accumulation of lipid peroxides, which are highly consistent with the molecular biology of the brain in clinical neurodegenerative disease patients. The lipid content of the mammalian brain is higher than that of other organs, accounting for 50–60% of brain dry weight (Yoon et al. 2022). The abundant bisallyl structures in brain PUFA-PLs are highly sensitive to free radical oxidation, explaining why the brain is prone to phospholipid peroxidation to generate primary, secondary, and final oxidation products and toxic protein covalently modified complexes (Frankel 1980). In addition, the uninterrupted work of the brain also requires a large amount of oxygen supply, which consumes about 20% of the oxygen in the breath, while at the same time, the ratio of membrane surface area to cytoplasmic volume is relatively higher, not to mention abundant redox transition metals (Halliwell 1992), and the antioxidant system is relatively weaker (Evans 1993). Therefore, oxidative damage and neuroinflammation caused by phospholipid peroxidation are intrinsic molecular mechanisms that cannot be underestimated in the occurrence and development of neurodegenerative diseases.

19.6.1.1 Alzheimer's Disease (AD)

AD is a neurodegenerative disease with a relatively high incidence rate, with insidious onset and slow progression. The clinical manifestations are memory loss, and progressive cognitive decline, accompanied by various mental behavioral abnormalities and personality changes, which seriously affect the quality of life of patients (Scheltens et al. 2016). The main pathological and diagnostic feature of AD is the accumulation of two proteins: amyloid beta peptide (Aβ), which aggregates into extracellular plaques, and hyperphosphorylated tau, which forms intracellular neurofibrillary tangles. Amyloid precursor protein in the transmembrane region is cleaved to generate Aβ, which can form cytotoxic oligomers that assemble into amyloid fibrils (Mattson 2004), thereby assembling into amyloid fibrils, which can specifically bind to the cell membrane phospholipid layer with relatively high affinity. Phospholipids are amphipathic and consist of two hydrophobic fatty acids bonded to carbon atoms in glycerol. Thus, the phospholipid bilayer provides an

extensive surface for amyloid interactions, and the composition of membrane phospholipids, such as the head or fatty acid chains, as well as the redox state of the fatty acid chains, can alter membrane-amyloid interactions and toxicity (Williams and Serpell 2011).

At present, the pathogenesis of AD is not very clear, and there are no effective means to prevent the occurrence of the disease or delay its progress of the disease. Recently, increasing evidence has shown that lipid peroxidation is one of the important factors in the pathogenesis of AD. Postmortem studies of brain regions have shown marked glutathione depletion and accumulation of lipid peroxidation in the brains of AD patients (Selkoe 2001). In addition, the high iron and oxidative stress environment in the AD brain can also promote the occurrence of phospholipid peroxidation. High concentrations of free 4-HNE and its protein complexes can be detected in brain regions (such as the amygdala, hippocampus, parahippocampal gyrus) and cerebrospinal fluid of AD patients (Sayre et al. 1997). Among them, many proteins related to energy metabolism and antioxidation can be targeted and interfered with by 4-HNE, causing the disorder of important cell functions, and then leading to the death of neuronal cells (Di Domenico et al. 2017). In addition, data have shown that MDA levels in the brains of AD patients are significantly higher than in healthy subjects (Dare et al. 2020). MDA that accumulates in the brain can covalently bind to a variety of proteins and promote the formation of abnormal protein adducts that severely disrupt the function of multiple brain regions, such as the frontal, temporal, occipital, and hippocampal structures as well as the nerve signals (Lopez-Riquelme et al. 2016).

NRF2 is a transcription factor and master regulator of the cellular antioxidant defense system. Increasing the expression of NRF2 in the pathological process of AD can reduce Aβ-related oxidative stress, thereby delaying the pathological process of AD (Kanninen et al. 2008), whereas loss or mutation of NRF2 leads to memory and impairment and worsening of Aβ pathology (Bahn and Jo 2019). In addition, the study found that *Gpx4*-specific knockout mice in the cerebral cortex and hippocampus exhibited cognitive decline and degeneration of hippocampal neurons in the water maze test. After being given a diet rich in vitamin E or a ferroptosis inhibitor such as liproxstatin-1, the degeneration of neurons in mice was significantly alleviated, suggesting that phospholipid peroxidation played an important role in the process of AD (Hambright et al. 2017). At present, increasing studies have proved the therapeutic effect of ferroptosis inhibitors such as iron chelators, dexmedetomidine, and antioxidants on AD (Jakaria et al. 2021).

19.6.1.2 Parkinson's Disease (PD)

PD is also a progressive neurodegenerative disease, clinically manifested as uncontrollable tremors, slow movements, muscle stiffness, and postural instability caused by dopamine (DA) deficiency, and pathologically manifested as loss of dopaminergic neurons in the substantia nigra, DA content decreases and α-synuclein (α-Syn) accumulates to form Lewy bodies (Reich and Savitt 2019). Current studies suggest that apoptosis, necroptosis, and autophagic death are all involved in the degenerative loss of dopaminergic neurons (Moujalled et al. 2021). Studies have found that these

forms of cell death share a common feature, that is, lipid peroxidation damage of dopaminergic neuron cells. The latest research proposes that ferroptosis is a neuron death mode closely related to lipid peroxidation in the development of PD (Sun et al. 2021). The main characteristics of ferroptosis are a load of iron and the accumulation of a large number of lipid peroxides, which are highly consistent with the molecular biological characteristics of brain changes in clinical Parkinson's patients. Previous studies have found that increased levels of lipid peroxidation products (Lim et al. 2019) and abnormally elevated iron concentrations (Dexter et al. 1987) were observed in the substantia nigra of PD patients. Although the pathological feature of lipid peroxidation has been widely recognized, the detailed molecular mechanism has not been well resolved, and the drugs that simply remove peroxides have not made optimistic progress in clinical trials.

Our research group also used multiple models to prove that phospholipid peroxidation increases the susceptibility to PD. First, we found the accumulation of 4-HNE, MDA, and phospholipid oxidation products in the classic PD model *SNCA*^{A53T} transgenic mice and 6-OHDA rat models (Sun et al. 2021). The burden of lipid peroxidation has been shown to significantly increase the susceptibility of animal models to PD, and the lipid peroxidation inhibitor trolox can significantly alleviate Parkinsonism behavioral disorders (Jiang et al. 2020). In addition, the analysis results of ox-PLs showed that the key proteins of phospholipid remodeling, such as GPX4 and iPLA$_2\beta$, are closely related to the occurrence and development of PD. The deletion or mutation of these proteins can directly cause the accumulation of ox-PLs in the cell membrane, thereby triggering the development of dopaminergic neurons, leading to neuroinflammation and neurodegeneration (Sun et al. 2021). Taken together, phospholipids and their oxidation products mediated-oxidative stress and inflammation play an important role in the pathogenesis of PD, and strategies targeting ferroptosis inhibition have also been reported as potential directions for the development of anti-PD drugs.

19.6.1.3 Amyotrophic Lateral Sclerosis (ALS)

ALS is the most common motor neuron disease characterized by the progressive degeneration of motor neurons in the brain and spinal cord, leading to muscle wasting, paralysis, and death (van Es et al. 2017). The pathogenesis of ALS is associated with excitotoxicity, mitochondrial dysfunction, endoplasmic reticulum stress, neuroinflammation, and oxidative stress (Pasinelli and Brown 2006; Paez-Colasante et al. 2015; Cook and Petrucelli 2019). Accumulating evidence has implicated phospholipid peroxidation and its derived ferroptosis in ALS. For example, GPX4, the known phospholipid hydroperoxides specifically catalyzing the reduction of phospholipid peroxides, was found to be an essential enzyme for the health and survival of motor neurons (Chen et al. 2015; Yant et al. 2003). Conditional ablation of *Gpx4* in adult mouse neurons triggers rapid degeneration of spinal motor neurons and accelerates paralysis in ALS mice (Chen et al. 2015). Moreover, motor neuron degeneration induced by *Gpx4* ablation was also characterized by iron enrichment (Chen et al. 2015). The rapid death of motor neurons induced by *Gpx4*

ablation suggests that spinal motor neurons are particularly susceptible to ferroptosis if the anti-ferroptotic defense system is compromised.

The study also found elevated lipid peroxidation in cerebrospinal fluid and plasma of ALS patients (Simpson et al. 2004). An in vitro experiment demonstrated that treatment of motor neuron cells with highly oxidized polyunsaturated fatty acids increased cellular lipid peroxidation and exacerbated motor neuron death due to mitochondrial dysfunction. Improving the antioxidant function of mitochondria can protect motor neurons, suggesting that blocking oxidative stress may be helpful for the clinical treatment of ALS (Liu et al. 2002). Southon et al. found that lipid peroxidation is involved in the pathogenesis of ALS, and reducing lipid peroxidation can improve neuronal degeneration in ALS model mice by inhibiting ferroptosis and delaying disease progression (Southon et al. 2020). Our research group found that the expression of phospholipid peroxide scavenging enzyme GPX4 decreased in the lumbar spinal cord of ALS model mice. Supplementing the expression of *Gpx4* by intrathecal injection of *Gpx4*-AAV can significantly alleviate the symptoms and disease progression of ALS (Tu et al. 2023). In addition, Wang et al. also found depletion of GPX4 in the spinal cord of both sporadic and familial ALS patients, which is commonly found in transgenic animal models of ALS (including superoxide dismutase 1 mutation ($SOD1^{G93A}$), TDP-43, and C9orf72 transgenic mice) early characterization of the spinal cord and brain. In conclusion, the current study reveals that ferroptosis mediates the death of motor neurons in ALS (Wang et al. 2022). All these findings highlight the anti-ferroptosis therapeutic strategies, such as targeting phospholipid peroxide clearance pathways such as GPX4 activation, may be the key to alleviating the loss of spinal cord motor neurons and a new direction in the treatment of ALS.

19.6.1.4 Multiple Sclerosis (MS)

MS is a neuroinflammatory disease characterized by central nervous system demyelination and focal lesions (Compston and Coles 2008). The diagnosis of MS depends on typical clinical symptoms and supportive laboratory and radiological examination, and the etiology is not clear. The etiology of MS is currently thought to be complex and diverse, possibly including immunodeficiency and lipotoxicity due to genetic and environmental causes (Compston and Coles 2008). Haider et al. found that oxidized lipids and DNA were highly enriched in sclerotic plaques of MS patients. MDA and oxidized phospholipid epitopes were observed in the cytoplasm of oligodendrocytes and astrocytes. Axon spheroids and neurons in gray matter lesions also have a large amount of oxidized phospholipid accumulation, with signs of degeneration, indicating that the production of ox-PLs is closely related to the damage of multiple sclerosis axons or neurons (Haider et al. 2011). Western blot analysis with EO6 mAb confirms the presence of ox-PC in the brains of MS patients, which is considered a marker of neuroinflammation in MS lesions (Qin et al. 2007). This evidence suggests that phospholipid peroxidation may be a key factor in increasing susceptibility to MS.

In addition, iron homeostasis is important to maintain phospholipid peroxidation levels. Excess iron will catalyze the Fenton reaction to generate ROS, which will

mediate a chain reaction to generate PLOOH. At the same time, iron-dependent enzymes, such as LOXs that use iron as the basic catalytic center, also catalyze the production of PLOOH and trigger ferroptosis (Kagan et al. 2017). Studies have shown that iron deposits were found in MS brains, and biochemical changes and pathological features of ferroptosis were found in MS animal model experimental autoimmune encephalomyelitis (EAE) mice, suggesting that ferroptosis may be associated with MS (Hametner et al. 2013). Luoqian et al. found that ACSL4-mediated ferroptosis can activate T cells in the central nervous system in the EAE mouse model while inhibiting ACSL4 or ferroptosis can effectively reduce the neurological damage in mice (Luoqian et al. 2022). Histone methyltransferase G9a represses the expression of anti-ferroptosis genes and reduces intracellular glutathione levels, thereby exacerbating oxidative stress in neurons and inducing neuronal ferroptosis (Rothammer et al. 2022). Hu et al. found that in the spinal cord neurons of EAE model mice, both *Gpx4* mRNA and protein expression were reduced. Increased lipid peroxidation products and decreased levels of polyunsaturated fatty acids and markers of ferroptosis, such as characteristic damage to mitochondria, appeared in the spinal cord of EAE animal models (Hu et al. 2019). These findings provide the basis for the potential application of ferroptosis inhibitors or GPX4 inducers/activators in the treatment of inflammatory demyelinating diseases such as MS.

19.6.2 Immune Function and Tumor

Phospholipid peroxidation also plays a crucial role in determining the survival and function of various cell types, including macrophages, neutrophils, T cells, and B cells, under different pathological conditions. Elevated levels of ROS are generated in response to physical, chemical, and pathogenic stimuli that activate inflammatory cells and induce oxidation (Coussens and Werb 2002; Hussain et al. 2003). ROS-mediated oxidation of phospholipids results in the formation of peroxidation products that can modulate immunity by modifying DNA bases and sustaining a lipid-dependent inflammatory cascade (Chung et al. 1996; Clemente et al. 2020). Ferroptotic cell death tumor cells release $KRAS^{G12D}$ protein, leading to the transformation of macrophages into an M2-like tumor-promoting phenotype through STAT3-dependent fatty acid oxidation (Dai et al. 2020a). Meanwhile, the induction of phospholipid peroxidation by high-iron diets or *Gpx4* depletion in KRAS-driven pancreatic ductal adenocarcinoma evokes the release of oxidized nucleobase 8-OHG and thus activates tumor-associated macrophages infiltration and M2 polarization, which results in promoting the pancreatic tumorigenesis (Dai et al. 2020b).

It is worth noted the accumulation of lipid hydroperoxides in the tumor microenvironment macrophages also can evoke tumor progression (Su et al. 2020; Luo et al. 2020). Interestingly, compared with M2 macrophages, M1 macrophages possess higher levels of iNOS, and the nitric oxide produced by iNOS can interact with lipid intermediates generated by 15-LOX to prevent the lipid peroxidation process, which makes them more resistant to phospholipid peroxidation (Kapralov et al. 2020). M1 macrophages with high iNOS expression readily spread and exert long-range

protection against ferroptotic cell death. However, the high expression of iNOS will produce resistance to cisplatin-induced tumor cell apoptosis, and promote tumor proliferation and metastasis (Kielbik et al. 2019). Other studies have found that repressing the iPLA$_2\beta$ can promote the polarization of macrophage toward the M2 phenotype, leading to the accumulation of inflammation (Ashley et al. 2016). All these findings mentioned above indicated that phospholipid peroxidation accumulated in macrophage influences its polarization, contributing to tumorigenesis and progression.

An interesting finding revealed that cytotoxic CD8$^+$ T cells released IFN-γ activates ACSL4 which converts AA to AA-CoA. The insertion of AA-CoA into the C16 and C18 acyl chain-containing phospholipids in the plasma membrane changed the phospholipids composition of tumor cells, thereby causing phospholipids peroxidation and leading to tumor cell death (Liao et al. 2022). Besides, the released IFN-γ from cytotoxic CD8$^+$ T cells repressed the activation of system x_c-, accumulated the phospholipid peroxidation, and resulted in sensitizing hepatocellular carcinoma cells to ferroptosis (Kong et al. 2021). Exhausted CD8$^+$ T cells decrease cytotoxicity due to the accumulation of long-chain fatty acids or cholesterol. For example, the uptake of Ox-LDL by CD8$^+$ T cells through CD36 will cause the loss of immune effector function due to lipid peroxidation (Xu et al. 2021). The overexpression of *GPX4*, a pivotal gene in the clearance of lipid peroxidation, can promote T cell function (Xu et al. 2021). Phospholipid peroxidation also affects the function of other immune cells. 4-HNE, as a main end product of phospholipids peroxidation, was found to inhibit the phagocytic function of neutrophils via the modification of specific proteins (Chacko et al. 2016). Phospholipid peroxidation can negatively feedback regulate the maturation of dendritic cells in the inflammatory response (Blüml et al. 2005), and affect the antigen cross-presentation of dendritic cells (Ramakrishnan et al. 2014).

