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PERSPECTIVE

# Mitochondrial regulation of ferroptosis

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**Ferroptosis is a form of iron-dependent regulated cell death driven by uncontrolled lipid peroxidation. Mitochondria are double-membrane organelles that have essential roles in energy production, cellular metabolism, and cell death regulation. However, their role in ferroptosis has been unclear and somewhat controversial. In this Perspective, I summarize the diverse metabolic processes in mitochondria that actively drive ferroptosis, discuss recently discovered mitochondria-localized defense systems that detoxify mitochondrial lipid peroxides and protect against ferroptosis, present new evidence for the roles of mitochondria in regulating ferroptosis, and outline outstanding questions on this fascinating topic for future investigations. An in-depth understanding of mitochondria functions in ferroptosis will have important implications for both fundamental cell biology and disease treatment.**

## Introduction

Ferroptosis is an iron-dependent form of regulated cell death that is triggered by the toxic buildup of lipid peroxides on cellular membranes (Hadian and Stockwell, 2020; Jiang et al., 2021; Stockwell et al., 2017). While this form of cell death had been implicated from prior studies (Bannai et al., 1977; Eagle, 1955a; Eagle, 1955b; Seiler et al., 2008), the term ferroptosis was coined by Stockwell and colleagues in 2012 (Dixon et al., 2012), and in recent years, it has attracted considerable interest in scientific communities, partly because ferroptosis represents a novel cell death mechanism that is morphologically and mechanistically distinctive from other known forms of regulated cell death such as apoptosis and necroptosis (Galluzzi et al., 2018).

Recent studies have further linked ferroptosis to various pathological conditions and diseases. Ferroptosis has been recognized as an important tumor-suppression mechanism, and impaired ferroptosis has been shown to contribute to tumor development (Jiang et al., 2015; Zhang et al., 2018). In contrast, excessive ferroptosis has been causally associated with diverse degenerative diseases, ischemia/reperfusion–induced organ injury, myocardial infarction, stroke, and acute kidney injury (Belavgeni et al., 2020; Jiang et al., 2021; Ratan, 2020; Stockwell et al., 2020). Hence, great interest has been expressed in exploring ferroptosis inducers (FINs) or ferroptosis inhibitors (see text box) to treat these diseases. Specifically, FINs have been proposed to treat certain cancers that are particularly susceptible to ferroptosis (such as therapy-resistant cancers or *NF2* mutant mesothelioma; Hangauer et al., 2017; Viswanathan et al., 2017; Wu et al., 2019) or be combined with immunotherapy, radiotherapy, and targeted therapies to improve therapeutic efficacy in cancer treatment (Lang et al., 2019; Lei et al., 2021a; Lei et al., 2020; Wang et al., 2019; Ye et al., 2020; Yi et al., 2020; Zhang et al., 2021). Conversely, ferroptosis inhibitors have been nominated to treat those diseases caused by excessive ferroptosis (Belavgeni et al., 2020; Hassannia et al., 2019; Jiang et al., 2021; Ratan, 2020; Stockwell et al., 2020; Zou and Schreiber, 2020).

Mitochondria are double membrane–bound organelles that conduct oxidative phosphorylation and generate most of the energy (in the form of ATP) in eukaryotic cells. Through a series of electron transport chain (ETC) complexes localized in the mitochondrial inner membrane, including complexes I, II, III, and IV, electrons are transferred from electron donors to electron acceptors and ultimately to oxygen. This electron transfer is coupled with proton pumping from mitochondrial matrix into the intermembrane space to establish proton motive force, which eventually drives ATP synthesis through complex V (ATP synthase). The mitochondrion is also the major organelle to produce reactive oxygen species (ROS) and hosts many other important metabolism processes, such as the tricarboxylic acid (TCA) cycle (Friedman and Nunnari, 2014); in addition, mitochondria have vital roles in governing multiple forms of regulated cell death (Chipuk et al., 2021).

Considering the central role of mitochondria in cell death regulation (Chipuk et al., 2021; Green and Kroemer, 2004; Green and Reed, 1998; Li et al., 2004; Newmeyer and Ferguson-Miller, 2003) and the intimate link between ferroptosis and cellular metabolism (Gao and Jiang, 2018; Gao et al., 2015; Gao et al., 2019; Lee et al., 2020; Stockwell et al., 2017; Zheng and Conrad, 2020), it has been postulated since the discovery of ferroptosis that the mitochondrion might also participate in governing ferroptosis. Indeed, the most notable morphological

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**Ferroptosis inhibitors and FINs**

Most currently identified ferroptosis inhibitors are radical-trapping antioxidants, which can trap chain-carrying radicals and thereby block propagation of the radical chain reactions during lipid peroxidation. For example, α-tocopherol and ubiquinol (which can be produced by FSP1 or DHODH from ubiquinone, as discussed in the main text) are two naturally occurring common radical-trapping antioxidants that are capable of inhibiting ferroptosis. Ferrostatin-1 and liproxstatin-1 are radical-trapping antioxidants that were identified from screenings as potent ferroptosis inhibitors (Dixon et al., 2012; Friedmann Angeli et al., 2014). These two ferroptosis inhibitors have been widely used in ferroptosis research to examine whether any given cell death is indeed ferroptosis and hold great potential for treating diseases caused by excessive ferroptosis. Readers are referred to a recent review for a detailed description of chemical structures and mechanisms of these ferroptosis inhibitors as radical-trapping antioxidants (Conrad and Pratt, 2019).

