Ferroptosis: a regulated cell death nexus linking metabolism, redox biology, and disease

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Summary

Ferroptosis is a form of regulated cell death characterized by the iron-dependent accumulation of lipid hydroperoxides to lethal levels. Emerging evidence suggests that ferroptosis represents an ancient vulnerability caused by the incorporation of polyunsaturated fatty acids into cellular membranes, and that cells have developed complex systems that exploit and defend against this vulnerability in different contexts. The control of ferroptosis is tightly linked to the regulation of numerous biological processes, including amino acid, iron and polyunsaturated fatty acid metabolism, and the biosynthesis of glutathione, phospholipids, NADPH and coenzyme Q10. Ferroptosis has been implicated in the pathological cell death associated with degenerative diseases (*i.e.*, Alzheimer's, Huntington's, and Parkinson's diseases), carcinogenesis, stroke, intracerebral hemorrhage, traumatic brain injury, ischemia-reperfusion injury, and kidney degeneration in mammals and is also implicated in heat stress in plants. Ferroptosis may also have a tumor suppressor function that could be harnessed for cancer therapy. This *Primer* reviews the mechanisms underlying ferroptosis, highlights connections to other areas of biology and medicine, and recommends tools and guidelines for studying this emerging form of regulated cell death.

Key words

Ferroptosis; cell death; iron; metabolism; ROS; peroxidation; glutathione; neurodegeneration; cancer; PUFA

Introduction

Cell death is critical in diverse aspects of mammalian development, homeostasis, and disease, and it is tightly integrated with other biological processes. The Nomenclature Committee on Cell Death (NCCD) recognizes a distinction between *accidental cell death*, which is caused by severe physical, chemical and mechanical insults and cannot be reversed by molecular perturbations, and *regulated cell death*, which can be modulated pharmacologically and genetically, and is therefore under the control of specific biological mechanisms; moreover, the NCCD notes that programmed cell death is a subset of regulated cell death that is predestined to occur in normal physiological contexts such as development (Galluzzi et al., 2015). Caspase-dependent apoptosis was the first regulated and programmed form of cell death to be characterized at the molecular level. In recent years, there has been a growing appreciation for the importance of regulated cell death mechanisms beyond apoptosis in explaining the molecular processes that control the demise of cells (Conrad et al., 2016), although the extent to which these other forms of regulated cell death are also programmed to occur under normal physiological conditions remains largely unknown. We focus in this *Primer* on ferroptosis, an iron-dependent form of regulated cell death that involves lethal, iron-catalyzed lipid damage.

The term ferroptosis was coined in 2012 (Dixon et al., 2012) to describe the form of cell death induced by the small molecule erastin, which inhibits the import of cystine, leading to glutathione depletion and inactivation of the phospholipid peroxidase glutathione peroxidase 4 (GPX4) (Yang et al., 2014). GPX4 converts potentially toxic lipid

hydroperoxides (L-OOH) to non-toxic lipid alcohols (L-OH) (Ursini et al., 1982) (Figure 1). Inactivation of GPX4 through depletion of GSH with erastin, or with the direct GPX4 inhibitor (1*S*,3*R*)-RSL3 (hereafter referred to as RSL3), ultimately results in overwhelming lipid peroxidation that causes cell death (Table 1). Subsequently, additional compounds and regulatory mechanisms governing ferroptosis have been identified (Xie et al., 2016a; Yang and Stockwell, 2016) (Tables 1 and 2), and this process has been implicated in a variety of pathological contexts and therapeutic strategies, described below.

Definition of ferroptosis and potential physiological functions

Ferroptosis describes a form of regulated cell death that occurs as a consequence of lethal lipid peroxidation. Cell death occurring exclusively by ferroptosis can be suppressed by iron chelators, lipophilic antioxidants, inhibitors of lipid peroxidation, and depletion of polyunsaturated fatty acids (PUFAs), and correlates with the accumulation of markers of lipid peroxidation.