19.7 Conclusion

Phospholipid peroxidation is a crucial process that induces ferroptosis. This process also plays important roles in cellular signal transduction and has been implicated in various diseases, including neurodegenerative diseases and cancer. Thus, targeting phospholipid peroxidation holds promise as a novel therapeutic strategy for these diseases. However, several critical questions remain unanswered. First, the specific cellular membranes that are relevant to phospholipid peroxidation-induced cell death have yet to be fully elucidated. Second, the precise mechanism by which phospholipid peroxidation-induced ferroptotic cell death is still unclear. Third, the threshold of cell death induced by lipid peroxidation remains to be determined, although the degree of phospholipid peroxidation accumulation is related to the occurrence of ferroptosis. Finally, a precise molecular understanding of the relationship between phospholipid peroxidation and various diseases requires further exploration. Addressing these questions will enhance our knowledge of phospholipid peroxidation and its impact on health. This understanding may provide the foundation for

developing prevention and treatment strategies, as well as identifying and developing targeted drugs that modulate phospholipid peroxidation.

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PKCβII–ACSL4 Axis Triggers Ferroptosis and Its Potential Implication in Ferroptosis-Related Diseases

20

Yi-Qing Guo, Hai-Liang Zhang, Rong Deng, and Xiao-Feng Zhu

Abstract

Ferroptosis is a regulated cell death process independent of apoptosis, triggered by excessive peroxidation of polyunsaturated fatty acids (PUFAs) in the presence of iron. This mode of cell death is closely linked to various pathological conditions, such as cancers, neurodegenerative diseases, cardiovascular diseases, and ischemia/reperfusion injury. The enzymes ACSL4 and LPCAT3 play crucial roles in incorporating free PUFAs into cellular membranes. ACSL4 facilitates the conversion of long-chain fatty acids to their active form called acyl-CoA, enabling lipid synthesis, while LPCAT3 promotes the subsequent integration of these fatty acids into phosphatidylethanolamine, a vital component of cell membranes. We recently uncovered a lipid peroxidation–PKCβII–ACSL4 positive-feedback loop that drives ferroptosis, particularly in the context of cancer immunotherapy. We observed that increased phosphorylation of ACSL4 at Thr328 during ferroptosis may serve as a potential biomarker for identifying ferroptosis in different physiological and pathological contexts. In this chapter, we delve into the significance of the PKCβII–ACSL4 axis in ferroptosis and its relevance to related diseases.

Distinct from apoptosis, ferroptosis is a recently identified form of cell death that is primarily driven by excessive peroxidation of polyunsaturated fatty acids (PUFAs) in an iron-catalyzed manner (Dixon et al. 2012). Ferroptosis is characterized by the accumulation of reactive oxygen species (ROS) and can potentially be prevented

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through the use of iron chelators, antioxidants, or genetic ablation of iron uptake (Yang and Stockwell 2008; Yagoda et al. 2007). As a regulatory cell death mechanism, ferroptosis is classified separately from other known forms of regulatory cell death based on genetic, biochemical, and morphological distinctions, according to the Nomenclature Committee on Cell Death (NCCD) (Tang et al. 2019). Distinct morphological characteristics of ferroptosis can be observed using transmission electron microscopy, including shrunken mitochondria with decreased crista, increased membrane density, and membrane rupture (Dixon et al. 2012; Friedmann Angeli et al. 2014). Biochemical features such as iron and ROS accumulation, decreased cystine uptake, glutathione (GSH) exhaustion, and excessive lipid peroxidation have been identified in ferroptosis (Zheng and Conrad 2020; Xie et al. 2016). Unlike inhibitors that effectively prevent apoptosis, necrosis, and autophagic cell death, traditional inhibitors have shown limited success in impeding ferroptosis, indicating that ferroptosis follows a distinct pathway (Dixon et al. 2012). However, it should be noted that recent studies suggest a potential link between autophagy and ferroptosis, indicating that autophagy can promote ferroptosis in certain circumstances (Chen et al. 2023a). Ferroptosis is regulated by complex intracellular metabolic events and multiple signaling pathways (Chen et al. 2021c). It has been demonstrated to play a significant role in various pathological contexts, including cancers, neurodegenerative diseases, cardiovascular diseases, and ischemia/reperfusion injury (IRI) (Pefanis et al. 2019; Reichert et al. 2020; Chen et al. 2021b; Jiang et al. 2021). Consequently, targeting ferroptosis holds promise as a strategy for managing these life-threatening diseases.

The term "ferroptosis" was coined in 2012 by the research group led by Brent R. Stockwell. They observed a distinct form of cell death when treating cells with erastin, a selective lethal compound that targets RAS-mutant tumors. Since many tumors rely heavily on iron for their progression (Torti et al. 2018), accumulating evidence suggests that ferroptosis plays a crucial role in suppressing tumor growth. Therefore, ferroptosis is considered a potential vulnerability, or Achilles' heel, in cancer. Numerous ferroptosis inducers (FINs), including experimental compounds and clinical drugs, have been shown to combat cancer by suppressing tumorigenesis and metastasis through the induction of ferroptosis. Excessive lipid peroxidation, a hallmark of ferroptosis, results in the breakdown of membrane integrity and subsequent cell death. Among various fatty acids (FAs), polyunsaturated fatty acids (PUFAs) have been identified as the most susceptible lipid species to peroxidation and are crucial for the execution of ferroptosis when incorporated into membrane phospholipids (Stockwell 2022).

Fatty acids (FAs) can be acquired either directly from the surrounding environment or through de novo synthesis. Exogenous FAs are obtained by a group of transporters, including the fatty acid transport protein family (FATPs, also known as solute carrier protein family 27 or SLC27), CD36 (also referred to as fatty acid translocase or FAT), and plasma membrane fatty acid-binding proteins (FABP) (Su and Abumrad 2009). In terms of de novo lipogenesis, cells utilize carbon sources such as glucose, glutamine, and acetate to synthesize citrate. ATP-citrate lyase (ACLY) then converts citrate into acetyl-CoA. Acetyl-CoA carboxylases (ACCs)

irreversibly carboxylate acetyl-CoA into malonyl-CoA, which serves as the substrate for fatty acid synthesis. Fatty acid synthase catalyzes the condensation of seven malonyl-CoA molecules and one acetyl-CoA molecule, ultimately generating palmitate, a saturated 16-carbon FA. Palmitate serves as the building block for various FAs as it can undergo desaturation and elongation through the enzymatic activities of stearoyl-CoA desaturase-1 (SCD1), fatty acid desaturase 2 (FADS2), and elongation of very long-chain fatty acid protein (ELOVLs) (Kuhajda et al. 1994; Currie et al. 2013). Alternatively, excess FAs are stored in cytoplasmic organelles called lipid droplets (LDs) in the form of triacylglycerides (TAGs) and sterol esters (Olzmann and Carvalho 2019). LDs help maintain lipid homeostasis and provide acetyl-CoA and NADPH through β-oxidation when cells experience metabolic stress.

FAs are a rich source of energy and can generate ATP through fatty acid oxidation (FAO), also known as β-oxidation (Lehner and Quiroga 2016; Bian et al. 2021). Firstly, FAs are activated in the cytosol by fatty acyl-CoA synthetases (ACSs) to form fatty acyl-CoA. To support FAO in the mitochondria, fatty acyl-CoA needs to be transported into the mitochondrial matrix. In the outer mitochondrial membrane, fatty acyl-CoA is converted to fatty acylcarnitine by carnitine palmitoyltransferase 1 (CPT1) and then transferred to the inner mitochondrial membrane by carnitine/acylcarnitine translocase (CACT). On the matrix side of the inner membrane, acyl-CoA is converted back from acylcarnitine and subsequently undergoes a series of four sequential enzymatic steps for breakdown. The enzymes involved in β-oxidation include acyl-CoA dehydrogenase, enoyl-CoA hydratase, L-3-hydroxyacyl-CoA dehydrogenase, and β-ketothiolase. This four-step cycle is repeated until all the carbon atoms of the fatty acyl-CoA molecule are converted into acetyl-CoA. Energy is generated from the breakdown products of FAO. Acetyl-CoA enters the tricarboxylic acid (TCA) cycle, and the other products, NADH and FADH2, serve as coenzymes in the electron transport chain to generate ATP. In addition to generating ATP, FAs are also involved in the synthesis of more complex lipids (Koundouros and Poulogiannis 2020).

In the context of ferroptosis, phosphatidylethanolamine (PE)-containing arachidonic acids (AA) and adrenic acids (AdA) act as preferred substrates for oxidation, fueling the execution of ferroptosis (Kagan et al. 2017). Mechanistically, the carbon–hydrogen (C–H) bonds of the bis-allylic methylene groups, found between conjugated dienes, are known to be the weakest among all C–H bonds. Due to the vulnerability of these specific C–H bonds, the bis-allylic hydrogen atom in polyunsaturated fatty acids (PUFAs), such as AA and AdA, is susceptible to abstraction. This leads to the formation of a peroxyl radical when in the presence of oxygen, particularly under conditions rich in iron and ROS (Lin et al. 2021). This process can occur through the Fenton reaction or enzymatic activities, such as lipoxygenases (LOXs), ultimately contributing to ferroptosis (Conrad and Pratt 2019; Yang et al. 2016). Enzymes involved in fatty acid metabolism have been shown to play a role in ferroptosis. For example, knockdown of stearoyl-CoA desaturase (SCD) inhibits lipid peroxidation and ferroptosis (Vriens et al. 2019; Liu et al. 2023). Inhibiting acetyl-CoA carboxylase (ACC) also confers resistance to

ferroptosis (Lee et al. 2020). Impairment of fatty acid oxidation (FAO) results in the accumulation of PUFAs, thereby promoting ferroptosis (Venkatesh et al. 2020; Nassar et al. 2020).

LOXs are non-heme iron-dependent enzymes that directly oxygenate PUFAs in cell membranes (Kuhn et al. 2015). Among PUFAs, AA and linoleic acid (LA) are the most common substrates for LOXs (Gaschler and Stockwell 2017). Inhibition or deletion of ALOX12 or ALOX15 impedes ferroptosis in various pathological contexts, including neurodegenerative diseases and cancers (van Leyen et al. 2006; Shah et al. 2018). It is worth noting that LOXs may play a more prominent role in specific circumstances (Kuang et al. 2021). ALOX12 is essential in p53-mediated ferroptosis that occurs independently of ACSL4 (Chu et al. 2019). Phosphatidylethanolamine-binding protein 1 (PEBP1), a scaffold protein and inhibitor of protein kinase cascades, complexes with LOXs and enables ferroptosis (Wenzel et al. 2017). Additionally, cytochrome p450 oxidoreductase (POR) has been reported to induce ferroptosis by promoting lipid peroxidation as an electron donor (Ghosh et al. 1997; Zou et al. 2020).

Although lipid peroxidation is a well-established hallmark of ferroptosis, the precise mechanisms by which lipid peroxidation is sensed and triggers ferroptosis are not fully understood. In our study, we have elucidated a positive-feedback loop known as the lipid peroxidation-PKCβII-ACSL4 loop, which plays a critical role in driving ferroptosis. Mechanistically, PKCβII serves as a sensor of lipid peroxidation and becomes activated in response to elevated levels of lipid peroxides. Subsequently, PKCβII phosphorylates ACSL4 at Thr328, leading to the activation of ACSL4 through dimerization. This activation of ACSL4 further amplifies the lipid peroxidation process, thereby promoting ferroptosis. In our research, we aim to provide a comprehensive understanding of the contribution and underlying mechanisms of lipid metabolism in the regulation of ferroptosis across various contexts. Additionally, we propose that targeting the lipid peroxidation-PKCβII-ACSL4 amplification loop could be a potential therapeutic strategy for diseases associated with ferroptosis.

ACSL4, encoded by the ACSL4 gene, is located on human chromosome Xq23. It was initially reported and named in 1997 and found to be expressed in various tissues including the adrenal gland (where it is most abundant), brain, liver, and other tissues with low tissue specificity (Kang et al. 1997). ACSL4 is a single-pass type III membrane protein and is found in different subcellular locations, including the outer membrane of mitochondria, peroxisome membrane, endoplasmic reticulum, and cell membrane (Ohkuni et al. 2013; Xu et al. 2021). Structurally, ACSL4 consists of an AMP-dependent synthesis/ligase domain, and the absence of 50 amino acids in its N-terminus may contribute to its distinct preference for fatty acids compared to other members of its family (Fig. 20.1) (Kang et al. 1997). As an isozyme of the long-chain fatty acid-CoA ligase family, ACSL4 converts and activates free PUFAs, particularly arachidonic acid (AA) and eicosapentaenoic acid (EPA), by attaching CoA to them, forming acyl-CoA esters. These active acyl-CoA esters further contribute to membrane alterations and fatty acid metabolism, including lipid synthesis and degradation through β-oxidation (Ohkuni et al. 2013; Nakahara et al. 2012;

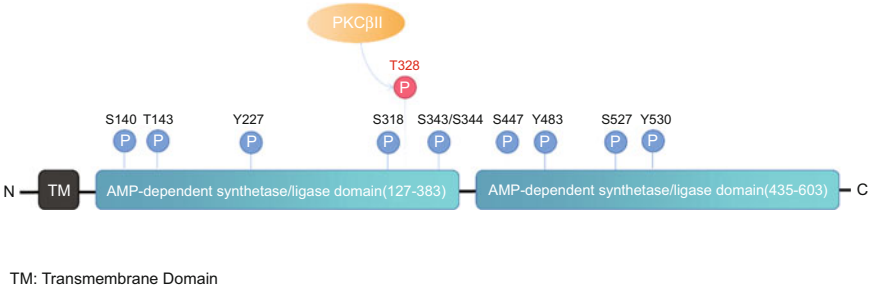

Fig. 20.1 Phosphorylation sites of ACSL4. Previous studies utilizing phosphoproteomics have identified several phosphorylation sites of ACSL4 (Smith et al. 2013; Yángüez et al. 2018; Osinalde et al. 2016; Beltrao et al. 2012; Olsen et al. 2010; Huang et al. 2017). However, the precise function and the kinases responsible for regulating these sites remain poorly understood. In our study, we aimed to address this knowledge gap. By mutating potential phosphorylation sites predicted by GPS and SCANSITE programs to non-phosphorylatable alanine (Ala), we successfully confirmed that PKCβII directly phosphorylates ACSL4 at Thr328. This phosphorylation event subsequently activates ACSL4 and plays a crucial role in fueling ferroptosis (Zhang et al. 2022)

Grevengoed et al. 2014). The absence of ACSL4 has been speculated to contribute to conditions such as Alport syndrome or mental retardation (Meloni et al. 2002). Furthermore, several lines of evidence indicate that ACSL4 expression is associated with carcinogenesis and aggressive phenotypes in colon adenocarcinoma, hormone-resistant prostate cancer, and breast cancer (Cao et al. 2001; Wu et al. 2015; Orlando et al. 2015).

Extensive research has clearly established the crucial role of ACSL4 in ferroptosis. Mechanistically, ACSL4 converts PUFA into PUFA-CoA, which is then incorporated into membrane phospholipids by LPCAT3, thereby fueling the execution of ferroptosis (Doll et al. 2017; Yuan et al. 2016; Lin et al. 2022). Additionally, ferroptosis is involved in cancer immunotherapy in an SLC7A11- or ACSL4-dependent manner. They also demonstrated that interferon (IFN)γ induces ACSL4 expression through the JAK/STAT1 signaling pathway, leading to increased incorporation of PUFA-CoA into membrane phospholipids and promoting ferroptosis (Liao et al. 2022; Wang et al. 2019).

In our study (Zhang et al. 2022), we employed two independent approaches: (1) a genome-wide CRISPR Cas9 screening, and (2) a screening of kinase inhibitors in combination with the ferroptosis inducer erastin. Interestingly, both approaches identified PKCβ as a potential uncharacterized pro-ferroptotic gene. The protein kinase C (PKC) family comprises a group of phospholipid-dependent Ser/Thr kinases, consisting of eight isoenzymes (PKCα, PKCβI, PKCβII, PKCγ, PKCδ, PKCε, PKCθ, and PKCη). These kinases have been implicated in a wide range of signal transduction pathways activated by hormones and growth factors, exerting their effects by phosphorylating target proteins (Kang et al. 2012; Mochly-Rosen et al. 2012). Evidence from several studies reveals an intimate correlation between PKCs and oxidative stress (Nakajima 2008; Gopalakrishna and Jaken 2000; Steinberg 2015).