FINs are compounds or treatments that are capable of inducing ferroptosis. FINs can be categorized into at least four classes based on their mechanisms of action (Feng and Stockwell, 2018). Class 1 FINs operate by blocking SLC7A11-mediated cystine uptake and include erastin and erastin analogues such as imidazole ketone erastin, as well as sulfasalazine and sorafenib. Glutathione, the cofactor of GPX4, is built up from cysteine, glutamate, and glycine, among which cysteine is the rate-limiting precursor for glutathione. Most cells obtain intracellular cysteine mainly through taking up cystine (the oxidized dimeric form of cysteine) from the extracellular milieu via SLC7A11 (Koppula et al., 2021a; Fig. 1). Consequently, cystine starvation or blockade of SLC7A11 cystine transporter activity by class 1 FINs depletes intracellular glutathione levels and induces ferroptosis. Class 2 FINs act by directly inhibiting the enzymatic activity of GPX4 and include RSL3, ML162, and ML210. Class 3 FINs, such as FIN56, induce ferroptosis by depleting both GPX4 protein and CoQ (which, upon being reduced to $CoQH_2$, can serve as a radical-trapping antioxidant to suppress ferroptosis; Shimada et al., 2016), whereas FINO2, the only class 4 FIN, acts by oxidizing iron and indirectly inactivating GPX4 (Gaschler et al., 2018a).

Notably, these FINs have different effects on the induction of mitochondrial lipid peroxidation. Class 2 FINs typically do not induce, while class 1 and 3 FINs can induce, potent mitochondrial lipid peroxidation (Gao et al., 2019; Mao et al., 2021). As discussed in the main text, mitochondrial GPX4 and DHODH constitute two main defense arms to suppress mitochondrial lipid peroxidation. Upon GPX4 inactivation by class 2 FINs, DHODH can still provide the other defense arm to detoxify mitochondrial lipid peroxides; consequently, cells treated with class 2 FINs die of ferroptosis mainly induced by nonmitochondrial lipid peroxidation, which explains the lack of strong mitochondrial lipid peroxidation in response to class 2 FINs. Because DHODH suppresses mitochondrial lipid peroxidation through reducing CoQ to $CoQH_2$, class 3 FINs (such as FIN56), by depleting both GPX4 and ubiquinone, disable both defense arms in mitochondria and induce potent mitochondrial lipid peroxidation. Considering that both classes 1 and 2 FINs target the SLC7A11–glutathione–GPX4 signaling axis (Fig. 1), it remains unclear how class 1 FINs (such as erastin) can induce mitochondrial lipid peroxidation (Gao et al., 2019), which suggests that, in addition to GPX4, other downstream effectors of class 1 FINs may exist to regulate mitochondrial lipid peroxidation. Consistent with this, a recent CRISPR screen identified very different profiles of ferroptosis modifiers upon cystine deprivation and GPX4 inhibition (Soula et al., 2020). Whether FINO2 induces mitochondrial lipid peroxidation remains unknown.

feature of ferroptotic cells under electron microscopy is the change in mitochondrial morphology; ferroptotic cells typically contain shrunken mitochondria with increased membrane density (Dixon et al., 2012). However, early findings suggested that cells lacking mitochondrial DNA (so-called $\rho^0$ cells) are as sensitive to ferroptosis as parental cells with intact mitochondria DNA (Dixon et al., 2012). Further, using similar mitophagy protocols to deplete mitochondria in cells, one study showed that cells depleted of mitochondria are still capable of undergoing ferroptosis (Gaschler et al., 2018b), while the other concluded that mitochondria-depleted cells exhibit significantly attenuated ferroptosis induced by class 1 FINs (erastin treatment or cystine starvation; see text box; Gao et al., 2019). (To put this in the context, using similar methods, earlier studies showed that mitochondria depletion via mitophagy does not appear to compromise RIPK3-dependent necroptosis [Tait et al., 2013]; thus, the observations that mitochondria depletion affects ferroptosis, but not other forms of necrotic cell death such as necroptosis, have stimulated a lot of interests in the ferroptosis field.) Likewise, treatment with ETC complex I inhibitors was shown to promote lipid peroxidation and ferroptosis (Basit et al., 2017), yet another study revealed that inhibiting complex I either does not affect ferroptosis induced by class 2 FINs (such as RSL3; see text box) or blocks ferroptosis induced by class 1 FINs (Gao et al., 2019).

Cells have defense mechanisms in place to detoxify lipid peroxides on cellular membranes, prominent among which is glutathione peroxidase 4 (GPX4), a peroxidase that uses reduced glutathione (GSH) as its cofactor to reduce lipid peroxides to lipid alcohols, thereby mitigating ferroptosis (Friedmann Angeli et al., 2014; Yang et al., 2014). Consequently, inactivation of GPX4, genetically or pharmacologically (by its inhibitors such as RSL3), curtails cellular antioxidant defenses against ferroptosis and unleashes potent ferroptotic cell death in many cell lines in vitro and in animals in vivo (Friedmann Angeli et al., 2014; Ingold et al., 2018; Matsushita et al., 2015; Yang et al., 2014). Lipid peroxidation is a hallmark of ferroptosis (Dixon et al., 2012; Stockwell et al., 2017). Notably, GPX4 inactivation induces potent lipid peroxidation in many cell lines (as revealed by C11-BODIPY staining) but typically does not induce strong mitochondrial lipid peroxidation under the same conditions (by staining of mitochondria-targeted version of C11-BODIPY), indicating that GPX4 inactivation–induced ferroptosis is mainly triggered by nonmitochondrial lipid peroxidation (Friedmann Angeli et al., 2014). In addition, the mitochondria-targeted antioxidant MitoTEMPO was reported to block doxorubicin-induced cardiac ferroptosis in mice, which provided strong in vivo evidence to link mitochondria to ferroptosis (Fang et al., 2019). However, another study showed that MitoTEMPO had a limited protective effect on ferroptosis induced by the combined treatment of cotylenin A and phenethyl isothiocyanate in pancreatic cancer cells (Kasukabe et al., 2016). These studies therefore suggest a context-dependent role of mitochondrial lipid peroxidation in ferroptosis.

Together, these studies suggest a complex and somewhat controversial role of mitochondria in ferroptosis. On the basis of a very recent study describing mitochondria-derived defense mechanisms against ferroptosis (Mao et al., 2021), this Perspective article aims to discuss both pro-ferroptosis functions of and ferroptosis defense mechanisms in mitochondria, reconcile current conflicting data on the role of mitochondria in ferroptosis regulation, and propose important unanswered questions on this fascinating topic for future investigations.