While a normal physiological function for ferroptosis as an adaptive and programmed form of cell death has not been established, one emerging view is that the incorporation of polyunsaturated fatty acids (PUFAs) into cell membranes during evolution was advantageous, but created a new vulnerability to lipid peroxidation. PUFAs allow for modulating membrane fluidity and adaptation of cells to environments with different temperatures, among other beneficial functions. However, the bis-allylic carbon between neighboring carbon-carbon double bonds in PUFAs is highly chemically

susceptible to peroxidation in the presence of metals, oxygen, radical species and specific enzymes. Investigations into the regulation of ferroptosis have revealed a complex set of processes, described below, that can drive or suppress lethal lipid peroxidation, suggesting that Nature regulates this vulnerability in numerous contexts. For example, some degenerative pathologies appear to be caused by overwhelming the lipid peroxidation repair systems that evolved in cells, resulting in cell death; emerging evidence also suggests that ferroptosis might serve a tumor suppressor function in removing cells that lack access to critical nutrients in their environment, or that have been compromised by infection or environmental stress, although these studies are still preliminary. The bulk of studies to date suggest that ferroptosis is triggered by degenerative processes or may be induced therapeutically in some cancers, but few studies have explored its natural functions. It is conceivable, but not yet demonstrated, that ferroptosis could be triggered during development or normal homeostatic tissue turnover by the accumulation of glutamate, iron, or PUFA-phospholipids, and/or by depletion of endogenous inhibitors of ferroptosis, such as GSH, NADPH, GPX4 or vitamin E. Examining such possibilities will be an important area of investigation in the future to determine whether ferroptosis is ever genetically programmed to occur, or is merely a vulnerability exploited by pathologies and potentially therapeutics.

Early history: studies linking thiol metabolism to oxidative cell death

Reduced glutathione (γ-L-glutamyl-L-cysteinylglycine, GSH) is an essential intracellular antioxidant synthesized from glutamate, cysteine and glycine in two steps by the ATP-dependent cytosolic enzymes glutamate-cysteine ligase (GCL) and glutathione

synthetase (GSS) (Figure 1); the rate of glutathione synthesis is limited by cysteine availability. Early studies identified extracellular cysteine and cystine as essential for growth of HeLa and other cells in culture (Eagle, 1955). Cysteine is required for cell growth, and to prevent cell death: human fibroblasts cultured in cystine-free medium die due to glutathione depletion, and this death is prevented by the lipophilic antioxidant α-tocopherol (Bannai et al., 1977). In addition, iron chelators, such as deferoxamine, prevent this cell death (Murphy et al., 1989). These results established the concept that extracellular cystine and intracellular cysteine are required to maintain the biosynthesis of glutathione and to suppress a type of cell death that is also preventable by treatment with iron chelators or lipophilic antioxidants. In addition, abortion of premature pollen due to abnormal microspore formation in the sterile rice plant *Oryza sativa L* has been reported to involve depletion of GSH and NADPH during meiosis, and consequent accumulation of ROS and cell death, which is intriguingly reminiscent of ferroptosis (Wan et al., 2007). However, when these historical studies were performed, knowledge of different types of cell death was lacking, and these observations were not yet interpreted as evidence for a unique form of regulated or programmed cell death.

The biochemical control of ferroptosis

Ferroptosis initiation and execution lies at the intersection of amino acid, lipid and iron metabolism (Figure 2). Ferroptosis can be activated when glutathione levels fall below a critical threshold, depriving GPX4 of its essential co-substrate. In the absence of GPX4 activity, iron-dependent processes acting on membrane PUFA-containing phospholipids result in membrane damage through PUFA peroxidation.

Amino acid and glutathione metabolism

Amino acid metabolism is tightly linked to the regulation of ferroptosis (Angeli et al., 2017). Because cysteine availability limits the biosynthesis of glutathione, some cells make use of the transsulfuration pathway to biosynthesize cysteine from methionine and therefore bypass the requirement for cystine import via the cystine/glutamate antiporter system x_c^-; consequently, these cells are resistant to ferroptosis induced by system x_c^- inhibitors (Figure 2). A genome-wide short interfering RNA (siRNA) screen for suppressors of erastin-induced ferroptosis revealed that knockdown of cysteinyl-tRNA synthetase (*CARS*) results in upregulation of the transsulfuration pathway and resistance to erastin-induced ferroptosis (Hayano et al., 2016). Knockdown of some, but not all, tRNA synthetases results in upregulation of the transsulfuration pathway, which can be detected by measuring the abundance of cystathionine beta synthase (*CBS*) mRNA (Hayano et al., 2016).