We conducted experiments to demonstrate the impact of PKC inhibitors on lipid peroxidation and ferroptosis induced by ferroptosis inducers (FINs). Specifically, the PKC inhibitors Go6983 and enzastaurin significantly inhibited lipid peroxidation and ferroptosis, indicating their role in inhibiting PKC rather than functioning as radical-trapping antioxidants. As Go6983 and enzastaurin are pan-PKC inhibitors, we further investigated the specificity of PKC isoforms in relation to ferroptosis vulnerability. To accomplish this, we generated models with genetic knockout of each PKC isoform and observed that deletion of PKC isoforms other than PKCβ had minimal effect on ferroptosis susceptibility. Through in-depth analysis, we discovered that only PKCβII, and not PKCβI, modulated ferroptosis. By examining the correlation between basal PKCβII expression and susceptibility to ferroptosis in a panel of cancer cell lines, we confirmed that PKCβII universally contributes to the susceptibility of cancer cells to ferroptosis. Notably, cells with high levels of PKCβII expression were found to be more susceptible to ferroptosis compared to cells with low PKCβII expression, suggesting that PKCβII may serve as a potential contributor and biomarker of ferroptosis.

Furthermore, we made an intriguing discovery that PKCβII might function as a sensor of lipid peroxidation during ferroptosis. The activation of PKCβII was induced by FINs, promoting its phosphorylation and membrane localization. In contrast, lipid peroxidation scavengers such as ferrostatin-1 and liprostatin-1 completely inhibited PKCβII activation. To further investigate the role of PKCβII, we transfected PKCβII kinase-dead mutants into PKCβ-knockout cells, which did not increase lipid peroxidation or ferroptosis. This suggests that the function of PKCβII in ferroptosis is dependent on its kinase activity.

Based on our findings, we put forth a hypothesis that PKCβII modulates ferroptosis by influencing the generation or scavenging of lipid peroxidation products. Since PKCβII did not have an impact on the expression of GPX4 (glutathione peroxidase 4) or cellular divalent iron levels, we further investigated the relationship between PKCβII and ACSL4. Through immunoprecipitation experiments, we observed an enhanced interaction between ACSL4 and PKCβII during ferroptosis, which was abolished upon treatment with PKCβII inhibitors. To support our hypothesis that PKCβII promotes ferroptosis by phosphorylating and activating ACSL4, we finally confirmed that PKCβII directly phosphorylates ACSL4 at Thr328. Moreover, we provided compelling evidence that PKCβII-mediated ACSL4 phosphorylation at Thr328 is a prerequisite for ACSL4 activation, lipid peroxide accumulation, and the execution of ferroptosis. In this process, we uncovered an unrecognized positive-feedback loop, namely the lipid peroxidation-PKCβII-ACSL4 loop, which plays a significant role in ferroptosis (Fig. 20.2). It is important to note that ferroptosis is involved in various pathological contexts, including cancer, neurodegenerative diseases, and ischemia/reperfusion injury (IRI) (Pefanis et al. 2019; Reichert et al. 2020; Chen et al. 2021b; Jiang et al. 2021). In our study, we also observed an increase in ACSL4 Thr328 phosphorylation during IRI, suggesting that the phosphorylation level of ACSL4 at Thr328 could serve as a potential biomarker for identifying ferroptosis in different physiological or pathological contexts.

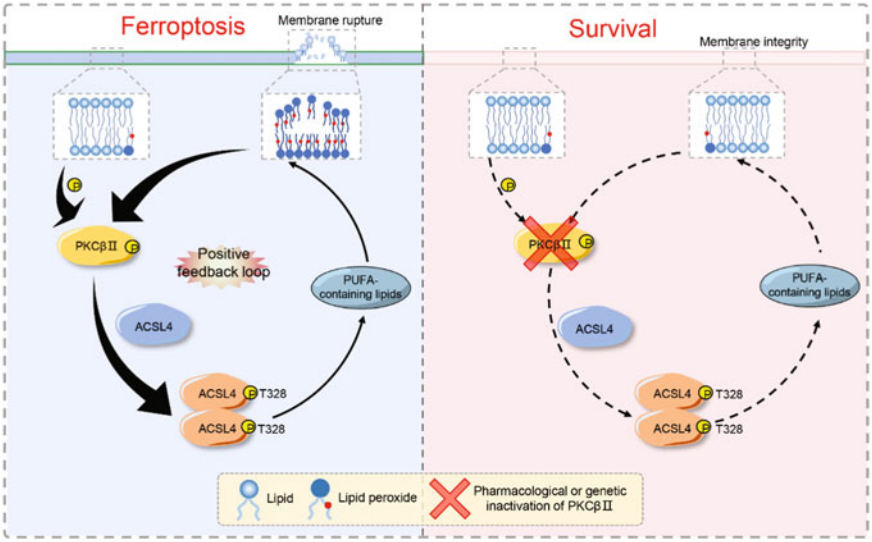

Fig. 20.2 Lipid peroxidation–PKCβII–ACSL4 positive feedback loop

Our study provides valuable insights into the execution process of ferroptosis by uncovering a self-enabling loop. Previous studies have predominantly considered ACSL4 as a passive participant in ferroptosis. However, our research demonstrates that ACSL4 plays a proactive role in driving ferroptosis through the lipid peroxidation-PKCβII-ACSL4 positive amplification loop. Nevertheless, our study raises several unanswered questions that require further investigation (Lei et al. 2022; Rodencal and Dixon 2022). Firstly, it remains unknown how PKCβII senses lipid peroxides and becomes activated. Additionally, it is unclear whether PKCβII can also sense other peroxides, such as PUFA-containing ether phospholipids. Importantly, we need to understand why the lipid peroxidation-PKCβII-ACSL4 positive-feedback cascade is necessary for ferroptosis. On one hand, we hypothesize that the inherent PUFA-containing phospholipids on the cell membrane may not be sufficient to generate lipid peroxidation to a lethal level. Therefore, we speculate that the lipid peroxidation-PKCβII-ACSL4 positive-feedback loop provides additional PUFA-containing phospholipid substrates, facilitating the propagation of lipid peroxidation throughout the cellular membranes and accelerating ferroptosis. Moreover, our study suggests that the lipid peroxidation-PKCβII-ACSL4 feedforward signal amplification loop may account for the "all-or-nothing" manner of ACSL4 in ferroptosis (Doll et al. 2017). In other words, even a limited amount of ACSL4 is sufficient to trigger ferroptosis in the presence of the lipid peroxidation-PKCβII-ACSL4 feedforward signal amplification loop. On the other hand, there is a competition at the membrane between processes that promote or counteract lipid peroxidation and ferroptosis. For instance, phospholipases prevent lipid peroxidation and ferroptosis by cleaving oxidized phospholipid acyl chains from the membrane (Beharier et al. 2020; Chen et al. 2021a; Sun et al. 2021), and ACSL3, another

isozyme in the ACSL family, retards ferroptosis by activating monounsaturated fatty acids (MUFAs) that can substitute PUFAs in membrane phospholipids (Magtanong et al. 2019; Ubellacker et al. 2020). Furthermore, membrane repair mediated by the endosomal sorting complexes required for transport (ESCRT) hinders the execution of ferroptosis by removing oxidatively damaged membrane components (Dai et al. 2020). The lipid peroxidation-PKCβII-ACSL4 feedforward signal amplification loop may serve to counteract these protective mechanisms mentioned above, highlighting its significance in the regulation of ferroptosis.

Ferroptosis has been shown to play a significant role in CD8+ T cell-mediated cancer immunotherapy. IFNγ derived from CD8+ T cells induces ferroptosis in cancer cells by downregulating SLC7A11, leading to glutathione depletion (Wang et al. 2019). Additionally, they demonstrated that IFNγ stimulates ACSL4 expression, further enhancing ferroptosis. They proposed a novel approach for natural ferroptosis induction using a combination of PUFAs and CD8+ T cell-derived IFNγ (Liao et al. 2022; Wang et al. 2019). Furthermore, ACSL4-deficient cancer cells exhibited impaired T cell-mediated anti-tumor immunity, highlighting the crucial role of ACSL4 in the effectiveness of cancer immunotherapy by regulating ferroptosis in cancer cells. In our study, we confirmed that the PKCβII-ACSL4 axis functions as a crucial regulator of ferroptosis in the context of cancer immunotherapy, acting as a steering mechanism. Consequently, the PKCβII-ACSL4 axis may serve as an indicator of tumor response to immunotherapy in clinical applications. Modulating the PKCβII-ACSL4 axis could be a promising strategy to enhance ferroptosis, ultimately eradicating cancers.

In addition to cancers, there is substantial evidence indicating that ferroptosis contributes to cell death associated with IRI and neurodegenerative diseases (Smith et al. 2013; Cui et al. 2021; Wang et al. 2022; Tang et al. 2021). Studies have demonstrated that inhibiting ferroptosis using iron chelators significantly blocks cardiomyocyte cell death (Fang et al. 2019). ACSL4-mediated ferroptosis exacerbates neuroinflammation and stroke caused by IRI (Cui et al. 2021). Notably, inhibiting ferroptosis remarkably mitigates acute kidney injury (Wang et al. 2022). Similarly, our study revealed an increase in ACSL4 Thr328 phosphorylation during IRI, suggesting that interfering with the PKCβII-ACSL4 axis is a promising strategy to alleviate ferroptosis-associated diseases, such as IRI and neurodegenerative diseases. Furthermore, Zhao et al. demonstrated that human hematopoietic stem cells (HSCs) are vulnerable to ferroptosis, contributing to bone marrow failure (Zhao et al. 2023). A recent study also revealed that Mycobacterium tuberculosis exploits ferroptosis in host cells to promote its spread and pathogenicity (Qiang et al. 2023). Based on these findings, we hypothesize that blocking ferroptosis by interfering with the PKCβII-ACSL4 axis may be a promising therapeutic approach for bone marrow failure and tuberculosis. However, further exploration is needed to determine appropriate therapeutic windows for PKCβII activators to ensure better clinical application and minimize potential side effects. Furthermore, the identification of specific substrates within the PKC family is crucial for comprehending the phosphorylation modifications involved in ferroptosis (Sun et al. 2015; Chen et al. 2023b).

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Cancer Treatment with Ferroptosis by a Combination of Iron Nanoparticles and Gene Therapy

21

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Abstract

Despite advancements in cancer treatment, the efficacy of current clinical therapies is still limited. Ferroptosis, an iron-dependent form of regulated cell death induced by excessive lipid peroxidation, has emerged as a potential avenue for the treatment of drug-resistant cancer cells. However, the resistance to ferroptosis still poses a challenge to the effectiveness of current ferroptosis-based cancer therapies. This chapter reviews the strategies and challenges associated with the use of iron oxide nanoparticles and gene therapy for cancer ferroptosis treatment. Based on this review, a novel safety combination therapy of iron oxide nanoparticles and gene therapy is proposed as a potential solution for overcoming ferroptosis resistance. Finally, the superiority and future research trends of combinatorial ferroptosis therapy are highlighted.

21.1 Introduction

Cancer is a major public health challenge and the second leading cause of death worldwide. Despite the considerable advances in cancer therapies such as surgery, radiotherapy, chemotherapy, targeted therapy, and immunotherapy, tumor resistance remains a significant limitation to their effectiveness. This is reflected by the high number of cancer deaths worldwide, which reached almost ten million in 2020 (Sung et al. 2021). The macroeconomic cost of cancer also remains substantial for the future (Chen et al. 2023).

Ferroptosis is a type of programmed cell death driven by excessive lipid peroxidation, which is controlled by integrated oxidation and antioxidant systems that are

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catalyzed by iron (Dixon et al. 2012). Interestingly, ferroptosis has been linked to resistance to cancer therapies, and inducing ferroptosis has been shown to reverse drug resistance (Tong et al. 2022; Chen et al. 2021a). Glutathione peroxidase 4 (GPX4) is a key regulator of ferroptosis. Viswanathan et al. found that vulnerability to ferroptosis induced by inhibition of the GPX4-regulated lipid peroxidase pathway was a common feature of therapy-resistant cancer cells across diverse mesenchymal cell-state contexts (Viswanathan et al. 2017). Additionally, nuclear factor erythroid 2-related factor 2 (NRF2), solute carrier family 7 member 11 (SLC7A11), and ferroptosis suppressor protein 1 (FSP1) also play vital roles in the antioxidant system of ferroptosis (Doll et al. 2019; Koppula et al. 2021; Dodson et al. 2019; Sun et al. 2016; Song et al. 2018). Iron dysregulation is another important factor that can trigger lipid peroxidation, as Fe^{2+} can cause cellular oxidative stress through the Fenton reaction. Genes involved in iron metabolism include ferroportin (FPN), lipocalin 2 (LCN2), ferritin heavy chain 1 (FTH1), and transferrin receptor (TFR1) (Basuli et al. 2017; Yao et al. 2021; Kong et al. 2021; Liu et al. 2021).

Recently, anticancer drugs based on ferroptosis induction have received significant attention, and various ferroptosis-inducing agents have been identified or developed, including small molecules and iron oxide nanoparticles (IONs)-based nanomaterials (Liang et al. 2019). Gene therapy targeting ferroptosis pathways also provides new possibilities for cancer treatment. However, cancer ferroptosis therapy still faces challenges, including side effects and ferroptosis resistance (Zhou et al. 2022; Yang et al. 2022). This chapter summarizes the efficacy and limitations of current IONs-based nanomedicine and gene therapy for cancer ferroptosis treatment. Furthermore, a safety combination therapy of IONs and gene therapy is proposed for overcoming ferroptosis resistance. Two generations of combination therapy using IONs and gene interference technology have been developed, namely GIFT (Gene Interfered-Ferroptosis Therapy) and FAST (Ferroptosis Assassinates Tumor), respectively (Gao et al. 2021b; Luo et al. 2022). FAST therapy exhibits efficient tumor eradication capacity and in vivo biosafety. Lastly, the advantages and future research trends of ferroptosis-based combination therapy are highlighted.

21.2 IONs-Induced Ferroptosis for Cancer

21.2.1 IONs Therapy

IONs, or iron oxide nanoparticles, are widely recognized as biocompatible and safe structures with unique physical properties. Over the years, significant advancements have been made in the development and adoption of IONs for various biomedical applications. These applications include tumor imaging using magnetic resonance imaging (MRI), hyperthermia, drug delivery, gene therapy, and biomolecule separation (Nowak-Jary and Machnicka 2022). Moreover, IONs, which are rich in trace element iron, have emerged as potent ferroptosis inducers (Wang et al. 2018b). Excessive accumulation of IONs within cells results in the release of iron ions, which subsequently trigger the Fenton reaction, leading to the production of reactive

oxygen species (ROS) and lipid peroxidation (Zheng et al. 2021). With a better understanding of the ferroptosis mechanism (Dixon et al. 2012), IONs have taken on a new role in anticancer strategies as non-canonical ferroptosis inducers (Hassannia et al. 2019). Gao et al. have synthesized ultrasmall superparamagnetic iron oxide nanoparticles (USPIO) capable of generating abundant toxic ROS intracellularly. These nanoparticles deplete glutathione (GSH) through lipid peroxidation, demonstrating their ability to induce ferroptosis in a time-dependent manner. It is worth noting that different-sized IONs exhibit varying antitumor effects (Gao et al. 2021a; Tian et al. 2022; Nowak-Jary and Machnicka 2022). Ultrasmall IONs (<5 nm) are particularly efficient in enhancing the toxicity of H_2O_2 due to their rapid release of Fe^{2+} (Tian et al. 2022). On the other hand, 10 nm IONs have been identified as the optimal size for tumor eradication, given their balanced abilities in tumor accumulation, intratumoral penetration, and cellular internalization (Tian et al. 2022). Consequently, the size of IONs is a crucial factor to consider in the development of IONs-based nanomedicine.

Pharmacokinetics, biodistribution, and in vivo biosafety are critical considerations in IONs-based ferroptosis therapy. The efficacy of IONs as a cancer therapeutic relies on their ability to specifically target tumors while minimizing toxicity to surrounding tissues and vital organs. The pharmacokinetics, biodistribution, and in vivo biosafety of IONs are influenced by their physicochemical properties, including size, shape, coating molecules, and surface charge. Therefore, it is important to conduct dedicated research on each designed nanostructure (Nowak-Jary and Machnicka 2022). The duration of IONs' circulation in the bloodstream serves as a crucial indicator of their pharmacokinetics, directly affecting the extent of nanomedicine accumulation in tumors. For instance, Lin et al. developed IONs with an extended blood circulation time, facilitating the accumulation of Fe^{2+} in tumors. This approach demonstrated an excellent therapeutic effect against tumors (Lin et al. 2022).