## An overview of the ferroptosis pathway

Polyunsaturated fatty acids (PUFAs) are fatty acids that contain more than one double bond. Incorporating PUFA-containing

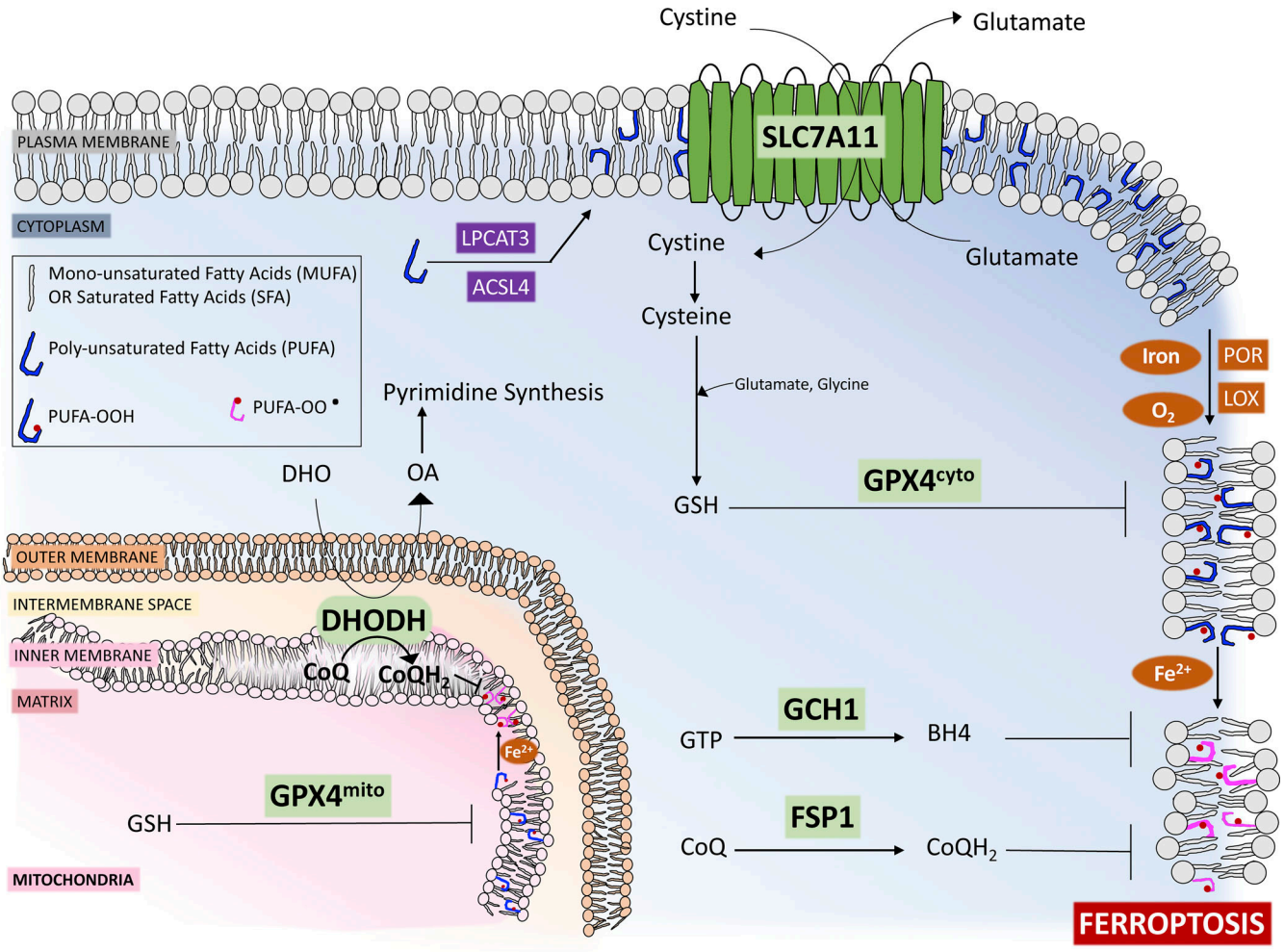


Figure 1. **The ferroptosis pathway.** ACSL4 and LPCAT3 mediate the biosynthesis of PUFA-containing PLs, which are susceptible to peroxidation through both nonenzymatic and enzymatic mechanisms. Excessive accumulation of lipid peroxides on cellular membranes can result in ferroptosis. Cells have evolved at least four defense systems with different subcellular localization to keep lipid peroxides in check and thereby protect cells against ferroptosis, including (1) cytosolic and mitochondrial GPX4, (2) FSP1 on plasma membrane, (3) DHODH in mitochondria, and (4) GCH1 (whose exact subcellular localization remains unclear). BH4, tetrahydrobiopterin; cyto, cytosolic; DHO, dihydroorotate; mito, mitochondrial; OA, orotate.

phospholipids (PLs) into cellular membranes is critical for maintaining membrane fluidity and signaling in cells. PUFA-PL biosynthesis requires lipid metabolism enzymes such as acyl-coenzyme A (CoA) synthetase long-chain family member 4 (ACSL4) and lysophosphatidylcholine acyltransferase 3 (LPCAT3; Fig. 1). However, PUFAs can also lead to adverse effects in cells; PUFAs often contain bis-allylic moieties with the methylene groups flanked by the C-C double bonds. The C-H bonds in such methylene groups are among the weakest ones and are very susceptible to oxidation (Conrad and Pratt, 2019). The oxidation of PUFA-PLs occurs through both nonenzymatic free-radical chain reactions called autoxidation (which requires oxygen and Fenton reactions) and enzymatic reactions (Conrad and Pratt, 2019; Yin et al., 2011; Fig. 1). Whereas enzymatic PUFA-PL oxidation was initially thought to be mainly mediated by lipoxygenases (LOXs; such as ALOX12 and ALOX15; Wenzel et al., 2017; Yang et al., 2016), more recent studies challenged this model and showed that PUFA-PL oxidation in most cancer cells is primarily mediated by cytochrome P450 oxidoreductase (POR; Koppula et al., 2021b; Shah et al., 2018; Yan et al., 2021; Zou et al., 2020b). Consequently, iron chelation or inactivation of ACSL4, LPCAT3, or POR blocks or significantly attenuates ferroptosis (Dixon et al., 2012; Dixon et al., 2015; Doll et al., 2017; Kagan et al., 2017; Yan et al., 2021; Zou et al., 2020b).