Glutamate and glutamine are also important regulators of ferroptosis (Gao et al., 2015). Glutamate is exchanged for cystine in a 1:1 ratio by system x_c^-, so glutamate levels impact system x_c^-. Indeed, high extracellular concentrations of glutamate inhibit system x_c^- and induce ferroptosis, perhaps explaining toxic effects of glutamate when it accumulates to high concentrations in the nervous system (Dixon et al., 2012). Of note, system x_c^- knockout mice are protected from neurotoxic insults due to decreased extracellular brain glutamate levels (Massie et al., 2011). Thus, accumulation of

extracellular glutamate could serve as a natural trigger for inducing ferroptosis in physiological contexts.

Glutamine is naturally present at high concentrations in human tissues and plasma, and its degradation (via glutaminolysis) provides fuel for the tricarboxylic acid (TCA) cycle and building blocks for essential biosynthetic processes, such as lipid biosynthesis. In the absence of glutamine, or when glutaminolysis is inhibited, cystine starvation and blockage of cystine import fail to induce the accumulation of reactive oxygen species (ROS), lipid peroxidation, and ferroptosis. This observation may be explained by the fact that α-ketoglutarate (αKG), a product of glutaminolysis, is required for ferroptosis (Gao et al., 2015).

Not all routes of glutaminolysis fuel ferroptosis, however. The first step of glutaminolysis involves conversion of glutamine into glutamate, a reaction catalyzed by the glutaminases GLS1 and GLS2. Although these enzymes are structurally and enzymatically similar, only GLS2 is required for ferroptosis (Gao et al., 2015). The *GLS2* gene, but not *GLS1*, is a transcriptional target of the tumor suppressor p53, and upregulation of *GLS2* contributes to p53-dependent ferroptosis (Jennis et al., 2016). Because ferroptosis has been suggested to be a relevant cell death mechanism in tissue injury (Conrad et al., 2016; Linkermann et al., 2014), glutaminolysis-targeted therapy may be effective in treating organ damage mediated by ferroptosis. Indeed, inhibition of glutaminolysis has been shown to attenuate ischemia/reperfusion-induced

heart and kidney damage and brain hemorrhage in experimental models (Gao et al., 2015; Li et al., 2017; Linkermann et al., 2014).

Lipid metabolism

Lipid metabolism is also intimately involved in determining cellular sensitivity to ferroptosis. PUFAs, which contain bis-allylic hydrogen atoms that can be readily abstracted, are chemically susceptible to lipid peroxidation and are necessary for the execution of ferroptosis (Yang et al., 2016). Thus, the abundance and localization of PUFAs determine the degree of lipid peroxidation that occurs in cells, and hence the extent to which ferroptosis is operative. Free PUFAs are substrates for synthesis of lipid signaling mediators, yet they must be esterified into membrane phospholipids and undergo oxidation to become ferroptotic signals (Kagan et al., 2017). Lipidomic studies suggest that phosphatidylethanolamines (PEs) containing arachidonic acid (C20:4) or its elongation product, adrenic acid (C22:4), are key phospholipids that undergo oxidation and drive cells towards ferroptotic death (Doll et al., 2017; Kagan et al., 2017). Therefore, formation of coenzyme-A-derivatives of these PUFAs and their insertion into phospholipids are necessary for the production of ferroptotic death signals. This is another potential point of regulation of ferroptosis, and future investigations may suggest physiological contexts in which ferroptosis is triggered or blocked by modulating enzymes involved in the biosynthesis of PUFA-containing membrane phospholipids.