21.2.2 Challenges of IONs Therapy

While the intrinsic therapeutic potential of bare IONs has garnered significant interest, the issue of resistance to IONs remains a critical challenge in this field. For instance, Huang et al. developed zero-valent iron nanoparticles (ZVI NPs) that induced mitochondrial lipid peroxidation and reduced glutathione peroxidase levels within subcellular organelles (Huang et al. 2019). However, they observed that resistant cancer cells were capable of attenuating the oxidative stress, mitochondrial dysfunction, and subsequent ferroptosis induced by ZVI NPs. A recent report compared the bioeffects of IONs coated with different types of molecules, including bare IONs, humic acids (HA)-IONs, and 3-aminopropyltriethoxysilane (APTES)-IONs (Kicheeva et al. 2023). The study revealed that HA-IONs released the least amount of Fe^{2+} ions, whereas APTES-IONs released the most. Thus, chemical modification of the IONs' surface plays a crucial role in ferroptosis. Various coating molecules can be employed for the functionalization of IONs, including inorganic

compounds (such as silica, metals, and metal oxides), natural polymers, macromolecules, monolayers, and small molecules (Nowak-Jary and Machnicka 2022). The appropriate selection of chemical moieties on the surface of IONs can contribute to inducing ferroptosis in cancer cells. For example, researchers investigated the anticancer activity of IONs functionalized with gallic acid (GA) and polyacrylic acid (PAA) (Fernandez-Acosta et al. 2022). Their findings demonstrated that IONs-GA/PAA treatment efficiently induced ferroptosis in glioblastoma, neuroblastoma, and fibrosarcoma cells. In another study, Yu et al. synthesized polypyrrole (PPy)@IONs using a portable method and observed that PPy@IONs significantly promoted ferroptosis while inhibiting the growth and invasion of colorectal cancer cells (Yu et al. 2022).

However, surface chemical modification of IONs alone is insufficient to overcome nanomedicine resistance, particularly in refractory cancer cells. IONs-resistant cancer cells exhibit heightened mitochondrial respiration function and enhanced ROS detoxification, allowing them to evade ferroptosis (Huang et al. 2019). To address this limitation, the combination of IONs with existing therapies has been explored as a potential treatment modality for cancer, including chemotherapy (Liu et al. 2023; Zhu et al. 2022), radiotherapy (Zhang et al. 2022), photothermal therapy (PTT) (Yu et al. 2023; Luo et al. 2023), and gene therapy (Gao et al. 2021b). For instance, doxorubicin (Dox), a conventional chemotherapy drug, has been utilized in combination with IONs. Zhu et al. developed a novel nanoplatform, ipGdIO-Dox, for MRI-guided chemo- and ferroptosis synergistic cancer therapies through the incorporation of gadolinium-engineered magnetic iron oxide loaded with Dox (Zhu et al. 2022). Upon cellular internalization, ipGdIO-Dox released Dox and abundant Fe^{2+} following degradation of the nanoplatform. The released Fe^{2+} generated highly toxic ROS, resulting in a more potent anticancer effect through the synergistic theranostic system.

Another chemotherapy drug, sorafenib, which possesses ferroptosis-inducing properties, has also been investigated. Liu et al. reported Sorafenib@Fe^{III}TA nanoparticles that respond to the acidic environment of lysosomes, facilitating Sorafenib release to inhibit the GPX4 enzyme and initiate ferroptosis (Liu et al. 2018). The Sorafenib@Fe^{III}TA-mediated cell death significantly inhibited tumor proliferation in vivo.

Despite these advancements, it is important to recognize that the use of IONs alone may still face challenges in overcoming ultimate drug resistance due to the robust systems maintaining iron and redox homeostasis within cells. Therefore, the combination of IONs with other therapeutic modalities offers a promising approach to enhance the efficacy of cancer treatment and overcome resistance mechanisms.

21.3 Gene Therapy-Induced Ferroptosis for Cancer

21.3.1 Gene Therapy

In recent years, gene therapy has emerged as a promising approach for the treatment of various diseases by modifying and regulating gene expression. This field offers an alternative to conventional therapies that often face limitations such as chemotherapy resistance and the inability to address genetic defects. Globally, there have been at least 943 clinical trials conducted to explore the potential of gene therapy (Duan et al. 2021). Gene therapy encompasses three main aspects: RNA interference (RNAi) technology, gene replacement therapy, and gene editing-based therapy.

RNAi technology utilizes molecules such as antisense oligonucleotides (ASO), microRNAs (miRNA), small interfering RNAs (siRNA), and short hairpin RNAs (shRNA) to mediate gene silencing at the mRNA level (Xu et al. 2019). This approach holds the potential to selectively inhibit the expression of specific genes implicated in disease processes.

Gene replacement therapy involves delivering plasmid DNA or viral vectors into cells to achieve target gene overexpression (Sayed et al. 2022). By introducing these genetic constructs, transient or persistent production of proteins that are abnormal or deficient in diseased bodies can be promoted, thereby restoring proper cellular function.

Gene editing-based therapy employs engineered or bacterial nucleases to facilitate targeted modification of genomic sequences in eukaryotic organisms. Programmable nucleases, including zinc finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), and cluster regulatory interval short tandem repeat (CRISPR)/CRISPR-associated protein (Cas)-associated nucleases, enable precise genomic alterations (Lehmann et al. 2017).

Efficient gene delivery to target tissues is a crucial step in gene therapy, and it is accomplished through the use of gene delivery carriers. These carriers can be categorized into two types: non-viral and viral carriers. While significant efforts and research have been dedicated to non-viral vectors, they have not yet reached the pinnacle of success. Over the past decade, the utilization of non-viral vectors for gene therapy has gained momentum, with peptides, liposomes, polymers, inorganic nanoparticles, and hybrid systems (combinations of different types) being popular in this field (Zu and Gao 2021). Among non-viral strategies, lipofection remains dominant in clinical trials (Sayed et al. 2022). In contrast, viral vectors have made notable strides in the gene therapy field, with several drugs based on viral vectors receiving regulatory approval in the past 5 years. Viral vectors are currently at the forefront of clinical trials, with adeno-associated virus (AAV) being the preferred choice (Au et al. 2021). AAVs are small, non-enveloped viruses that package a linear single-stranded DNA (ssDNA). The AAV genome, which has a size of 4.7 kb, consists of a central region containing the replicase (Rep) and capsid (Cap) genes, flanked by inverted terminal repeats (ITR) of 145 bases (Colella et al. 2018). Recombinant AAV (rAAV) has been engineered by deleting all viral protein genes and retaining only the terminal ITR sequences associated with viral replication and

packaging (Duan et al. 2021). This modification maximizes the transgene capacity of AAV while reducing immunogenicity and cytotoxicity. AAV has emerged as the primary vector in gene therapy due to its low immunogenicity, non-pathogenic nature, diverse capsid proteins, and ease of stable production.

21.3.2 Gene Therapy-Induced Ferroptosis for Cancer

Several gene therapy-based strategies have been developed to induce ferroptosis and eliminate cancer cells. These strategies can be categorized into four types: (1) SiRNA: SiRNA-mediated gene silencing is a promising therapeutic approach. For example, Zhou et al. utilized a cationic covalent framework to deliver SLC7A11 siRNA, leading to effective fibrosarcoma treatment (Zhou et al. 2022). Li et al. employed exosome-mediated delivery of multi-siRNAs to silence two ferroptosis suppressor genes (GPX4 and DHODH) and overcome drug resistance in hepatocellular carcinoma (Chen et al. 2022). (2) MiRNA: Systemic delivery of miRNAs in vivo has demonstrated significant therapeutic effects on cancer by promoting ferroptosis. Examples include miR-21-3p and miR-101-3p (Guo et al. 2022; Luo et al. 2021). (3) LncRNA: LncRNA-mediated ferroptosis therapies are currently limited but hold promise. Gai et al. reported a folate-modified liposome-mediated delivery of lncRNA therapy for the treatment of non-small-cell lung cancer. Their study demonstrated that lncRNA MT1DP (metallothionein 1D pseudogene) attenuated NRF2 expression, thereby aggravating oxidative stress (Gai et al. 2020). (4) ShRNA: Researchers have proposed a multi-shRNAs-based gene cocktail therapy. Yang et al. co-delivered shRNAs targeting GPX4 and methylenetetrahydrofolate dehydrogenase 2 (MTHFD2) to enhance the combined anticancer efficacy of ferroptosis and apoptosis. Downregulation of GPX4 mediated by shGPX4 and GSH depletion induced by apoptosis-inducing plasmid shMTHFD2 synergistically amplified the ferroptosis effect (Yang et al. 2022). These strategies highlight the potential of ferroptosis-based gene cocktail therapies and combination therapies involving multiple forms of cell death to achieve enhanced anticancer effects. However, it is important to note that gene therapy alone may not be sufficient to overcome drug resistance due to the lack of effective ferroptosis inducers and the presence of multiple antioxidation pathways in cells (Tang and Kroemer 2020). Further research and the development of novel ferroptosis inducers are necessary to address these challenges and improve the efficacy of ferroptosis-based gene therapies.

21.4 IONs and Gene Therapy Co-Induced Ferroptosis for Cancer

Current cancer therapies still face several key limitations, including drug resistance, side effects, low response rates, and the possibility of cancer recurrence. In light of these challenges, ferroptosis has emerged as a promising approach due to its potential to induce cell death in drug-resistant cancer cells (Liu et al. 2022c). As a

result, there is increasing interest in developing ferroptosis-driven nanotherapeutics and gene therapies for cancer treatment. However, it is important to recognize that neither IONs nor gene therapy alone can completely eradicate tumors. Cancer cells have various mechanisms of resistance to ferroptosis that are not fully addressed by IONs or gene therapy alone (Liu et al. 2022a).

Cells exhibit sensitivity to iron concentration, and even small fluctuations in concentration can trigger significant cellular responses (Mleczko-Sanecka and Silvestri 2021). Given the vital role of iron in cellular processes, cells have evolved mechanisms to maintain intracellular iron homeostasis (Trujillo-Alonso et al. 2019; Anderson and Frazer 2017; Katsarou and Pantopoulos 2020). Intracellular iron levels are intricately regulated to ensure iron homeostasis (Luo et al. 2020). Cells possess effective mechanisms to store and export excess intracellular iron ions released from internalized IONs. Consequently, the induction of ferroptosis by a single IONs treatment is insufficient for effective cancer treatment in clinical settings.

Furthermore, ferroptosis is a complex and tightly regulated process involving iron accumulation, lipid peroxidation, and mitochondrial membrane disruption. Lipid peroxidation mediated by reactive oxygen species (ROS) is a crucial step in ferroptosis. Cells possess six known antioxidant systems, including the System XC-glutathione (GSH)-GPX4 pathway, transsulfuration pathway, mevalonate pathway, FSP1-Coenzyme Q10 (CoQ10) pathway, dihydroorotate dehydrogenase (DHODH)-dihydroubiquinone (CoQH2) pathway, and GTP cyclohydrolase-1 (GCH1)-tetrahydrobiopterin (BH4) pathway (Liu et al. 2022b). Additionally, the level of the labile iron pool (LIP) or oxidized iron also influences lipid peroxidation through the Fenton reaction (Lv et al. 2022). Previous gene therapies have not comprehensively targeted all the key genes involved in these ferroptosis resistance pathways. Cancer cells can resist ROS-mediated lipid peroxidation through their own regulatory mechanisms, leading to the persistence of ferroptosis-resistant cells. To overcome this limitation, our research group has developed two generations of combination therapies utilizing IONs and gene interference technology. The first generation, known as GIFT (gene interfered-ferroptosis therapy) was reported by Gao et al. (Gao et al. 2021b), while the second generation, with improved antitumor efficacy, is called FAST (Ferroptosis Assassinates Tumor) (Luo et al. 2022). These combination therapies aim to address the complex network of ferroptosis resistance pathways by utilizing both IONs and gene interference technology. By targeting multiple key genes involved in ferroptosis resistance, these approaches seek to enhance the efficacy of ferroptosis-based cancer treatments.

21.4.1 GIFT Strategy

The principle of GIFT is depicted in Fig. 21.1. GIFT involves the utilization of a gene-interfering vector (GIV) and DMSA-coated Fe3O4 nanoparticles (FeNPs). The GIV comprises a promoter known as DMP (NF-κB-specific promoter) and a downstream effector gene. DMP consists of a NF-κB decoy sequence and a minimal

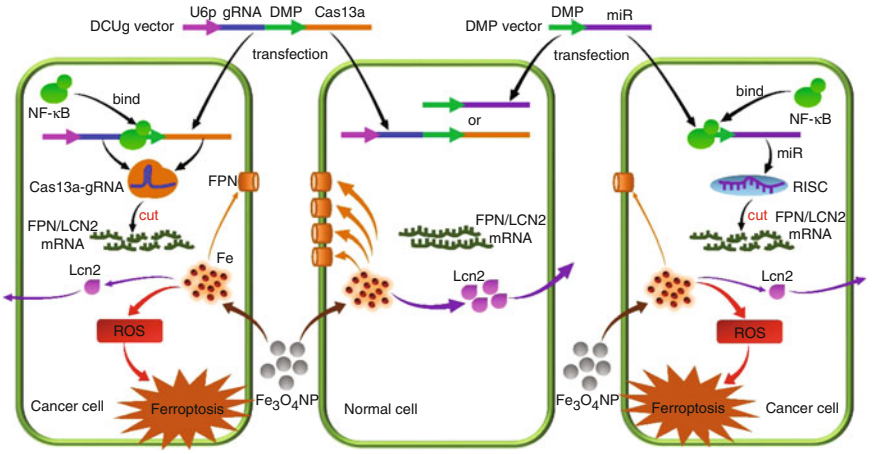

Fig. 21.1 GIFT strategy. Schematic show of CRISPR/Cas13a- and miRNA-based GIFT. DCUg DMP-Cas13a-U6-gRNA, DM DMP-miRNA, U6p U6 promoter, gRNA guide RNA, DMP NF-κB decoy-minimal promoter

promoter. NF-κB is a transcription factor that is commonly overactivated in cancer cells (Ben-Neriah and Karin 2011), enabling the activation of the effector gene specifically in cancer cells through NF-κB binding to DMP (Wang et al. 2018a). Conversely, in normal cells lacking NF-κB activity, the effector gene remains unexpressed (Dai et al. 2020; Gao et al. 2020). Hence, DMP acts as a cancer cell-specific promoter.

In GIFT, when the GIV containing DMP-controlled CRISPR/Cas13a or miRNA is transfected into cancer cells, the expressed Cas13a protein or miRNAs are produced. Cas13a can bind with guide RNA (gRNA) transcribed from a U6 promoter to form the Cas13a-gRNA complex. Alternatively, the expressed miRNAs can associate with the RNA-induced silencing complex (RISC). Both the Cas13a-gRNA and miRNA-RISC complexes have the ability to target the mRNA of interest, resulting in the suppression of the target gene expression specifically in cancer cells.

In this study, we selected two iron metabolic genes, FPN and LCN2, as the target genes. Both FPN and LCN2 play roles in the efflux of iron ions within cells (Yao et al. 2021; Sabelli et al. 2017). Our previous research revealed that the expression of these two genes was significantly upregulated in cells treated with FeNPs (Liu and Wang 2013). Based on this finding, we hypothesized that knocking down the expression of FPN and LCN2 in cancer cells using transfected GIVs of DMP-controlled CRISPR/Cas13a or miRNA would hinder the export of intracellular iron ions generated by internalized FeNPs. This would result in the accumulation of iron ions within the cells, leading to a substantial increase in intracellular ROS levels and triggering significant ferroptosis in cancer cells.

In normal cells, Cas13a or miRNA production is absent, thereby allowing the unaffected expression of FPN and LCN2 genes. Consequently, normal cells can actively export intracellular iron ions produced by FeNPs, thereby maintaining iron

homeostasis and minimizing the impact of FeNPs treatment. As a result, significant ferroptosis is induced in a wide range of cancer cells, while normal cells remain minimally affected. By delivering the therapeutic genes using AAV vectors, we achieved significant inhibition of tumor growth and long-lasting cures in mice. In mouse models of leukemia, colon cancer, and lung metastatic melanoma, the GIFT therapy successfully eradicated tumors in 40%, 30%, and 10% of mice, respectively, with no recurrence observed for up to 250 days post-treatment. Furthermore, in vivo safety experiments demonstrated that the GIFT reagents did not cause significant toxicity to the body. It is worth noting that both IONs and AAV are approved materials for clinical use (Soetaert et al. 2020; Wang et al. 2019), which brings GIFT cancer therapy closer to clinical application. The successful outcomes obtained from this study, along with the safety profiles of IONs and AAV, emphasize the potential of GIFT therapy as a promising approach for cancer treatment, with the possibility of transitioning from the laboratory to bedside.