As noted above, lipid peroxidation requires iron-mediated Fenton reactions. In addition, LOXs and POR, which drive lipid peroxidation, are also iron-dependent enzymes (Wenzel et al., 2017; Yang et al., 2016; Zou et al., 2020b). These together at least partly explain the iron-dependent nature of ferroptotic cell death. Iron metabolism is subjected to tight regulation via its uptake, storage, utilization, and export. Iron is taken up into cells mainly via transferrin receptor 1 (TFR1; which transports ferritin-bound iron through receptor-mediated endocytosis). Transferrin and TFR1 have been shown to be required for ferroptosis under different contexts (Gao et al., 2015; Yu et al., 2020). Interestingly, TFR1 was also recently identified and validated as a ferroptosis marker (Feng et al., 2020; Lei et al., 2021b). Cytosolic iron is stored in ferritin, and iron can be released from

ferritin through nuclear receptor coactivator 4 (NCOA4)–mediated ferritinophagy (an autophagic degradation of ferritin). Likewise, deficiency of NCOA4 or other autophagy genes, such as ATG5 and ATG7, suppressed ferroptosis by limiting labile iron pool available for lipid peroxidation (Gao et al., 2016; Hou et al., 2016). Iron export is primarily mediated by ferroportin 1 and further facilitated by prominin 2, which promotes the formation of ferritin-containing exosomes that export iron out of cells. Deficiency of ferroportin 1 or prominin 2 promotes ferroptosis by increasing intracellular iron labile pools (Brown et al., 2019; Geng et al., 2018). Therefore, iron metabolism has an essential role in ferroptosis governance through multiple mechanisms (Chen et al., 2020).

To counteract these adverse effects caused by ferroptosis-inducing metabolic activities, cells have evolved various defense mechanisms to quench toxic lipid peroxides and therefore ensure appropriate cell survival. Until very recently, three such defense mechanisms have been identified. The primary cellular defense pathway against ferroptosis is the solute carrier family 7 member 11 (SLC7A11)–GPX4 signaling axis, wherein GPX4 uses GSH as its cofactor to quench lipid hydroperoxides and SLC7A11 imports cystine for GSH biosynthesis (Dixon et al., 2012; Friedmann Angeli et al., 2014; Koppula et al., 2018; Koppula et al., 2021a; Stockwell et al., 2017; Yang et al., 2014; Fig. 1). Inactivation of GPX4 or SLC7A11 by corresponding FINs (such as RSL3 and erastin, respectively) or cystine starvation induces potent ferroptosis in many cancer cells (Dixon et al., 2012; Friedmann Angeli et al., 2014; Jiang et al., 2015; Yang et al., 2014; Zhang et al., 2018).

Recent studies identified ferroptosis suppressor protein 1 (FSP1; also called AIFM2) as another ferroptosis inhibitor that acts independently of GPX4 to suppress ferroptosis (Bersuker et al., 2019; Doll et al., 2019). Mechanistically, FSP1 acts as an oxidoreductase mainly localized on the plasma membrane and to reduce ubiquinone (coenzyme Q, or CoQ) to ubiquinol ($CoQH_2$; Bersuker et al., 2019; Doll et al., 2019; Fig. 1). CoQ is a lipophilic metabolite that consists of a redox active quinone head group and a long polyisoprenoid lipid tail and serves as a critical electron transport carrier in mitochondrial ETCs (Stefely and Pagliarini, 2017). Its fully reduced form, $CoQH_2$, can also serve as a radical-trapping antioxidant to detoxify lipid peroxyl radicals. Therefore, FSP1 was proposed to suppress ferroptosis through generating $CoQH_2$ on the plasma membrane (Bersuker et al., 2019; Doll et al., 2019). Finally, GTP cyclohydrolase 1 (GCH1) was recently identified as yet another ferroptosis suppressor through its functions in regulating the levels of PLs with two PUFA tails and generating tetrahydrobiopterin ($BH_4$), another radical-trapping antioxidant with anti-ferroptosis activity (Kraft et al., 2020; Soula et al., 2020; Fig. 1). It appears that, unlike GPX4 inactivation, inactivation of FSP1 or GCH1 alone is insufficient to trigger strong ferroptosis in most cell lines with adequate expression of GPX4 (that is, unlike *GPX4* knockout [KO] cells, *FSP1* KO or *GCH1* KO cells are generally viable); rather, their inactivation weakens cellular defense systems against ferroptosis and thereby significantly sensitizes cells to GPX4 inactivation–induced ferroptosis (Bersuker et al., 2019; Doll et al., 2019; Kraft et al., 2020; Soula et al., 2020).