For example, two enzymes, ACSL4 and LPCAT3 (Table 2), are involved in the biosynthesis and remodeling of PUFA-PEs in cellular membranes. Loss of these gene

products depletes the substrates for lipid peroxidation and increases resistance to ferroptosis (Dixon et al., 2015; Doll et al., 2017; Kagan et al., 2017; Yuan et al., 2016b). Conversely, cells that are supplemented with arachidonic acid or other PUFAs are sensitized to ferroptosis (Yang et al., 2016). Hydroperoxy derivatives of PUFA-PEs also cause ferroptotic death when added to cells with inactivated GPX4 (Kagan et al., 2017). Intriguingly, proper development of the fetal immune system in humans depends on adequate PUFA dietary intake, suggesting a possible role for ferroptosis in this process, although other explanations are also possible (Enke et al., 2008).

Enzymatic effectors, such as non-heme, iron-containing proteins, including lipoxygenases (LOXs), can mediate ferroptotic peroxidation (Kagan et al., 2017; Seiler et al., 2008; Yang et al., 2016). Free PUFAs, rather than PUFA-containing phospholipids, are the preferred substrates of LOXs (Kuhn et al., 2015); PE phospholipids can, however, form a non-bilayer arrangement (van den Brink-van der Laan et al., 2004) that may facilitate pro-ferroptotic oxidation of PUFA-containing PE phospholipids by LOXs, rather than free PUFAs. Indeed, genetic depletion of LOXs protects against erastin-induced ferroptosis (Yang et al., 2016), suggesting LOXs contribute to ferroptosis. Several ferroptosis inhibitors, including members of the vitamin E family (tocopherols and tocotrienols) and flavonoids, can inhibit LOX activity in some contexts (Khanna et al., 2003; Xie et al., 2016b). Some lipoxygenases are required for normal embryonic development in vertebrates; for example, 12S-lipoxgenase is essential for the development of numerous tissues in zebrafish, suggesting that

ferroptosis could be involved in these developmental processes, although further study is required to test this possibility (Haas et al., 2011).

The execution phase of ferroptosis may be a direct result of lipid peroxidation. Lipid peroxides decompose into reactive derivatives, including aldehydes and Michael acceptors, which can react with proteins and nucleic acids (Gaschler and Stockwell, 2017). The hypothesis that these reactive intermediates are responsible for cell death is supported by the observation that a cell line selected for erastin resistance showed several-hundred-fold upregulation of *AKR1C* family genes (Dixon et al., 2014), which encode aldoketoreductases that, among other functions, reduce reactive end-products of lipid peroxidation to unreactive compounds (MacLeod et al., 2009).

Iron metabolism

Iron is required for the accumulation of lipid peroxides and the execution of ferroptosis. Thus, iron import, export, storage, and turnover impact ferroptosis sensitivity. Notably, transferrin and transferrin receptor, which import iron from the extracellular environment, are required for ferroptosis (Gao et al., 2015; Yang and Stockwell, 2008), while silencing of the iron metabolism master regulator *IREB2* decreases sensitivity to ferroptosis (Dixon, 2012). Within cells, autophagy can modulate sensitivity to ferroptosis through its impact on iron metabolism (Gao et al., 2015; Hou et al., 2016). Selective autophagy of ferritin, referred to as ferritinophagy, modulates ferroptosis sensitivity, possibly by controlling iron availability (Gao et al., 2016; Hou et al., 2016; Mancias et al., 2014; Wang et al., 2016b). Ferritin is recognized by the specific cargo receptor NCOA4, which

recruits ferritin to autophagosomes for lysosomal degradation and the release of free iron (Mancias et al., 2014). Other proteins impacting iron metabolism in the cell (*e.g.,* HSPB1 and CISD1) impact ferroptosis sensitivity (Sun et al., 2015; Yuan et al., 2016a). Thus, regulation of iron metabolism and ferritinophagy are additional potential points of control of ferroptosis.