21.4.2 FAST Strategy

GIFT therapy has demonstrated broad antitumor activity, high cancer specificity, potential for tumor eradication, and biosafety in treating various cancer cells and tumors in mice. However, it should be noted that GIFT achieved tumor eradication only in a limited proportion of mice. We observed significant upregulation of antioxidant genes (such as SLC7A11, GCLM, NQO1) in cells treated with FeNPs, indicating the important regulatory role of the six antioxidant systems mentioned earlier in FeNPs-induced ferroptosis (Luo et al. 2020). Therefore, further knockdown of key antioxidant genes could enhance the antitumor efficacy of GIFT therapy.

Furthermore, RNA-seq data revealed a significant increase in the expression of FTL and FTH1 genes in cells treated with FeNPs (Luo et al. 2020). These genes encode subunits of ferritin, which stores Fe^{3+} in cells and contributes to ferroptosis resistance (Chen et al. 2020). Moreover, FTH1 possesses ferroxidase activity, enabling the conversion of Fe^{2+} to Fe^{3+} and facilitating subsequent iron entry into the ferritin mineral core, with assistance from FTL. Therefore, it is conceivable that knocking down FTH1 in combination with GIFT therapy would further increase the cellular labile iron pool, potentially enhancing the efficacy of ferroptosis.

To address the ferroptosis resistance observed in residual cancer cells after GIFT treatment, we have developed FAST therapy. FAST therapy involves the knockdown of five additional genes that are distributed in the glutathione-dependent pathway (GPX4, SLC7A11, NRF2), COQ-dependent pathway (FSP1), and iron storage (FTH1). The principle of cancer specificity in FAST therapy is similar to GIFT therapy, as both are based on the overactivation of NF-κB in cancer cells (Fig. 21.2). Seven miRNA expression cassettes, each targeting different genes, are co-delivered into the body using a single AAV vector. These miRNAs work synergistically with FeNPs to promote potent ferroptosis. As a result, FAST therapy exhibits significantly greater antitumor activity compared to GIFT therapy. In

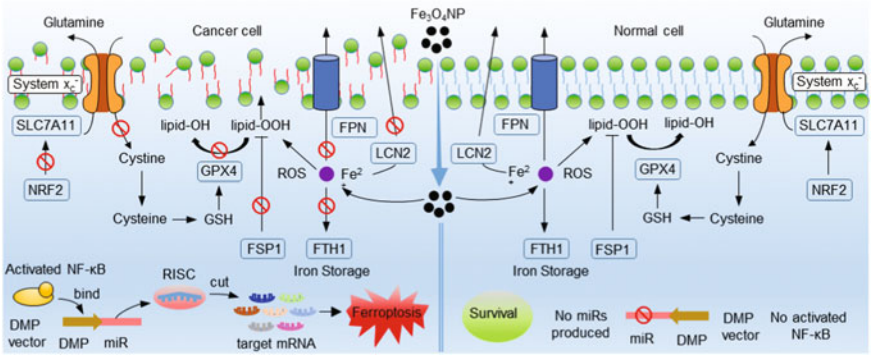

Fig. 21.2 FAST strategy. Schematic diagram of FAST eradicating tumor

particular, FAST therapy achieved tumor eradication in over 50% of mice with three different types of tumors (leukemia, colon cancer, and lung metastatic melanoma), resulting in long-term survival without tumor relapse for up to 250 days. FAST therapy demonstrates broad antitumor activity, high cancer specificity, and in vivo biosafety.

The underlying mechanism of FAST therapy involves the induction of catastrophic ferroptosis through intense lipid peroxidation triggered by the Fe^{2+} ions released from FeNPs. FAST therapy represents a new paradigm in advanced materials, combining IONs and AAV to create an unprecedented cancer assassin. This advanced combinatorial material transforms conventional IONs into a powerful weapon against cancer.

21.4.3 Advantages of FAST

Ferroptosis can be triggered by four classes of ferroptosis-inducing compounds (FINs) (Hassannia et al. 2019; Feng and Stockwell 2018). Class I FINs reduce the levels of glutathione (GSH) (e.g., erastin). Class II FINs directly interact with GPX4 (e.g., RSL3). Class III FINs target the mevalonate pathway (e.g., FIN56). Class IV FINs elevate the levels of the labile iron pool (LIP) (e.g., Fe^{2+}). FAST therapy encompasses all the ferroptosis-inducing functions of these four classes of FINs. Specifically, miSLC7A11 and miNRF2 belong to class I, miGPX4 belongs to class II, miFSP1 belongs to class III, and miFTH1, miFPN, and miLCN2 belong to class IV. Additionally, FeNPs serve as a favorable source of Fe^{2+} to augment the LIP levels. Through the combined effects of these four classes of FINs, the major antioxidant pathways in tumor cells are disrupted, while the FeNPs-induced accumulation of Fe^{2+} and excessive lipid peroxidation synergistically eliminate ferroptosis-resistant cancer cells. To achieve optimal antitumor effects, 10 nm FeNPs were chosen for this synergistic treatment, as it represents the optimal size

for high tumor accumulation of FeNPs (Tian et al. 2022). Consequently, FAST therapy exhibits highly efficient tumor elimination capabilities.

Another major advantage of FAST therapy is its excellent in vivo therapeutic safety. AAV vectors, used in FAST therapy, have the highest safety level (RG1) for gene therapy. FAST therapy combines the expression cassettes of seven miRNAs into a single AAV particle, enabling synergistic systemic treatment. Importantly, each miRNA expression is individually controlled by a cancer-specific promoter (DMP). DMP ensures cancer cell-specific gene expression by relying on the over-activity of NF-κB in cancer cells. This design feature ensures that AAV-based DMP-controlled miRNA therapy is not toxic to normal tissues. Furthermore, IONs, the metallic nanoparticles used in FAST therapy, have already been approved for clinical use and have demonstrated safety. The combination of AAV-based gene therapy and FeNPs in FAST therapy provides a high level of biosafety.

Additionally, the administration of FAST therapy is straightforward, as FeNPs and AAV vectors can be mixed together for intravenous injection. The FAST reagents effectively induce significant ferroptosis in a wide variety of tumors due to the constitutive over-activity of NF-κB in cancer cells. In summary, FAST therapy exhibits pan-cancer antitumor activity, high tumor selectivity, a high rate of tumor eradication, and remarkable in vivo biosafety. As a result, FAST therapy represents a promising alternative to current cancer treatments, holding significant potential for improving patient outcomes.

21.5 Conclusions and Perspectives

The ferroptosis pathway is closely linked to drug resistance and metastasis in cancer (Ubellacker et al. 2020; Chen et al. 2021a). Inducing ferroptosis can restore the sensitivity of drug-resistant cancer cells to standard treatments. However, the signaling pathways involved in ferroptosis are highly complex and encompass six antioxidant systems (Chen et al. 2021b; Liu et al. 2022a). The presence of these multiple antioxidant systems allows some residual ferroptosis-resistant cancer cells to evade ferroptosis induction through IONs-based or gene therapy approaches. Ferroptosis resistance contributes to the challenges associated with tumor eradication and recurrence. Combinatorial therapy involving IONs and gene therapy presents a potential solution to overcome ferroptosis resistance. An example of such therapy is FAST therapy, which combines nanomedicine and gene interference technology. In FAST therapy, IONs serve as Fe^{2+} donors, while AAV vectors target multiple antioxidant systems to trigger intense ferroptosis and eradicate tumors. FAST therapy also offers excellent in vivo safety, ease of drug administration, and the potential to treat various types of cancer.

However, one significant barrier to AAV-based gene therapy is the rapid clearance of AAV vectors by anti-AAV neutralizing antibodies in the body. Additionally, challenges such as the affordability of the drug product, package size limitations, and off-tumor infection need to be addressed in AAV-based gene therapy. To address these challenges, the use of biocompatible IONs-based vectors loaded with nucleic

acids to induce ferroptosis can be an effective alternative to AAV-based gene therapy. For instance, GIFT therapy utilized a non-viral vector called PEI-modified IONs (FeNC) to transfer miRNA-expressing plasmids (Gao et al. 2021b). However, the treatment efficacy of this approach still falls behind that of viral vector-based AAV therapy. Therefore, it is crucial to explore more effective non-viral DNA vectors based on iron nanoparticles in the future. The development of such a dual-role nanoparticle would represent an ideal ferroptosis inducer for combinatorial therapy, addressing the limitations of current approaches and offering enhanced treatment outcomes.

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Inhibitors of Oxytosis/Ferroptosis: A New Class of Therapeutics for Alzheimer's Disease

22

Pamela Maher

Abstract

Alzheimer's disease is a progressive neurological disorder that affects the brain, causing problems with memory, thinking, and behavior. It is the most common cause of dementia, a group of brain disorders that affect a person's ability to function independently. Oxytosis is a form of non-apoptotic regulated cell death characterized by glutathione depletion and dysregulated production of free radicals from mitochondria, resulting in lethal lipid peroxidation. The major steps that define oxytosis were first detailed in 2001, and since then, other laboratories have provided additional key insights into the pathway. In 2012, the pathway was redescribed in fibroblasts as an iron-dependent, non-apoptotic form of cell death named ferroptosis. This chapter discusses oxytosis/ferroptosis as a potential therapeutic target in Alzheimer's disease.

22.1 Introduction

Alzheimer's disease (AD) is one of the most devastating brain disorders. It slowly erodes memory and thinking skills over the course of many years as it causes neuronal dysfunction and eventually death. An estimated 6.5 million Americans are currently living with AD and nearly 13 million are projected to have the disease by 2050 as the population ages (Association A 2022). In 2022, AD and other dementias cost the USA more than $320 billion and this cost is projected to reach $1 trillion by 2050 (Association A 2022).

AD was first described over 100 years ago by Alois Alzheimer who identified the histological alterations that today are known as amyloid-beta (Aβ) plaques and

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neurofibrillary tangles (NFTs) of the microtubule-associated protein tau (MAPT). Most of the research in AD has been carried out around these two pathological hallmarks, namely targeting the hypothesis that the plaques are the culprit causing the disease. But in the last decade, as we have further understood the molecular drivers of AD, a need for therapeutics that target pathways alternative to the amyloid hypothesis has been mounting. The probability of developing AD grows disproportionally with age and even though aging is the biggest risk factor for the disease, there is still a lack of understanding of how aging contributes to AD. This is because aging is not regarded as a disease and historically there has been a disconnection between the two fields. This limitation is increasingly being acknowledged and the science is now moving toward addressing the role of aging in AD. Therefore, these are exciting times, as this research is also uncovering knowledge about basic biological mechanisms, such as those related to metabolism, inflammation, and regeneration.

In 2021, the Food and Drug Administration (FDA) approved the first ever disease-modifying therapy (DMT) for AD—Aducanumab. Aducanumab is an anti-amyloid monoclonal antibody and while its efficacy has generated controversy and is still being fully evaluated, Aducanumab set a pace to develop DMTs for AD that is only expected to intensify. It should be noted that even though a therapy that mildly improves AD will likely benefit the patients, it is very unlikely that it will avoid the financial strain of the disease on the healthcare systems. This is because the patients will still require expensive, specialized care. The only way to really beat AD is to develop a therapy, or a combination of therapies, with a truly meaningful impact on the disease. This will require support for the development of new therapies that target the disease from multiple angles. In fact, these therapies, depending on how they work, could have their use extended to other forms of age-related dementia, because they would not be focused on the amyloid pathway which is specific to AD.

Other major obstacles to drug development for AD include the cost and duration of the clinical trials as well as patient enrolment in the trials. If societies are truly invested in treating or curing AD, there must be a collective effort to overcome these barriers. The successful development of drugs and vaccines for COVID-19 in the last 2 years has proven that such an endeavor is possible. In this chapter, we will discuss the current therapeutic landscape in the AD field. We will focus on a mechanism of neurodegeneration called oxytosis/ferroptosis whose relevance for aging and AD is starting to be understood. The drug discovery, preclinical studies, and clinical development of therapeutics that target oxytosis/ferroptosis in the AD brain will be described in detail with the example of two inhibitors of this pathway developed in our laboratory that are currently in Phase I clinical trials for AD.

22.2 The Dire Situation with the AD Therapeutics Available

For the last 30 years, AD patients have been treated with a combination of two types of therapeutics, acetylcholinesterase inhibitors [tacrine (discontinued), donepezil, rivastigmine, and galantamine], and the *N*-methyl-D-aspartate (NMDA) receptor

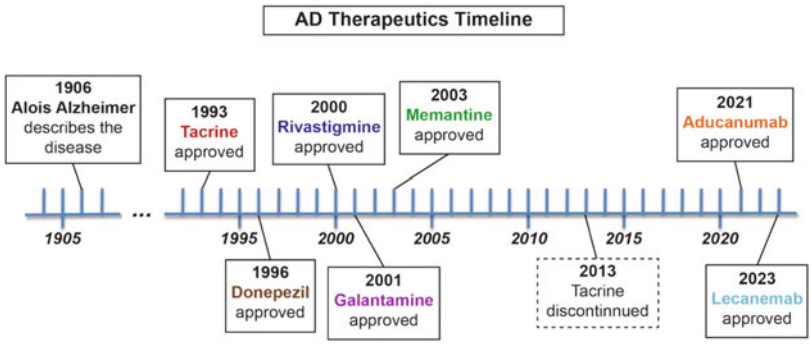

Fig. 22.1 Timeline of current therapeutics approved by the FDA for AD

antagonist memantine (Fig. 22.1) (Masters et al. 2015; Jeremic et al. 2021). Both cholinergic and glutamatergic signaling are involved in learning and memory processes and are impaired in AD (Parsons et al. 2013). Acetylcholine is reduced in forebrain neurons at early stages of the disease (Mufson et al. 2008) and acetylcholinesterase inhibitors work by delaying the extrasynaptic metabolism of acetylcholine thereby increasing its availability for neurotransmission and enhancing postsynaptic stimulation (Masters et al. 2015). Acetylcholinesterase inhibitors are used to treat symptoms of mild-to-moderate AD. Memantine prevents glutamate excitotoxicity and is used to treat symptoms of moderate to severe AD. NMDA receptors are over-activated in AD, leading to sustained Ca^{2+} influx and gradual neurodegeneration (Parsons et al. 2013). Acetylcholinesterase inhibitors and memantine are often combined, as they can have additive effects (Masters et al. 2015).

However, all of these approved drugs are only cognition-enhancing agents. This means that, although they can temporarily alleviate memory symptoms and other cognitive changes for varying lengths of time, they do not alter the main pathological processes of AD (Association A 2022). Moreover, for nearly 20 years since the approval of memantine by the Food and Drug Administration (FDA) in 2003, there was no other drug introduced onto the market for treating AD. Only in 2021 was aducanumab (developed by Biogen and Eisai) conditionally approved by the FDA, also becoming the first ever drug to address the underlying biology of AD rather than the symptoms (Sevigny et al. 2016). Aducanumab is an anti-amyloid monoclonal antibody that targets the aggregated forms of Aβ thereby reducing Aβ plaque build-up in the brain through the activation of microglial phagocytosis (Sevigny et al. 2016). The drug was studied in people with early AD [mild cognitive impairment (MCI) and mild AD] and it was shown to reduce visible Aβ plaques in the brain as determined by amyloid PET imaging but not to noticeably improve cognitive function or quality of life. Aducanumab received the greenlight from the FDA through the accelerated approval pathway, which provides individuals with a serious disease earlier access to drugs when there is an expectation that the drug will have a clinical benefit (Association A 2022). The company is still required to verify clinical

benefit in a post-approval trial which is now ongoing. The drug is expensive ($28,200, down from its initial list price of $56,000), is associated with potentially dangerous cases of brain swelling and bleeding [called Amyloid Related Imaging Abnormalities (ARIA)], and has to be administered via an infusion directly into the vein once a month.