## The pro-ferroptosis functions of mitochondria

Unlike their role in apoptosis regulation, wherein mitochondria mainly serve as a passive reservoir for various apoptosis-inducing factors such as cytochrome *c* and Smac (Green and Reed, 1998; Jiang and Wang, 2004; Li et al., 2004; Newmeyer and Ferguson-Miller, 2003), current studies indicate that diverse metabolic activities in mitochondria have more active roles in inducing ferroptosis. First, the mitochondrion is a major source of cellular ROS (Murphy, 2009). Specifically, the electron leakage from ETC complexes I and III produces superoxide ($O_2^{\bullet -}$), which is subsequently converted to hydrogen peroxide ($H_2O_2$) through superoxide dismutase (SOD)–mediated dismutation (Murphy, 2009). $H_2O_2$ can react with ferrous ion ($Fe^{2+}$) to generate hydroxyl radicals ($^{\bullet}OH$), which then abstract the bis-allylic hydrogen in PUFAs to generate PUFA radicals (PUFA$^{\bullet}$). These unstable carbon-centered radicals react rapidly with oxygen to form PUFA peroxyl radicals (PUFA-OO$^{\bullet}$), which ultimately form PUFA hydroperoxides (PUFA-OOH; Murphy, 2009; Zheng and Conrad, 2020; Fig. 2). Therefore, mitochondrial ROS production likely contributes to ferroptosis induction through promoting lipid peroxidation. Consistent with this, treatment with MitoQ (a mitochondria-targeted version of the lipophilic antioxidant decylubiquinone) was found to significantly rescue GPX4 inactivation–induced ferroptosis (Jelinek et al., 2018). However, another study reported that more than two orders of magnitude higher concentrations of MitoQ are needed to achieve a ferroptosis-rescuing effect similar to that of decylubiquinone (Friedmann Angeli et al., 2014). Because MitoQ is not exclusively targeted to mitochondria, it is possible that the ferroptosis-rescuing effect of MitoQ at high concentrations relates to its ability to quench lipid peroxides on other nonmitochondrial membranes.

The mitochondrion is also the major organelle to generate ATP, the cellular energy currency. Electron transport through the ETC complexes generates proton motive force, which is coupled with energy production through ATP synthase (Friedman and Nunnari, 2014; Vasan et al., 2020). Notably, recent studies showed that energy depletion (such as glucose starvation) activates the energy sensor AMP-activated protein kinase (AMPK), which potently suppresses the synthesis of some PUFAs and ferroptosis through phosphorylating and inactivating acetyl-CoA carboxylase (ACC; a rate-limiting enzyme in fatty acid biosynthesis), whereas AMPK inactivation promotes ferroptosis (Lee et al., 2020; Li et al., 2020). Consistent with this, treatment with various ETC complex inhibitors or mitochondrial uncoupling agents was shown to significantly block ferroptosis induced by class 1 FINs, suggesting that electron transport and proton pumping in mitochondria play important roles in inducing ferroptosis (Gao et al., 2019), possibly through generating ATP and subsequently inactivating AMPK (Fig. 2).

Finally, mitochondria also have a biosynthetic role in cellular metabolism. The mitochondrion houses the TCA cycle and various anaplerotic reactions that replenish the TCA cycle, such as glutaminolysis (Friedman and Nunnari, 2014). Glutaminolysis inhibition or glutamine starvation was shown to significantly suppress ferroptosis induced by class 1 FINs (Dixon et al., 2012; Gao et al., 2015); supplementation of α-ketoglutarate (αKG; a TCA metabolite linked to the glutaminolysis pathway) as well as