Other metabolic pathways controlling ferroptosis sensitivity

Several other metabolic pathways modulate cellular sensitivity to ferroptosis. The mevalonate pathway leads to the production of coenzyme Q10 (CoQ_{10}), which is required to shuttle electrons as part of the mitochondrial electron transport chain; however, this function is not relevant to ferroptosis (Dixon et al., 2012). Rather, CoQ_{10} moonlights as an endogenous inhibitor of ferroptosis by serving an antioxidant function in membranes (Shimada et al., 2016b). The ferroptosis-inducing compound FIN56 depletes CoQ_{10} by modulating squalene synthase activity (SQS), which in part drives accumulation of lethal lipid peroxidation (Shimada et al., 2016b). Statin drugs, which inhibit HMG CoA reductase, the rate-limiting enzyme of the mevalonate pathway, sensitize cells to ferroptosis, presumably by depleting CoQ_{10}, and possibly by also inhibiting downstream tRNA isopentenylation via TRIT1, which is required for the biosynthesis of GPX4 (Fradejas et al., 2013; Shimada et al., 2016b; Viswanathan et al., 2017).

NADPH and selenium abundance also impact ferroptosis sensitivity. NADPH is an essential intracellular reductant needed to eliminate lipid hydroperoxides. Indeed,

NADPH levels are a biomarker of ferroptosis sensitivity across many cancer cell lines (Shimada et al., 2016a). Selenium is required for the biosynthesis of GPX4, which has an active-site selenocysteine (Cardoso et al., 2017). Thus, selenium supplementation promotes ferroptosis resistance, while selenium depletion promotes ferroptosis sensitivity, presumably by modulating GPX4 abundance and activity (Cardoso et al., 2017). A number of other genes have been implicated as modulators of sensitivity to ferroptosis or markers of ferroptosis in diverse contexts: *SAT1*, which lies downstream of p53 (Ou et al., 2016) is involved in polyamine metabolism; *kiss of death (KOD)* in *Arabidopsis* (Distefano et al., 2017) and FANCD2 in bone marrow stromal cells are induced during ferroptosis (Song et al., 2016); *TTC35*, *CS*, *ATP5G3*, and *RPL8* are involved in diverse processes in human cancer cells and suppress erastin-induced ferroptosis upon knockdown (Dixon et al., 2012).

The NRF2 transcription factor is also a modulator of ferroptosis sensitivity (Sun et al., 2016b): NRF2 controls the expression of *AKR1C*, the metal-binding protein MT-1G (Sun et al., 2016a), and other antioxidant genes, the expression of key proteins in iron signaling, including ferritin and ferroportin, and the expression of enzymes in the pentose phosphate pathway, which generate the bulk of cellular NADPH. Additionally, genes encoding proteins responsible for glutathione synthesis, including SLC7A11, GCLC/GLCM, and GSS are NRF2 target genes (Kerins and Ooi, 2017).

Links between ferroptosis and pathology

Recent discoveries have revealed connections between ferroptosis and degenerative and neoplastic diseases (Conrad et al., 2016; Toyokuni et al., 2017). Historically, regulated cell death was assumed to be apoptotic in models of ischemic injury to the brain, heart, liver, kidney and intestine. When necroptosis was discovered as a RIPK3-and-MLKL-dependent form of regulated necrosis, it was suggested that this pathway is a primary contributor to ischemic injury in the heart and kidney (Linkermann et al., 2013; Newton et al., 2016). Only later was it discovered that ferroptosis is a primary driver of ischemic injury in some models (Tonnus and Linkermann, 2017).

Inhibitors of ferroptosis, such as ferrostatins and liproxstatins, protect from ischemic injury in mouse models in the liver, kidney, brain, and heart (Friedmann Angeli et al., 2014; Gao et al., 2015; Linkermann et al., 2014; Skouta et al., 2014; Tuo et al., 2017). These inhibitors are also protective in models of degenerative brain disorders, including Parkinson´s, Huntington´s, and Alzheimer´s Diseases, as well as in other forms of neurodegeneration and traumatic and hemorrhagic brain injury (Chen et al., 2015; Do Van et al., 2016; Gascon et al., 2016; Guiney et al., 2017; Hambright et al., 2017; Li et al., 2017; Skouta et al., 2014; Zille et al., 2017). Ferroptosis in some other tissues and diseases has been examined, including liver hemochromatosis (Wang et al., 2017). A number of clinicopathological features of dementia are consistent with ferroptosis (Table 3). Similar features are manifest in other neurodegenerative diseases (Belaidi and Bush, 2016).