The current AD drug development pipeline was recently reviewed [January 2022 (Cummings et al. 2022b)]. Of a total of 143 agents in trials for AD, the vast majority are DMTs (119 agents). The rest are symptomatic agents, including 14 targeting cognitive enhancement and 10 to treat neuropsychiatric and behavioral symptoms. Of the 119 DMTs, 40 are biologics and 79 are small molecules. Twenty DMTs have amyloid, 13 have tau, 23 have inflammation, and 19 have synaptic plasticity/neuroprotection as their primary mechanistic targets. Of all the agents being tested, 31 are in Phase 3 trials. In addition to aducanumab, four other monoclonal antibodies (donanemab, gantenerumab, lecanemab, and solanezumab) are being tested. Results for donanemab (developed by Eli Lilly), which recognizes Aβ aggregated in plaques, are expected sometime in 2023. In September 2022, Biogen and Eisai reported positive findings for lecanemab (Biogen 2022). Lecanemab reduced cognitive decline by 27% in people with early-stage AD at 18 months in a Phase 2 trial, which would make it the first of its kind to show a significant cognitive benefit in a robust trial. Although to a lesser degree than aducanumab, lecanemab also had ARIA side effects. The FDA has recently finished reviewing lecanemab and approved it under the accelerated approval pathway. Unlike aducanumab that primarily binds to amyloid protein after it has aggregated, lecanemab acts at an earlier stage targeting amyloid protofibrils. Gantenerumab (developed by Roche) was designed to bind Aβ fibrils and readouts were announced in November 2022 with failure in two large, late-stage clinical trials with MCI or mild AD (Roche 2022). Solanezumab (developed by Eli Lilly), which recognizes soluble monomeric but not fibrillar Aβ, failed multiple times when tested in patients with mild-to-moderate AD and it is now being tested to determine whether it can slow cognitive decline in cognitively unimpaired older adults with already elevated levels of Aβ. Overall, some of these trials are looking promising but more information on how patients perform in the long-term, especially with respect to maintenance of cognitive function and quality of life, with amyloid immunotherapy is required.

The other DMTs in Phase 3 trials are all small molecules (Cummings et al. 2022b): AGB101 (AgeneBio), a SV2A modulator that reduces Aβ-induced neuronal hyperactivity; atuzaginstat (Cortexyme), an inhibitor of bacterial proteases of P. gingivalis that cause neuroinflammation; blarcamesine (from Anavex), a sigma-1 receptor agonist that targets oxidative stress, protein misfolding, mitochondrial dysfunction and inflammation; GV-971 (Shanghai Greenvalley), an algae-derived agent that alters the microbiome to reduce peripheral and central inflammation; hydralazine, a free-radical scavenger; two forms of omega-3 fatty acids being tested as prevention in older cognitively healthy adults; effects of aerobic exercise training and intensive pharmacological reduction of vascular risk factors (losartan+amlodipine+atorvastatin) on cognitive performance in older adults who have a high risk for AD; metformin, which is being tested in individuals with MCI hoping

that it will increase insulin sensitivity and improve glucose metabolism in the brain; NE3107 (Neurmedix), a small molecule that binds to the extracellular signal-regulated kinase (ERK) and selectively inhibits inflammation-driven ERK- and NF-κB-induced inflammatory mediators that is being tested in mild-to-moderate probable AD; Nilotinib (KeifeRx), a tyrosine kinase inhibitor that induces autophagy and promotes the clearance of Aβ and tau; semaglutide (Novo Nordisk), a glucagon-like peptide-1 (GLP-1) agonist that reduces inflammation and improves insulin signaling in the brain; simufilam (Cassava Sciences), a filamin A protein inhibitor that prevents the interaction of soluble Aβ and the α7 nicotinic acetylcholine receptor, which has been reported to trigger tau phosphorylation and synaptic dysfunction; tricaprilin (Cerecin), a formulation of caprylic triglyceride, which induces ketosis to improve mitochondrial metabolism and neuronal function; TRx0237 (TauRx Therapeutics), a tau protein aggregation inhibitor; and valiltramiprosate (Alzheon), a prodrug of tramiprosate that inhibits Aβ peptide aggregation into toxic oligomers (Cummings et al. 2022b).

In drug development for AD a distinction can be made between Aβ-targeting approaches and non-Aβ approaches that target alternative pathways. While amyloid-targeting therapies still predominate, there is increasing recognition that to effectively treat the disease, it may be necessary to look at other mechanisms. With the advances in biomarker research, the diagnosis of AD in a patient can now be made based on the presence of Aβ and phosphorylated tau [positron emission tomography (PET) and body fluids] and not just clinical measurements (Scheltens et al. 2021; Masters et al. 2015). This is important, because the clinical stages in AD can range from cognitively normal to MCI and dementia, underscoring a continuum which spans a period of years (Scheltens et al. 2021). However, it must be stressed that the pathological hallmarks of AD are associated with other key pathological changes in the brain, which, as discussed above, are becoming the focus of new therapeutic approaches under development. During the preclinical phase of AD, alterations in neurons, microglia, and astroglia drive the progression of the disease before cognitive impairment is observed. Neuroinflammation, alterations in the vessels, and dysfunction of the glymphatic system act upstream or in parallel to accumulating Aβ (Scheltens et al. 2021). Non-amyloid therapies, whether acting upstream or downstream of Aβ toxicity, can be combined with anti-amyloid-targeting therapies. Because these events can be autocatalytic, a combination of therapies with multiple targets could offer a more efficient way of halting pathology.

The greatest known risk factor for AD is increasing age (Scheltens et al. 2021; Association A 2022). About 1 in 9 people age 65 and older has AD, and the prevalence is 1 in 3 people over the age of 85 (Association A 2022). It would be expected that old age offers the best targets for treating AD. However, the degenerative processes associated with aging are still poorly understood as are their roles in AD. Aging is a complex process characterized by damage to a number of different molecular systems, including DNA, RNA, epigenome, protein, lipids, and metabolites. Moreover, it is the accumulation of damage in the form of various molecular species that may define aging through their combinatorial effect (Gladyshev et al. 2021). At a functional level, this damage can lead to altered

intercellular communication (inflammation), genomic instability, telomere attrition, epigenetic alterations, loss of proteostasis, deregulated nutrient-sensing, mitochondrial dysfunction, cellular senescence, and stem cell exhaustion (Lopez-Otin et al. 2013). The idea is that some of these changes might translate into a failure of specific processes in the brain thus contributing to AD. Next, we will describe how a unique mechanism of neurodegeneration called oxytosis/ferroptosis is associated with the aging process and offers the potential for developing an entirely new class of therapeutics for AD.

22.3 Oxytosis/Ferroptosis in AD, a Therapeutic Target Worth Pursuing

Oxytosis is a form of non-apoptotic regulated cell death characterized by glutathione (GSH) depletion and dysregulated production of free radicals from mitochondria that results in lethal lipid peroxidation (Tan et al. 1998; Tan et al. 2001; Lewerenz et al. 2018; Maher et al. 2020). The major steps that define oxytosis were first detailed in 2001 by Tan et al. (Tan et al. 2001). Since then, other laboratories have provided additional key insights into the pathway (Albrecht et al. 2010; Seiler et al. 2008; Dolga et al. 2018; Ratan 2020), which was later redescribed in fibroblasts in 2012 as an iron-dependent, non-apoptotic form of cell death named ferroptosis (Dixon et al. 2012). But because the mechanistic steps are essentially the same, we will be calling it oxytosis/ferroptosis (Lewerenz et al. 2018; Maher et al. 2020; Soriano-Castell et al. 2021c).

Oxytosis/ferroptosis can be triggered by inhibiting cystine uptake via system x_c^- with either glutamate or erastin, which subsequently depletes intracellular GSH (Tan et al. 1998; Tan et al. 2001; Lewerenz et al. 2018; Maher et al. 2020). GSH depletion leads to inhibition of the GSH-dependent enzyme GSH peroxidase 4 (GPX4), activation of lipoxygenases (LOXs), and dysfunctional mitochondria. GPX4 can also be directly inhibited by the chemical RSL3. ROS and lipid peroxides are generated, which potentiate intracellular calcium (Ca^{2+}) influx through store-operated Ca^{2+} channels and cell death. Iron plays an important role in oxytosis/ferroptosis as iron chelators prevent oxytosis/ferroptosis-mediated cell death (Dixon et al. 2012; Maher et al. 2020). Iron can generate ROS via the Fenton reaction and also promote the activation of the non-heme iron-containing LOX enzymes (Eaton et al. 2020; Seiler et al. 2008; Bayir et al. 2020). Free polyunsaturated fatty acids (PUFAs) as well as lipids containing esterified PUFAs are particularly susceptible to peroxidation and are required for the execution of oxytosis/ferroptosis (Yang et al. 2016; Conrad et al. 2018; Maher et al. 2020). Oxidized PUFAs are not intrinsically toxic to cells, but the excessive accumulation of oxidized PUFA-containing lipids within cell membranes appears to drive oxytosis/ferroptosis (Stockwell 2022). As a result, the integrity, fluidity, permeability, and function of the biomembranes become compromised (El-Beltagi and Mohamed 2013; Alche 2019; Farmer and Mueller 2013). In addition, lipid peroxides can generate a variety of short-chain

reactive carbonyl species (RCS) that can further damage biomolecules such as proteins and DNA (Farmer and Mueller 2013; Alche 2019).

Importantly, all these processes are also observed in the central nervous system (CNS) with aging and are exacerbated in AD (Majernikova et al. 2021; Conrad et al. 2021; Zhang et al. 2021; Chen et al. 2021; Maher et al. 2020; Ashraf et al. 2020) (Fig. 22.2). The levels of GSH in the brain decrease with age in humans and are further reduced in AD (Ballatori et al. 2009; Ansari and Scheff 2010; Emir et al. 2011; Currais and Maher 2013; Mandal et al. 2015). The loss of GSH is associated with impairments in cognitive function, microglial activation, and endothelial dysfunction (Tan et al. 2001; Currais and Maher 2013). Decreases in blood GSH levels with age have also been observed (Maher 2005; Giustarini et al. 2006; Droge et al. 2006; Sekhar et al. 2011) and are exacerbated in MCI and AD (Bermejo et al. 2008; Rae and Williams 2017). Furthermore, genetic ablation of Gpx4 specifically in neurons causes neurodegeneration in mice associated with neuroinflammation (Seiler et al. 2008; Hambright et al. 2017). Interestingly, microglia appear to be more responsive to the induction of oxytosis/ferroptosis in co-cultures with neurons and astrocytes in a process that is mediated by the vesicle trafficking regulator SEC24B and leads to an environment that causes neurotoxicity (Ryan et al. 2023).

In addition, post-mortem brain samples from subjects with MCI already show increased levels of lipid peroxidation, and by-products of lipid peroxidation in plasma, urine, and cerebrospinal fluid (CSF) are elevated throughout the progression of the disease (Ashraf et al. 2020; Bradley et al. 2010; Sultana et al. 2013; Bradley-Whitman and Lovell 2015; Gaschler and Stockwell 2017). Consequently, RCS such as malondialdehyde (MDA) and 4-hydroxynonenal (HNE) have been implicated in the disease (Maher et al. 2020). Several LOXs have also been implicated in AD (Joshi and Pratico 2014; Joshi et al. 2015; Kuhn et al. 2015; Singh and Rao 2019). 12/15-LOX is upregulated in the brain regions that are most vulnerable to AD early in the disease (Joshi et al. 2015), and 5-LOX expression increases with age in the brain and is exacerbated in AD (Joshi and Pratico 2014).

A decline in cerebral energy metabolism is observed with aging and is one of the earliest events in AD (Currais 2015; Costantini et al. 2008; Cunnane et al. 2011; Yin et al. 2014). Abnormalities in mitochondria include reductions in electron transport chain (ETC) activity and respiration, increases in mitochondrial-derived oxidative stress, significant changes in the mitochondrial metabolite landscape, oxidative damage to mitochondrial DNA (mtDNA), RNA, lipid and protein species, and an accumulation of mtDNA mutations (Swerdlow and Khan 2004; Currais 2015; Lin and Beal 2006; Onyango et al. 2016; Yin et al. 2016).

Moreover, aging is associated with imbalances in iron metabolism and disruption in metal homeostasis is an important feature in AD (Gleason and Bush 2021; Ashraf et al. 2020; Hagemeier et al. 2012; Hare et al. 2013). Although iron plays a role in neurotransmitter synthesis, myelination, and mitochondrial function (Hare et al. 2013), its accumulation may contribute to AD pathology in a process similar to that found during oxytosis/ferroptosis. Selective accumulation of iron has been detected in neurofibrillary tangles (Good et al. 1992) as well as specific brain regions

Oxytosis/Ferroptosis and AD

	Oxytosis/ Ferroptosis	Alzheimer's Disease
GSH	Decreased GSH levels induced by X_c^- inhibition initiate oxytosis/ferroptosis	Decreased GSH levels in the brain and blood of patients
LOXs	Activated	Increased expression and activation in the brain
Iron	Required for the pathway	Accumulated in specific brain regions
GPX4	Inhibited by lack of GSH or directly with RSL3	Neuronal KO of Gpx4 in mice causes neurodegeneration and neuroinflammation
ROS	Increased	Oxidative modifications increased in the brain
Lipid peroxidation	Increased	Increased in brain, plasma and CSF
Mitochondria	Dysfunctional	Dysfunctional and deficits in energy metabolism
Ca^{2+}	Intracellular influx	Intracellular influx
Inflammation	Microglia are particularly responsive to oxytosis/ferrooptosis	Pro-inflammatory microglia and astrocytes
Aβ pathology	Intracellular aggregation of Aβ induces oxytosis/ferroptosis	Vascular, intracellular and plaques
Tau pathology	?	Tau hyperphosphorylation and NFTs
Synaptic loss	?	Degeneration and loss as the disease progresses
Cell death	Endpoint of the pathway	Progressive neuronal cell death

Fig. 22.2 The key pathological events in oxytosis/ferroptosis and AD

(Tao et al. 2014) and is associated with hippocampal damage (Raven et al. 2013) and the rate of cognitive decline in AD patients (Ayton et al. 2020a).

Although one of the hallmarks of AD is the accumulation of Aβ in extracellular plaques, Aβ also accumulates inside neurons in AD brain (Gouras et al. 2005; LaFerla et al. 2007; Wirths et al. 2004). It has been shown that intraneuronal accumulation of Aβ can induce oxytosis/ferroptosis associated with the expression of pro-inflammatory mediators and metabolic alterations characterized by deficits in glucose metabolism and mitochondrial bioenergetics (Huang et al. 2020).

Despite all of this evidence, the therapeutic value of oxytosis/ferroptosis for treating AD remains largely unexplored. Importantly, while cultured cells die rapidly following the induction of oxytosis/ferroptosis, it is likely that with aging and in AD patients oxytosis/ferroptosis takes place over an extended time period thereby leading to a slow degeneration of basic neuronal functions prior to cell death (Maher et al. 2020). This large window of opportunity would have huge implications for the development of therapies.

To date, a number of studies have been carried out that have tested the efficacy of anti-oxytotic/ferroptotic agents in animal models of AD as well as AD patients. Although these compounds were not initially developed with the specific purpose of inhibiting oxytosis/ferroptosis in AD (they target aspects of AD pathology that have come to be recognized as relevant to oxytosis/ferroptosis), studying them may provide clues on the role of oxytosis/ferroptosis in AD.

For instance, deferoxamine (DFO), an iron chelator that efficiently inhibits oxytosis/ferroptosis in cell culture and that is used to alleviate iron burden in thalassemia major and sickle cell patients, has been shown to decrease AD-related pathology, including cognitive loss and Aβ deposition in animals (Fine et al. 2012; Guo et al. 2013; Sripetchwandee et al. 2016; Farr and Xiong 2021). In clinical trials, DFO reduced the rate of decline of daily living skills in AD patients (Crapper McLachlan et al. 1991; McLachlan et al. 1993). However, DFO treatment is associated with systemic toxicities at high doses, a short half-life that leads to low patient compliance and possibly problems with crossing the blood–brain barrier (BBB) (Farr and Xiong 2021). Deferiprone (DFP) is another iron chelator that inhibits oxytosis/ferroptosis. DFP is capable of penetrating the BBB and removing iron from the brain. It is currently being evaluated in a phase 2 randomized placebo-controlled clinical trial with prodromal and mild AD patients (NCT03234686) (Ayton et al. 2020b). α-Lipoic acid (LA), a naturally occurring enzyme cofactor with iron chelator properties, rescued tauopathy and cognitive impairment in P301S Tau transgenic mice (Zhang et al. 2018a). Interestingly, LA blocked the iron overload, lipid peroxidation, and inflammation seen in these mice, while reducing the ROS content and increasing the expression of GPX4. In a limited study with AD patients (not double-blinded, placebo-controlled or randomized), LA appeared to delay the development of cognitive decline in moderate AD patients (Hager et al. 2007).