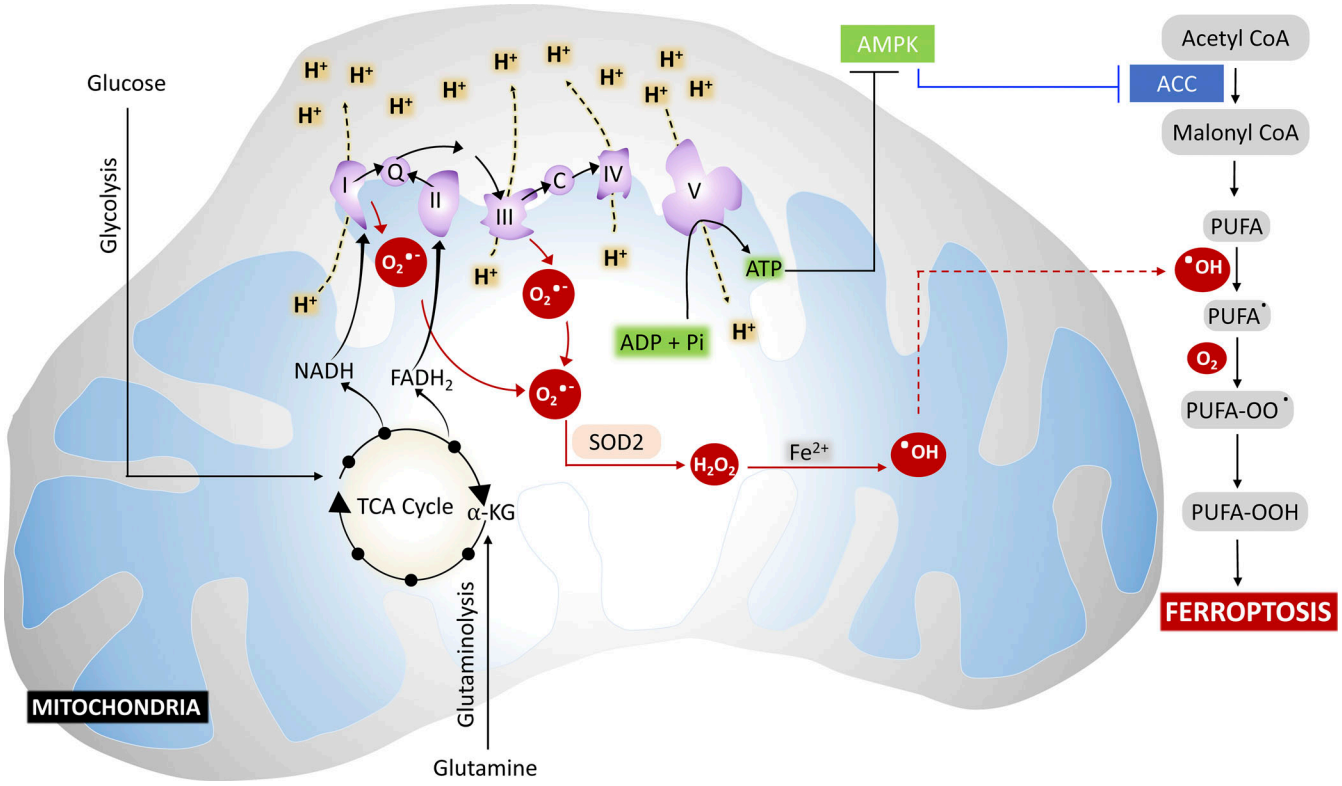


Figure 2. **The role of mitochondria in promoting ferroptosis.** Energy stress–mediated AMPK activation blocks ferroptosis by suppressing ACC-mediated conversion of acetyl-CoA to malonyl-CoA, a precursor for PUFA synthesis. ETCs in mitochondria drive proton motive force and ATP synthesis, which counteracts energy stress–induced AMPK activation and thereby promotes ferroptosis. In addition, the electron leakage from ETC complexes I and III produces $O_2^{\bullet-}$, which can promote PUFA peroxidation and thereby ferroptosis. Glutaminolysis and the TCA cycle in mitochondria can drive ETC activities and further promote ferroptosis. C, cytochrome *c*; $O_2^{\bullet-}$, superoxide; $^{\bullet}OH$, hydroxyl radicals; Q, ubiquinol.

other TCA intermediates downstream of αKG restores ferroptosis under glutamine starvation conditions (Gao et al., 2019), whereas inactivation of the αKG dehydrogenase complex suppresses cystine starvation–induced ferroptosis (Shin et al., 2020). The underlying mechanisms of the TCA cycle in regulating ferroptosis likely relate to its function in supporting electron transport and fatty acid biosynthesis (Fig. 2). Consistent with this, inactivating ACC blocks ferroptosis (Lee et al., 2020; Shimada et al., 2016). Therefore, current studies suggest that the diverse roles of mitochondria in bioenergetic, biosynthetic, and ROS regulation contribute to its pro-ferroptosis function (Fig. 2). It should be noted that ferroptosis has been demonstrated to spread to neighboring cells as a rapidly propagating wave, indicating cell–cell communication during ferroptosis (Katikaneni et al., 2020; Linkermann et al., 2014; Riegman et al., 2020). Notably, mitochondria released from necrotic cells can act as damage-associated molecular patterns to impact on neighboring cells (Krysko et al., 2011). Whether mitochondria released from ferroptotic cells can help spread ferroptosis to neighboring cells via damage-associated molecular patterns or other mechanisms is an interesting question for future investigations.

### Defense mechanisms against ferroptosis in mitochondria

Despite compelling evidence to support the importance of mitochondria in driving ferroptosis, the observation that GPX4 inactivation–induced ferroptosis is not accompanied by obvious mitochondrial lipid peroxidation (Friedmann Angeli et al., 2014) argues against mitochondrial lipid peroxidation having a prominent role in inducing ferroptosis. Further, cells have evolved both cytosolic and mitochondrial versions of GPX4; however, it is believed that cytosolic GPX4, but not the mitochondrial one, has a role in ferroptosis protection, largely because it has been shown that cytosolic, but not mitochondrial, GPX4 is essential for embryonic development in mouse (Liang et al., 2009; Schneider et al., 2009; Yant et al., 2003; Zheng and Conrad, 2020).

This conceptual hurdle was resolved by very recent findings showing that upon GPX4 inactivation, dihydroorotate dehydrogenase (DHODH) steps in to detoxify lipid peroxides and defend against ferroptosis in mitochondria; consequently, inactivation of both DHODH and mitochondrial GPX4 induces drastic mitochondrial lipid peroxidation and unleashes much more potent ferroptosis than does GPX4 inactivation alone (Mao et al., 2021). DHODH is a mitochondrial enzyme that is tethered to the outer face of the mitochondrial inner membrane and catalyzes the fourth enzymatic step in pyrimidine biosynthesis, wherein DHODH couples the oxidation of DHO to orotate (a precursor for pyrimidine synthesis) to the reduction of CoQ to $CoQH_2$ (Madak et al., 2019; Vasan et al., 2020), which subsequently acts as an radical-trapping antioxidant to quench lipid

peroxyl radicals in the mitochondrial inner membrane (Fig. 1). Therefore, this study proposes that mitochondrial GPX4 does play a role in ferroptosis defense; however, its anti-ferroptosis function can only be revealed under DHODH inactivation conditions. Notably, doxorubicin was shown to suppress mitochondrial GPX4 expression, and overexpression of mitochondrial GPX4 significantly rescued doxorubicin-induced ferroptosis (Tadokoro et al., 2020). Whether doxorubicin also suppresses the expression or activity of DHODH remains to be studied.

Importantly, CoQ is mainly synthesized in mitochondria (Stefely and Pagliarini, 2017); therefore, DHODH is in a perfect position to reduce CoQ to $CoQH_2$ and exert its anti-ferroptosis function in mitochondria. Other strong evidence to establish the anti-ferroptosis role of mitochondrial $CoQH_2$ is derived from studies of alternative oxidase (AOX), a mitochondrial inner membrane–localized protein that is used by plants and some lower organisms for electron transport in mitochondria; AOX directly transfers electrons from $CoQH_2$ to oxygen, thereby bypassing complex III and IV activities (Hakkaart et al., 2006). Overexpression of *Ciona intestinalis* AOX was shown to drastically promote mitochondrial lipid peroxidation and ferroptosis under RSL3 treatment (Mao et al., 2021). Because AOX exclusively acts on mitochondrial $CoQH_2$ pools and does not contribute to proton pumping or ATP or ROS generation, the only explanation for AOX's potent pro-ferroptosis effect seems to be its oxidation of $CoQH_2$ in mitochondria.