Brain iron levels inevitably rise during aging and in degenerative diseases, which can be detected both in post-mortem analysis as well as in living individuals; such increases could contribute to an age-dependent risk of ferroptosis (Belaidi and Bush, 2016; Buijs et al., 2017). Genetic evidence also links brain degeneration to ferroptosis: inducible deletion of *gpx4* in adult mice causes hippocampal neuronal loss with astrogliosis, as seen in AD (Yoo et al., 2012). The study of ferroptosis in nervous tissue is complicated by the presence of neuronal support cells, including astrocytes, microglia and oligodendrocytes. Damage incurred by a support cell undergoing ferroptosis could, in principle, be transmitted to neurons in a wave-like propagation of ferroptosis (Linkermann et al., 2014). In addition, treatment with the iron chelator deferiprone was recently reported to be beneficial in a randomized controlled trial for PD (Devos et al., 2014); dopamine is also reported to enhance stability of GPX4 (Wang et al., 2016a). Given the plethora of disease implications, the optimization of existing inhibitors or the identification of new inhibitors that block ferroptosis is a potential approach to treating a number of degenerative diseases.

Table 3. Features of dementia consistent with ferroptosis

Feature	Comment	References.
Decreased cortical GSH	Found in post-mortem and imaging assays; an association with amyloid pathology has been reported	(Chiang et al., 2017; Mandal et al., 2015)
Lipid peroxidation products	Hydroxynonenal, malondialdehyde, and acrolein are reported elevated; may be related to redox-catalysis by metal-Aβ	(Di Domenico et al., 2017; Williams et al., 2006)
Depletion of PUFAs	Decreased DHA associated with reduced levels of PE in AD hippocampus; loss of PUFAs in AD brains	(Prasad et al., 1998; Sydenham et al., 2012)
Excess extracellular glutamate	Glutamate trafficking/receptor systems altered in AD; NMDAR blocker, memantine, is an approved AD drug	(Greenamyre et al., 1985; Revett et al., 2013)
Pathology spreads	Neurodegeneration spreads along the pathways of neural connectivity; release of toxic factors (lipid radicals, PUFA oxidation products) possible	(Braak and Del Tredici, 2015; Brettschneider et al., 2015)
Decreased cortical GPX4	Human brain tissue data indirectly implicate GPX4 impairment	(Cardoso et al., 2017)
Increased cortical p53	Increased p53 and lipid peroxidation products in post-mortem human brain tissue	(Cenini et al., 2008)
Increased cortical iron	MRI studies show elevated cortical iron in AD; hippocampal iron accelerates cognitive deterioration in patients positive for amyloid pathology	(Ayton et al., 2017; Raven et al., 2013)
Increased 12/15 lipoxygenase activity	LOX activity explored as pharmacological target for AD	(Di Meco et al., 2017)

Clinical benefit of Vitamin E	A large randomized control trial of patients with mild-moderate AD showed that vitamin E slowed cognitive decline by 19% / yr; vitamin E is a low potency anti-ferroptotic agent	(Dysken et al., 2014)
Possible clinical benefit of iron chelation	In preclinical AD models, iron chelators rescue cognitive deficits; a phase 2 clinical trial reported that desferrioxamine delayed deterioration in AD	(Crapper McLachlan et al., 1991)
ACSL4 inhibitors decrease FTD in humans	Long-term use of pioglitazone, a recently discovered ACSL4 inhibitor, decreases frontotemporal dementia in patients	(Heneka et al., 2015)