Ferrostatin-1 and liproxstatin-1 were identified as two potent inhibitors of oxytosis/ferroptosis that prevent the accumulation of lipid hydroperoxides due to their reactivity as radical-trapping antioxidants (RTAs) (Zilka et al. 2017). They both

reduced neuronal death and memory loss induced by Aβ in a mouse model of stereotaxic infusion of oligomeric Aβ1-42 into the hippocampus (Bao et al. 2021). Ferrostatin-1 and liproxstatin-1 also protected primary neurons exposed to Aβ1-42 (Bao et al. 2021). There are no data on the effects of these agents in humans with AD and they appear to have poor BBB penetration (Keuters et al. 2021).

Other studies have been conducted that provide evidence that targeting additional aspects of oxytosis/ferroptosis may be beneficial. *N*-acetylcysteine (NAC), an antioxidant and a glutathione precursor, prevented Aβ toxicity in primary neurons (Paula-Lima et al. 2011) and attenuated memory deficits, synaptic plasticity loss, and lipid peroxidation in animals exposed to Aβ oligomers (Fu et al. 2006; More et al. 2018). Although the effects of administering NAC to a small group of probable AD patients for a short period of time (6 months) were not clear (Adair et al. 2001), a comprehensive clinical trial is still missing.

Selenium is an essential micronutrient that functions as a cofactor for the reduction of antioxidant enzymes, such as GPX4. Although the studies reporting on the concentrations of selenium in the brains of AD patients are inconsistent (Loef et al. 2011), supplementation with selenium reduced cognitive decline and decreased Aβ and tau pathologies in a transgenic mouse model of AD (Zhang et al. 2017; Zhang et al. 2018b). Several studies have assessed the effects of selenium in human AD subjects and reported positive outcomes (Pereira et al. 2022). However, these studies were only conducted for up to 6 months and with very small groups of participants. Therefore, additional studies are required.

Coenzyme Q10 (CoQ10) is a vitamin-like substance synthesized in the human body. In addition to its support role in mitochondrial oxidative phosphorylation, CoQ10 is a lipid-soluble antioxidant that protects the cell membranes from oxidative damage (Mantle et al. 2021). The inhibition of oxytosis/ferroptosis by CoQ10 is modulated by ferroptosis suppressor protein 1 (FSP1) (Doll et al. 2019; Bersuker et al. 2019). A water-soluble formulation of coenzyme Q10 (Ubisol-Q10) inhibited Aβ plaque formation and improved long-term memory in a transgenic mouse model of AD (Muthukumaran et al. 2018). In a small clinical trial with mild-to-moderate AD patients treated with CoQ10 for 4 months, no clinical benefit was reported (Galasko et al. 2012). Idebenone is a short-chain synthetic analog of CoQ10 which also protects cells against oxytosis/ferroptosis and can be regulated by FSP1 (Soriano-Castell et al. 2021b). Clinical studies with idebenone were mixed, reporting both modest cognitive improvements (Gutzmann and Hadler 1998; Gutzmann et al. 2002) as well as a failure to slow cognitive decline in patients (Thal et al. 2003).

Another low-potency inhibitor of oxytosis/ferroptosis that has been reasonably well-studied is vitamin E, which acts as a radical scavenger and whose circulating levels are decreased in AD patients. Multiple randomized trials for AD have been conducted but the data are inconsistent and inconclusive (Browne et al. 2019).

Overall, it is clear that many of the clinical trials performed with oxytosis/ferroptosis relevant compounds had profound limitations regarding the number of subjects and the length of treatment time. For instance, the current trials with monoclonal antibodies for Aβ are performed with thousands of patients and for durations of up to 18 months. So, much of the data on the efficacy of these inhibitors

of oxytosis/ferroptosis in AD patients lack robustness. In addition, there are more potent inhibitors of oxytosis/ferroptosis that target different aspects of the pathway that have not yet been tested in AD patients. Oxytosis/ferroptosis is becoming recognized as a new promising target for neurodegenerative diseases and multiple laboratories are now developing therapeutic strategies (Majernikova et al. 2021; Conrad et al. 2021; Zhang et al. 2021; Chen et al. 2021; Maher et al. 2020). Next, we will detail an approach to identify and develop novel inhibitors of oxytosis/ferroptosis.

22.4 Drug Discovery to Develop Inhibitors of Oxytosis/Ferroptosis

There are two main screening approaches to drug discovery that can be pursued; a target-based approach, which is focused on specific molecular targets, and a phenotypic-based approach, that aims to identify interventions that protect against a disease phenotype. Some well-known inhibitors of oxytosis/ferroptosis are RTAs and iron-chelating molecules. These were discussed above and they act upon general mechanisms of oxytosis/ferroptosis (Hadian and Stockwell 2021). A few specific molecular targets that can be modulated to inhibit oxytosis/ferroptosis in cells have been identified that should expand the possibilities for modulating oxytosis/ferroptosis in the future (Fig. 22.3).

Acyl-CoA synthetase long-chain family member 4 (ACSL4) is an enzyme that converts free long-chain fatty acids into fatty acyl-CoA esters so that they can be inserted into membrane phospholipids, a step catalyzed by lysophosphatidylcholine acyltransferase 3 (LPCAT3). The activation of long-chain polyunsaturated fatty acids (PUFAs) and consequent incorporation into cellular membranes increases the risk of oxytosis/ferroptosis and inhibition of either ACSL4 or LPCAT3 can prevent that (albeit to different extents) (Doll et al. 2017; Conrad et al. 2021; Dixon et al. 2015).

The lipid biosynthetic pathway has provided additional targets for inhibiting oxytosis/ferroptosis. It has been shown that inhibition of acetyl-CoA carboxylase 1 (ACC1), the rate-limiting enzyme that catalyzes the carboxylation of acetyl-CoA to malonyl-CoA, protected cells against oxytosis/ferroptosis associated with a decrease in the levels of free PUFAs (Currais et al. 2019). The inhibition of ACC1 can be mediated by the activation of AMP-activated protein kinase (AMPK) as a consequence of modulating the mitochondrial ATP synthase (Goldberg et al. 2018; Currais et al. 2019; Lee et al. 2020; Li et al. 2020a). More recently, it was demonstrated that inhibition of the downstream enzyme fatty acid synthase (FAS), which is required for the synthesis of fatty acids from malonyl-CoA, also prevents cell death induced by oxytosis/ferroptosis (Ates et al. 2020).

With regard to lipid peroxidation, as mentioned above, LOXs offer excellent therapeutic targets against oxytosis/ferroptosis. Although it has been shown that several of the commercially available inhibitors of LOXs are in fact good RTAs, both chemical and genetic manipulation of LOX activity in nerve cells has been

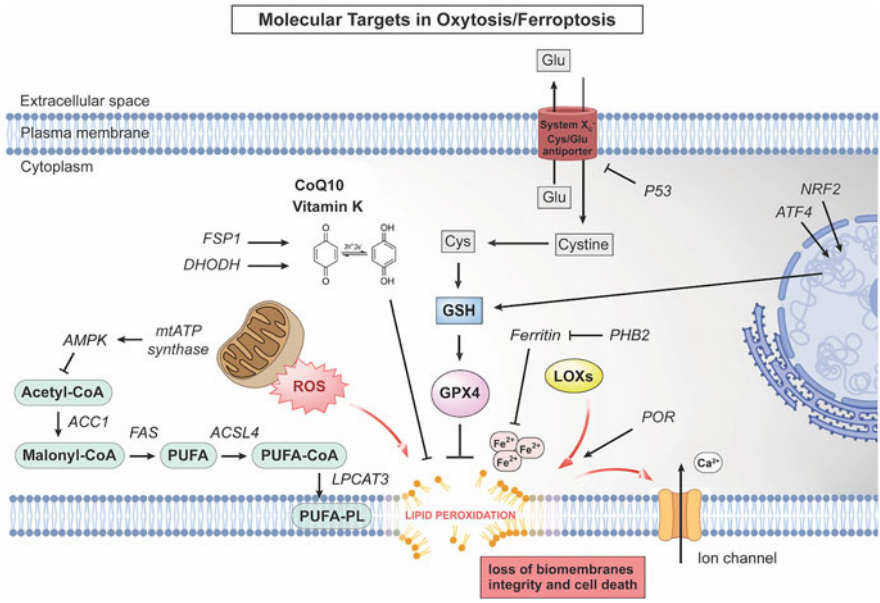

Fig. 22.3 Molecular targets for potential therapeutic intervention in oxytosis/ferroptosis. ACC1 acetyl-CoA carboxylase 1, ACSL4 acyl-CoA synthetase long-chain family member 4, AMPK AMP-activated protein kinase, ATF4 activating transcription factor 4, Ca^{2+} calcium, CoQ10 coenzyme Q10, Cys cysteine, DHODH dihydroorotate dehydrogenase, FAS fatty acid synthase, Fe^{2+} iron, FSP1 ferroptosis suppressor protein 1, Glu glutamate, GPX4 GSH peroxidase 4, GSH glutathione, LOXs lipoxygenases, LPCAT3 lysophosphatidylcholine acyltransferase 3, mtATP synthase mitochondrial ATP synthase, NRF2 nuclear factor erythroid 2-related factor 2, PHB2 prohibitin 2, POR cytochrome P450 oxidoreductase, PUFA polyunsaturated fatty acid, PUFA-CoA polyunsaturated fatty acid coenzyme A, PUFA-PL polyunsaturated fatty acid phospholipid

shown to interfere with oxytosis/ferroptosis (Shah et al. 2018; Maher et al. 2020). There are a number of LOX inhibitors in development or clinical use for other indications such as inflammation and some of these might be repurposed for AD (Hu and Ma 2018). Another enzyme that potentiates lipid peroxidation is cytochrome P450 oxidoreductase (POR) (Zou et al. 2020). POR mediates the transfer of electrons from NADPH to cytochrome P450 which is required in cellular redox homeostasis and detoxification. POR also donates electrons to other redox partners and it has been shown to up-regulate the peroxidation of membrane polyunsaturated phospholipids perhaps by accelerating the cycling between Fe(ii) and Fe(iii) in the heme component of cytochrome P450 (Zou et al. 2020).

The tumor suppressor p53 has also been linked to oxytosis/ferroptosis. p53 inhibits cystine uptake by repressing expression of SLC7A11 thereby sensitizing cells to ferroptosis (Jiang et al. 2015). However, the activation of p53 alone is not sufficient to induce ferroptosis by itself.

Accumulating studies indicate a crosstalk between autophagy and oxytosis/ferroptosis. Autophagic degradation of the cellular iron storage protein ferritin in

lysosomes (ferritinophagy) results in the release of iron and increased sensitivity to ferroptosis (Hou et al. 2016; Gao et al. 2016) and inhibition of ferritinophagy by blockage of autophagy prevented oxytosis/ferroptosis. However, the use of autophagy inhibitors in AD would be unwise given that autophagy has been associated with interventions that prevent age-related pathology. Furthermore, the role of this pathway in oxytosis/ferroptosis appears to be cell type dependent (Soriano-Castell et al. 2021a).

More recently, inhibition of prohibitin 2 (PHB2) was shown to promote the expression of the iron storage protein ferritin, reducing the iron content and decreasing the susceptibility to oxytosis/ferroptosis in multiple cell lines exposed to erastin (Yang et al. 2022).

Just as steps that potentiate oxytosis/ferroptosis can be negatively regulated to confer cytoprotection, there are molecular pathways that can be enhanced to reduce the progression of oxytosis/ferroptosis. A good example is the activation of nuclear factor erythroid 2-related factor 2 (NRF2), a master transcription factor for regulating cellular antioxidation responses that often culminate in the mitigation of lipid peroxidation (Song and Long 2020; Dodson et al. 2019). Some of its downstream genes also encode proteins involved in the synthesis and metabolism of GSH, the regulation of iron metabolism, and the detoxification of reactive intermediates. Numerous activators of Nrf2 have been described and several of these are in clinical trials for neurological and other diseases (Robledinos-Anton et al. 2019).

Another important transcription factor is activating transcription factor 4 (ATF4), which promotes the expression of a variety of genes involved in amino acid import, GSH biosynthesis, and resistance to oxytosis/ferroptosis (Harding et al. 2003; Lewerenz et al. 2012; Lewerenz et al. 2014). Data indicate that ATF4 plays a key role in regulating basal GSH levels in neuronal and non-neuronal cells, whereas NRF2 appears to function as a multiplier of ATF4 activity under conditions of oxidative stress (Lewerenz and Maher 2009).

There are other molecular pathways that negatively regulate oxytosis/ferroptosis but that have yet to be properly tested in models of neurodegeneration. One of these is FSP1 which can act on both CoQ10 (discussed above) (Doll et al. 2019; Bersuker et al. 2019) and vitamin K (Mishima et al. 2022; Soriano-Castell et al. 2021b) to generate their reduced hydroquinone forms which are potent RTAs and inhibit oxytosis/ferroptosis. The cytosolic lipid transfer protein STARD7 might play a role in this pathway as well by ensuring the synthesis of CoQ10 in mitochondria and its transport to the plasma membrane to be used by FSP1 (Deshwal et al. 2023). Dihydroorotate dehydrogenase (DHODH) is another enzyme that works in parallel with GPX4 to inhibit oxytosis/ferroptosis by reducing CoQ10 to ubiquinol (Mao et al. 2021).

Although all these molecular regulators of oxytosis/ferroptosis may have the potential to be therapeutically targeted, whether they offer effective treatment avenues for AD without negatively affecting vital cellular processes will require rigorous testing using inhibitors or activators of these regulators in animal models, as discussed below.

As an alternative to target-based approaches, phenotypic screening can be used to identify therapeutics without the requirement of a known molecular target. Phenotypic screening uses biological models of a disease to identify agents that modify the phenotype to generate a positive outcome (Prior et al. 2014). Because of that, it often leads to the discovery of new targets when the protective action of a therapeutic is later investigated.

Phenotypic screening assays can be used that expose cultured nerve cells to inducers of oxytosis/ferroptosis, such as cystine deprivation, glutamate, erastin, and RSL3 (Maher et al. 2020). This approach has identified inhibitors of oxytosis/ferroptosis from a variety of sources, including libraries of natural compounds and natural extracts (Fischer et al. 2019; Soriano-Castell et al. 2021b). Once protective compounds are singled out, they may be further improved via medicinal chemistry in order to generate compounds with lower EC50s against oxytosis/ferroptosis (more potent) that are safe (mutagenicity, chromosomal stability, and cardiac safety), present optimal metabolism and pharmacokinetics (DMPK), and have good brain penetrance. As a guideline, successful CNS drugs should have MW $\leq$ 400, CLogP $\leq$ 5, tPSA $\leq$ 90, HBD $\leq$ 3, and HBA $\leq$ 7 for best penetration of the BBB (Hitchcock and Pennington 2006) (Pajouhesh and Lenz 2005; Prior et al. 2014). The goal is to improve the drug-like properties of a compound prior to testing it in animal models of AD.

The selection of animal models to study age-associated dementia has been a challenge (Gotz et al. 2018). The majority of the models for AD have been developed to mimic the aggregation of Aβ and rely on the expression of mutations that are found in human familial AD (FAD), including the amyloid precursor protein (APP) and presenilins (PSEN1 and PSEN2). However, as mentioned above, AD patients develop additional pathological features associated with old age which are thought to play a major role in the disease. As such, a combination of models to investigate various molecular pathological mechanisms and to test therapeutic candidates may be required. Since aging is the greatest risk factor for sporadic AD, models that reflect the impact of aging in dementia should be incorporated into preclinical testing. An example is the SAMP8 non-transgenic mouse model of accelerated aging which exhibits an age-related deterioration in learning and memory and develops a number of brain alterations similar to those found in AD, including increased oxidative stress, inflammation, vascular impairment, gliosis, Aβ deposition, and tau hyperphosphorylation (Currais et al. 2015; Pallàs 2012; Pallas et al. 2008; Takeda 2009). Preclinical studies with AD models can be complemented with the Gpx4BIKO mouse model of neuronal oxytosis/ferroptosis which utilizes an inducible *Gpx4* inducible knockout in forebrain neurons (Hambright et al. 2017). When treated with tamoxifen, these mice exhibit significant deficits in spatial learning and memory function associated with hippocampal neurodegeneration, markers of oxytosis/ferroptosis (lipid peroxidation), and neuroinflammation (astrocytosis and microgliosis) when compared to control mice (Hambright et al. 2017). A candidate oxytosis/ferroptosis inhibitor can be tested in these models for its effects on cognition as well pathological, biochemical, and histological changes associated with both AD and oxytosis/ferroptosis. These data

can be complemented by omic analyses (transcriptomics, proteomics, and metabolomics) that will provide a more integrative understanding of how the compounds are working (Ates et al. 2020; Currais et al. 2015; Currais et al. 2019).