Notably, overexpression of mitochondrial GPX4, but not cytosolic GPX4 or FSP1, can rescue mitochondrial lipid peroxidation and ferroptotic cell death induced by RSL3 treatment in *DHODH* KO cells (Mao et al., 2021). (Whether GCH1 regulates mitochondrial lipid peroxidation remains unknown, but GCH1 is not known to be localized in mitochondria.) Likewise, treatment with mitochondria-targeted antioxidants such as $mitoQH_2$ or MitoTEMPO provided much more potent protective effects in RSL3-treated *DHODH* KO cells than in their WT counterparts treated with RSL3 (Mao et al., 2021). These findings together suggest a model in which cytosolic GPX4 and FSP1 versus mitochondrial GPX4 and DHODH act as two separate defense systems to quench nonmitochondrial and mitochondrial lipid peroxides, respectively (Figs. 1 and 3 A), which can result in three scenarios of ferroptosis-inducing conditions. (1) In WT cells treated with lethal doses of RSL3 (which inactivates both cytoplasmic and mitochondrial GPX4), ferroptosis is primarily induced by nonmitochondrial lipid peroxidation (under which condition DHODH-mediated anti-ferroptosis defense can keep mitochondrial lipid peroxidation in check; Fig. 3 B). (2) Although RSL3 treatment at sublethal doses does not induce obvious ferroptosis in WT cells, such treatments in *DHODH* KO cells can unleash potent ferroptosis primarily induced by mitochondrial lipid peroxidation (Fig. 3 C). (3) Further increasing RSL3 concentrations in *DHODH* KO cells triggers lipid peroxidation in both mitochondrial and nonmitochondrial compartments, resulting in ferroptosis induced by both mitochondrial and nonmitochondrial lipid peroxidation (Fig. 3 D).

In *DHODH* KO cells treated with sublethal doses of RSL3, how mitochondrial lipid peroxidation can trigger plasma membrane rupture and ferroptotic cell death remains unknown. In this regard, excessive mitochondrial ROS has been shown to induce mitochondrial permeability transition (referred to a dramatic increase of the permeability of mitochondrial inner membrane to low-molecular-weight solutes), which can trigger massive $Ca^{2+}$ release into cytosol and subsequent activation of hydrolytic enzymes, resulting in necrotic cell death (Bonora et al., 2015; Rasola and Bernardi, 2011). Whether mitochondrial lipid peroxidation–induced ferroptosis in this context has any relevance to mitochondrial permeability transition–induced cell death remains a fascinating question for future studies.

It is worth noting that mitochondrial iron metabolism also has an important role in governing ferroptosis. While Hmox1-dependent heme degradation and free iron overload promotes doxorubicin-induced ferroptosis and cardiomyopathy, possibly through Hmox-1's effect in mitochondria (Fang et al., 2019), overexpression of mitochondrial ferritin, an iron-storage protein localized in mitochondria, was shown to suppress erastin-induced ferroptosis likely via promoting iron storage in mitochondria (Wang et al., 2016). Therefore, multiple players might operate in mitochondria to control lipid peroxidation and ferroptosis.

So why would cells have evolved a separate defense system in mitochondria? As discussed in the preceding section, various metabolic activities render mitochondria particularly susceptible to lipid peroxidation; therefore, cells have a significant need to build up strong defense systems against ferroptosis in mitochondria. The mitochondrion is a unique organelle in that it contains double membranes. Consequently, defense systems localized in the cytosol and other cellular membranes (such as cytoplasmic GPX4 and FSP1) cannot enter into mitochondria and quench lipid peroxides accumulated in mitochondrial inner membranes, forcing cells to evolve a separate defense system in mitochondria. Notably, other antioxidant systems also have separate mitochondrial arms, such as SOD1 and thioredoxin reductase 1 in cytosol versus SOD2 and thioredoxin reductase 2 in mitochondria (Hayes et al., 2020). Therefore, this division appears to represent a common strategy used by cells to address the need for antioxidant defense in mitochondria.

## Concluding remarks and future perspectives

Overall, these recent studies suggest that although diverse metabolic activities in mitochondria facilitate ferroptosis, mitochondria are also equipped with strong anti-ferroptosis defense systems; therefore, it will be challenging to attribute a one-size-fits-all, either pro- or anti-ferroptotic, function to mitochondria as a whole organelle. In this author's view, unlike iron or PUFA-PL biosynthesis, mitochondria are likely not required for ferroptosis; that is, in mitochondria-depleted cells, a lethal accumulation of lipid peroxides on nonmitochondrial membranes would be sufficient to trigger ferroptosis. However, various metabolic processes in mitochondria actively contribute to ferroptosis; consequently, mitochondria-deficient cells are quantitatively more resistant to ferroptosis than their WT counterparts under the treatment of some FINs. This view might help to reconcile some of the conflicting data in the literature regarding the role of mitochondria in ferroptosis (Dixon et al., 2012; Gao et al., 2019; Gaschler et al., 2018b).