This phenotypic screening-based approach has generated two very neuroprotective AD drug candidates developed in our laboratory, CMS121 and J147, that are the result of chemical optimization of the natural compounds fisetin and curcumin, respectively (Chen et al. 2011; Chiruta et al. 2012). Both CMS121 and J147 are very potent inhibitors of oxytosis/ferroptosis in nerve cell culture and reduce the brain pathology when administered to transgenic AD and SAMP8 mice (Ates et al. 2020; Chen et al. 2011; Currais et al. 2019). They appear to be doing so by maintaining mitochondrial function with aging through activation of the AMPK/ACC1 pathway (Currais et al. 2019). Their molecular targets were only later identified and are two proteins new to the process of cell death in oxytosis/ferroptosis, the mitochondrial ATP synthase for J147 (Goldberg et al. 2018) and FAS for CMS121 (Ates et al. 2020). Both of the compounds have gone through Investigational New Drug (IND) approval and are in Phase I clinical trials for AD (NCT03838185 and NCT05318040). These steps are discussed next.

It is worth mentioning that it is important to have multiple candidates that inhibit oxytosis/ferroptosis in the pipeline in order to increase the chances of one succeeding in clinical trials. This is particularly relevant when they target different proteins in the pathway. Given that oxytosis/ferroptosis still remains relatively unexplored from a therapeutic perspective, the development of additional inhibitors should be highly encouraged.

22.5 Preclinical Studies

In the USA, one of the biggest hurdles that needs to be surmounted in order to test a new drug candidate, such as a novel inhibitor of oxytosis/ferroptosis, in human patients is obtaining IND approval from the Food and Drug Administration (FDA). The specific requirements for this process can be obtained from multiple documents available on the FDA website (https://www.fda.gov/regulatory-information/fda-rules-and-regulations). The overall goal of the studies required for IND approval is to establish that the drug candidate will not expose human subjects to unreasonable risks when used in short-term, early-stage clinical trials. The IND application requires information in three areas: animal pharmacology and toxicology studies, manufacturing information and clinical protocols and investigator information. The animal pharmacology and toxicology studies comprise a variety of different types of information on the drug candidates including (1) studies on the pharmacological effects and mechanism(s) of action of the drug candidate in animals including safety pharmacology (effects on respiratory, cardiac, and brain function) as well as any information on the absorption, distribution, metabolism, and excretion (ADME) of the drug candidate in animals and (2) studies on the toxicological effects of the drug candidate in animals (e.g., effects on cardiac function, respiration, kidney function) and in vitro (e.g., genotoxicity, mutagenic potential) as appropriate. For

age-associated diseases, the latter studies would likely not include assessment of the drug candidate's reproductive and fetal effects. However, it would include information on the drug candidate's potential genotoxicity.

A critical goal of the animal toxicity studies is to establish both the maximum tolerated dose (MTD) which is the highest dose that does not cause unacceptable side effects and the No Observed Adverse Effects Limit (NOAEL) dose which is the highest dose that does not cause any side effects for the drug candidate. This information will be used to determine the starting dose and the dose escalations for the first-in-human (FIH) study (Phase 1 clinical trial) and is determined from repeated-dose toxicity studies which need to be done in two species, one rodent (usually rats) and one non-rodent (often dog), for a minimum of 2 weeks. These studies must use the same route and schedule of administration as is planned for the clinical use of the drug candidate in humans. The duration of these studies depends on the length of the planned clinical trial. For a clinical trial longer than 6 months, the recommended minimum duration for repeated-dose toxicity studies is 6 months in rodents and 9 months in non-rodents.

The manufacturing information includes information on the composition, manufacture, and stability of the drug candidate as well as the controls used for manufacturing both the drug candidate itself and the drug product which would include any additional ingredients that are used to formulate the product that will be tested in humans.

The clinical protocols and investigator information consist primarily of a detailed protocol for the FIH study proposed for the drug candidate as well as information on the qualifications of the clinical investigators who will oversee the Phase 1 clinical trial. The IND application should also include detailed information on previous human experience with the drug candidate if it has been investigated or marketed previously either in the USA or other countries.

All drug candidates that are new chemical entities require IND approval. Furthermore, any drug candidate, regardless of its source (e.g., natural products), requires IND approval for human studies if the intention of the clinical trial(s) is to test its ability to cure, mitigate, treat, or prevent a disease. However, if the intention of the clinical trial is to test the ability of the drug candidate to affect the structure or function of the body but not to be used for a therapeutic purpose and the drug candidate is already used as a dietary supplement or food, then the clinical trial does not require IND approval. Once an IND application is submitted, the FDA has 30 days to review it for safety concerns. If no concerns are noted, then the investigator can proceed with the clinical trial.

22.6 The Clinical Testing Path, Challenges, and Hope

Clinical trials proceed in phases (Fig. 22.4). For excellent descriptions of all phases of clinical trials for AD see Alzheimer's Disease Drug Development (Cummings et al. 2022a).

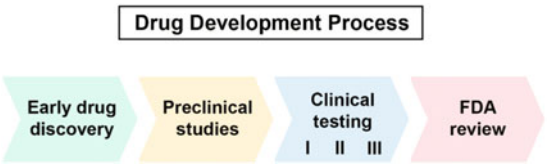

Fig. 22.4 The drug development process

Phase 1 trials are used to assess the safety and tolerability of a drug candidate in humans as well as to determine human pharmacokinetics. Young healthy male and female volunteers are usually used for these studies. There are generally two parts to these studies: a single ascending dose (SAD) study where single doses are given to small cohorts of participants and there is a series of increases in dose until the prespecified maximal dose is reached or safety and tolerability concerns require an early termination, and a multiple ascending dose (MAD) study where multiple doses are given to trial participants over a fixed period of time (usually 7 days) and there is a series of increases in dose until the prespecified maximal dose in reached or safety and tolerability concerns require an early termination. In both cases, a variety of physiological parameters are monitored to ensure that the drug candidate is not adversely affecting key organs. In addition, blood and sometimes urine samples are taken to determine pharmacokinetic parameters. Escalation to the next higher dose only occurs following evaluation of the physiological results from the previous dose to ensure the safety of the participants. The most critical aspect in initiating these studies is deciding on the starting dose which is based on the NOAEL level determined in the animal toxicology studies as discussed earlier.

If the drug candidate appears safe and is well tolerated by the participants in the Phase 1 trial, then a Phase 2 trial can be initiated. This is often the first time that the drug candidate is tested in the target population which in the case of AD can be people at risk of developing AD, people with mild cognitive impairment (MCI) or people at various stages of AD depending on how the drug candidate is expected to work. Phase 2 trials for AD frequently include multiple studies and can have several goals including determining the safety and tolerability of one or more doses as determined by the Phase 1 studies in the target population, establishing target engagement by using appropriate biomarkers and assessing early signals of drug efficacy which can be based either on biomarkers or on measures of relevant outcomes such as improvements in cognitive function and/or reductions in functional impairments. For AD drug candidates and particularly for those thought to be most effective at early stages of the disease, these latter two outcomes can take a relatively long period of time to be manifest so Phase 2 trials that last 12 months or longer are often needed. Phase 2 trials have a much better chance of success if the study population is relatively homogeneous based on strict inclusion and exclusion criteria since the numbers of subjects are typically small (100–300). Unlike the Phase 1 trials, the design of Phase 2 trials is not fixed and should be configured to fit both the drug candidate and the target population to be treated. Furthermore, Phase 2 trials provide room for experimentation, especially with respect to biomarker and clinical outcome readouts.

The goal of Phase 3 trials is to provide definitive evidence for drug efficacy at a predetermined dose. These trials include large numbers of subjects generally recruited over multiple sites and, for AD, usually last several years to ensure that there is a clear, drug-related effect on the disease. Previously, approval of a drug candidate for AD required clear indications of efficacy against the clinical hallmarks of the disease including decreases in cognitive function and/or increases in functional impairments. However, the two recent AD drug approvals by the FDA (see above) were based solely on the effects of the drugs on brain Aβ levels. Whether this will translate into real clinical benefits for AD patients remains to be determined.

22.7 Biomarkers

As noted above, biomarkers are becoming an increasingly important part of clinical trials. They are used both to select subjects for clinical trials as well as to determine the effects of potential disease-modifying therapies. For excellent overviews of several aspects of biomarkers for AD drug trials, see Alzheimer's Disease Drug Development (Cummings et al. 2022a). Biomarkers encompass both brain imaging biomarkers and fluid biomarkers in cerebrospinal fluid (CSF) and/or blood (plasma and/or serum). These can be used to monitor both specific aspects of AD pathology and markers of drug candidate target engagement which may not necessarily be the same if the immediate drug candidate target is not directly connected to AD pathology. For example, if it modulates oxytosis/ferroptosis.

The two main approaches to imaging for AD are magnetic resonance imaging (MRI) and positron emission tomography (PET). MRI provides high-resolution imaging of the brain and so can be used to determine the volume and integrity of different brain regions. In contrast to PET, it does not require radiation. Hippocampal atrophy, as measured by a decrease in volume using MRI, is seen early in AD and continues throughout the progression of the disease. Although it is not specific to AD, it can still provide important information regarding neuronal degeneration. MRI can also be used to measure brain GSH and iron levels. Although these two changes are not exclusive to oxytosis/ferroptosis, changes in their levels are characteristic of the activation of this pathway and so their measurement could provide some indication of whether a drug candidate was acting on this pathway.

PET imaging can detect the levels and brain location of a specific molecular target by using an injected target probe that is labeled with a positron-emitting isotope with a relatively short half-life (a few hours). PET can be used to detect a number of targets related to AD including amyloid and tau as well as alterations in glucose metabolism or inflammation. The levels of binding of PET probes for amyloid begin to increase early in disease development and then plateau in early AD while the levels of binding of PET probes for tau increase more continuously throughout the course of the disease. Interestingly, early increases in tau and amyloid deposition are seen in distinct parts of the brain and only begin to overlap later in disease development. Regional glucose metabolism is measured using FDG PET and is thought to be tightly coupled to synaptic function. It begins to decrease prior to clinical

diagnosis of AD and continues to decline thereafter. The tracers that have been used to monitor inflammation by PET imaging bind to the translocator protein (TSPO) which is an outer mitochondrial membrane protein that is upregulated in neuroinflammation and was thought to be specific for microglial activation although more recent evidence suggests upregulation in astrocytes and endothelial cells as well. These inflammation tracers are still in the experimental stage due to problems with affinity, signal-to-noise ratio, and blood–brain barrier permeability.

The core CSF AD biomarker profile includes Aβ42 and/or the Aβ42/Aβ40 ratio along with phospho tau (usually p-tau$_{181}$) and total tau. Decreases in Aβ42 and the Aβ42/Aβ40 ratio are seen in both prodromal AD and AD as are increases in both phospho and total tau. Markers of neurodegeneration such as neurogranin and neurofilament light chain (NfL) can also be measured in CSF and can provide additional information about disease progression although they are not specific to AD.

While both imaging and CSF biomarkers are very good at identifying AD pathology, they have some drawbacks including the need for special facilities, expense, limited availability, and, in the case of CSF, invasiveness. Thus, there has been an increasing emphasis on developing blood-based biomarkers [for review, see (Klyucherev et al. 2022)]. However, the levels of Aβ42, Aβ40 as well as phospho tau/tau in the blood are much lower than in CSF so measurement requires very sensitive techniques (e.g., SIMOA). Both neurogranin and NfL, the markers of neurodegeneration, can also be measured in blood and increases in plasma NfL levels are seen early in disease development and increase with disease progression. Blood markers of inflammation have the potential to provide additional information about disease status and progression but whether distinct patterns are specific for AD is still being investigated. Although there are no blood biomarkers specific to oxytosis/ferroptosis yet, changes in GSH, lipid peroxides, and by-products of lipid peroxidation are characteristic of pathway activation and could be used to determine if a specific drug candidate might be acting on this pathway.

In summary, while a number of biomarkers are available for use in clinical trials, what to use depends on the nature of the participants in the trials, the type of drug candidate and the goals for its use as well as cost and the level of invasiveness participants are willing to tolerate.

22.8 Discussion and Conclusion

Since it was first described, oxytosis/ferroptosis has emerged as an important toxicity pathway in a number of different human diseases. Importantly, the observation that it has well-defined molecular steps makes therapeutic interventions possible. In addition to AD, features of oxytosis/ferroptosis have been observed in heart, liver, vascular, and kidney diseases (Han et al. 2020; Li et al. 2020b; Fang et al. 2019; Gao et al. 2015; Friedmann Angeli et al. 2014; Martin-Sanchez et al. 2017; Wang et al. 2019; NaveenKumar et al. 2018). Therefore, the work outlined in this review is relevant to all human pathology in which oxytosis/ferroptosis is a driving force.

Ferroptosis was named in 2012 as an iron-dependent, non-apoptotic form of cell death in cancer cells (Dixon et al. 2012). While inducing oxytosis/ferroptosis or increasing susceptibility to it may treat certain cancer cells, unless highly specific to those cells, these approaches have the potential to accelerate age-related diseases that may be driven by oxytosis/ferroptosis, such as AD. Although the reverse situation also raises caution, i.e., using inhibitors of oxytosis/ferroptosis to treat AD that may accelerate cancer growth, the fact is that surprisingly some of these inhibitors actually possess anti-tumorigenic activity. This could be because some compounds are good inhibitors of lipid peroxidation while simultaneously increasing ROS production (Soriano-Castell et al. 2021b), which is lethal to cancer cells. Another possibility could be that the inhibition of metabolic reactions that lead to the protection against oxytosis/ferroptosis also limits cancer growth, such as the fatty acid biosynthetic pathway (ACC1 and FASN), as mentioned above. What perhaps should not be done is to use inducers of oxytosis/ferroptosis non-specifically as co-adjuvants in cancer treatments without understanding fully the implications to the rest of the body.

It is worth noting that an inverse relationship between the incidence of cancer and the incidence of AD has been detected by several cross-sectional studies (Demetrius and Simon 2012; Driver et al. 2012; Bowles et al. 2017). Not only is AD associated with a reduced risk of cancer, the incidence of cancer is associated with a reduced risk of AD (Demetrius and Simon 2012; Driver et al. 2012; Musicco et al. 2013; Plun-Favreau et al. 2010). This could be explained by metabolic pathways that are relevant to both cancer and neuroprotection. For instance, the hypoxia-inducible factor 1-alpha (HIF1α) and glycolysis pathways are pro-tumorigenic and have also been shown to be neuroprotective (Soucek et al. 2003; Majmundar et al. 2010). Clearly, the metabolic similarities and disparities between cancer and AD warrant further investigation.

A key idea taken from the studies discussed here is that a combination of therapies that target different aspects of oxytosis/ferroptosis might be the most effective method of treating the pathway in AD. This is because not only would it allow for synergy between drugs but also might make it feasible to use lower amounts of those drugs and thereby reduce any potential side effects. Another advantage is the possibility of the drugs targeting oxytosis/ferroptosis in different cell types, tissues, etc. This is of particular relevance in age-associated diseases such as AD, where the progressive dysfunction of peripheral organs with aging can contribute to the exacerbation of the disease in the CNS (Aczel et al. 2022). Although clinical trials with multiple drug candidates pose an additional layer of challenges, we think that this could be very much worthwhile.

In summary, considering the failure rate of AD drug candidates in clinical trials, there is clearly a need to identify additional compounds with therapeutic potential based on less explored target pathways. The knowledge regarding the molecular processes governing oxytosis/ferroptosis has come a long away in recent years and the evidence is strong in support of the hypothesis that the pathway may play a key pathological role in AD. We have discussed how potent inhibitors of oxytosis/ferroptosis can be developed to hasten the fight against this devastating disease.

Furthermore, since AD is highly heterogeneous and many drug candidates fail at the IND or clinical trial steps, multiple drug candidate inhibitors of oxytosis/ferroptosis that target the pathway at different regulatory steps will likely be required. Therefore, there are many opportunities for future studies.

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