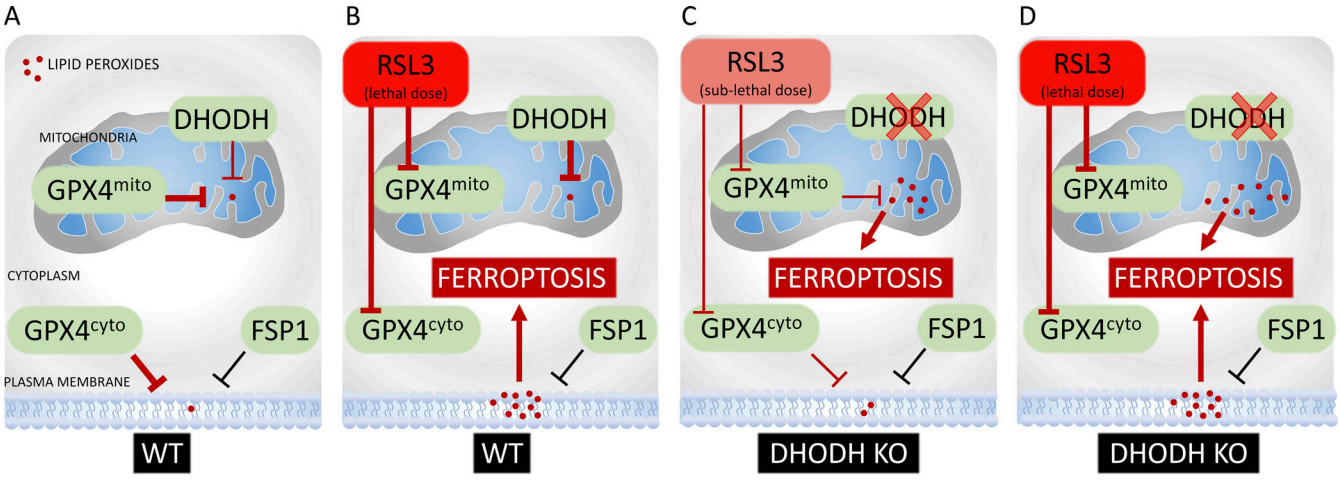


Figure 3. **GPX4$^{mito}$/DHODH and GPX4$^{cyto}$/FSP1 constitute two separate defense systems to detoxify lipid peroxides and suppress ferroptosis in cells. (A)** GPX4$^{mito}$ and DHODH suppress lipid peroxidation in mitochondria, whereas GPX4$^{cyto}$ and FSP1 detoxify lipid peroxides in nonmitochondrial compartments, including the plasma membrane. In each compartment, GPX4 has a more important role in neutralizing lipid peroxides than DHODH or FSP1. (GCH1 is not shown here because its subcellular localization currently remains elusive.) **(B–D)** Depending on RSL3 doses and DHODH status, RSL3 treatment can induce potent lipid peroxidation in nonmitochondrial compartments, mitochondria, or both compartments, resulting in ferroptosis. Note that RSL3 treatment significantly increases DHODH activity (B). cyto, cytosolic; mito, mitochondrial.

These findings also open up several important questions for future investigations. Several enzymes are located in the mitochondrial inner membrane that can reduce CoQ to $CoQH_2$, including complexes I and II and DHODH; indeed, it is believed that in most cells, complexes I and II should have more important roles in generating $CoQH_2$ than does DHODH. On the basis of this, similar to DHODH inactivation or AOX overexpression, inhibition of complex I or II is expected to sensitize cells to GPX4 inactivation–induced ferroptosis. Contradictory to this expectation, complex I or II inhibitors were found to not affect cellular sensitivity to RSL3 (or even suppresses ferroptosis induced by cystine starvation; Gao et al., 2019). This is likely because that complex I or II has a more complicated role in regulating ferroptosis; although $CoQH_2$ generated by these complexes can defend against ferroptosis in mitochondria, as discussed, their functions in generating ROS and/or ATP also strongly promote ferroptosis. It is therefore possible that these pro-ferroptosis functions by complex I or II may have offset or overridden their anti-ferroptosis function through generating $CoQH_2$. Notably, DHODH is not involved in ROS or ATP generation, thereby providing a unique advantage to mitochondria in their use of DHODH to defend against ferroptosis. Further studies are warranted to clarify the role of complexes I and II in ferroptosis regulation.

Because FSP1 can exert a strong anti-ferroptosis function on the plasma membrane (and likely other nonmitochondrial membranes) in the absence of GPX4 (Bersuker et al., 2019; Doll et al., 2019), it remains unclear why, upon GPX4 inactivation, the ferroptosis defense system in nonmitochondrial compartments collapses before that in mitochondria (as reflected by potent nonmitochondrial lipid peroxidation accumulated in GPX4-inactivated cells; Friedmann Angeli et al., 2014). The following speculation might help explain this observation. Both FSP1 and DHODH suppress ferroptosis by converting CoQ to $CoQH_2$ (Fig. 1). Because CoQ is synthesized in mitochondria (Stefely and Pagliarini, 2017), CoQ can be directly used by DHODH to defend against ferroptosis in mitochondria but needs to be transported from the mitochondria to the plasma membrane or other organelles for its use in ferroptosis defense in nonmitochondrial membranes. In addition, the membrane area in nonmitochondrial compartments is much larger than that in mitochondria. Consequently, the CoQ pool on nonmitochondrial membranes is likely much more limiting than that in mitochondria, resulting in a more rapid collapse of defense systems in nonmitochondrial compartments than that in mitochondria upon GPX4 inactivation. It will be interesting to compare the kinetics of CoQ and $CoQH_2$ levels between nonmitochondrial and mitochondrial fractions in response to ferroptotic stress. Additional studies will also illuminate the mechanisms underlying the differential effects of different classes of FINs in inducing mitochondrial lipid peroxidation (see text box).

Finally, these recent studies also suggest that ferroptosis defense systems can be compartmentalized in different subcellular compartments, thereby opening new directions to study ferroptosis regulation in other organelles, such as endoplasmic reticulum, Golgi apparatus, lysosomes, and peroxisomes. Interestingly, recent studies revealed that the synthesis of polyunsaturated ether PLs in peroxisomes drives ferroptosis (Cui et al., 2021; Zou et al., 2020a), suggesting that there might also exist peroxisome-localized defense mechanisms. As discussed, double membrane–bound organelles would require separate defense systems to quench lipid peroxides accumulated in their inner membranes. Notably, ferroptosis-like cell death has been described in plants (Conrad et al., 2018; Distéfano et al., 2017). This raises the question of whether plant cells have evolved their separate ferroptosis defense systems in the chloroplast, another double-membrane organelle. A deeper understanding of ferroptosis regulation in mitochondria and other subcellular

compartments will not only advance our fundamental understanding of this intriguing cell death mechanism but also identify new therapeutic opportunities to target ferroptosis in disease treatment.

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B. Gan has filed a patent relating to the use of DHODH inhibitors to target ferroptosis in cancer therapy.

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