Ferroptosis applications in neoplastic diseases

Cancer cells are susceptible to perturbations of thiol metabolism, whereas oxidative stress via excess iron is associated with carcinogenesis (Toyokuni et al., 2017). Agents that inhibit cystine uptake via the cystine/glutamate antiporter (system x_c^-), such as sulfasalazine, arrest tumor growth and can induce ferroptosis in some circumstances (Dixon et al., 2014; Toyokuni et al., 2017). Likewise, direct depletion of cystine from plasma using an engineered cystine-degrading enzyme conjugate (*i.e.,* cyst(e)inase) arrests tumor growth and triggers cell death (Cramer et al., 2017). Agents that conjugate to glutathione, such as APR-246, as well as chemical or genetic inhibition of glutathione biosynthesis, disrupt tumor cell growth and induce a ferroptosis-like form of cell death (Liu et al., 2017). Typically, elevated levels of ROS are detected in response to perturbation of cysteine or glutathione metabolism and, where tested, cell death is prevented by antioxidant treatment.

Erastin and RSL3 were originally identified in phenotypic screens for compounds that are selectively lethal to engineered tumor cells. Improved analogs of erastin with increased solubility, selectivity, and potency have been created, and some have shown efficacy in xenograft tumor studies (Yang et al., 2014). Nanoparticles that induce ferroptosis have also demonstrated efficacy in xenograft studies (Kim et al., 2016). The

tumor suppressor p53 has been reported to repress *SLC7A11*, a component of system x_c^-, thus inducing ferroptosis in some contexts (Jiang et al., 2015). In addition, CD44v, a cancer stem cell marker, associates with system x_c^-, and stabilizes this complex; this observation suggests that CD44v may be a biomarker for tumors that are sensitive to system x_c^- inhibitors that induce ferroptosis (Toyokuni et al., 2017).

Model systems in which ferroptosis has been observed

A variety of experimental settings—from cell culture to mice and plants—can be used to explore mechanisms of ferroptosis. Careful consideration must be given to the selection of appropriate cell lines, however, because not all cell lines and experimental systems are susceptible to this process. In addition, *ex vivo* cultures have been shown to be sensitive to ferroptosis, including hippocampal postnatal rat brain slices treated with glutamate (Dixon et al., 2012), striatal rat brain slices with ectopic mutant huntingtin expression (Skouta et al., 2014), and freshly isolated renal tubules (Linkermann et al., 2014).

A large-scale characterization of ferroptosis sensitivity of cancer cell lines found that cancer cell lines have highly varied sensitivity to ferroptosis (Yang et al., 2014). Cell lines such as HT-1080 fibrosarcoma cells and Panc-1 pancreatic cancer cells have robust ferroptotic responses and are frequently used as model systems to study ferroptosis mechanisms. In addition, mouse embryonic fibroblasts (MEFs) are generally sensitive to ferroptosis, even when lacking the apoptotic regulators BAX and BAK (Friedmann Angeli et al., 2014; Wolpaw et al., 2011). HT-22 hippocampal neuronal cells

have been used as a model for neuronal sensitivity to ferroptosis (Xie et al., 2016a), and U937 monocytes have been used as models for studying ferroptosis in immune cells (Conrad et al., 2016). Some primary cell systems have also been used for studies of ferroptosis, including HRPTEpiCs (primary human renal proximal tubule epithelial cells), HK2 cells, mouse lung epithelial cells, human bronchial epithelial cells, and spinal motor neurons. In addition, *Arabidopsis* seedlings exposed to 55 °C heat stress have been used as a model for ferroptotic-like cell death in plants (Distefano et al., 2017).

Commonly used reagents for studying ferroptosis

There are four classes of ferroptosis inducers currently in use (Yang and Stockwell, 2016): (i) system x_c^- inhibitors (erastin and its analogs, sulfasalazine, glutamate, and sorafenib); (ii) GPX4 inhibitors, such as RSL3 and ML162; (iii) FIN56, which depletes GPX4 protein, and the lipophilic antioxidant CoQ10 (Shimada et al., 2016b); and (iv) FINO2, which indirectly inhibits GPX4 activity and stimulates lipid peroxidation (Abrams et al., 2016) (Table 1). In addition to these canonical ferroptosis inducers, several other reagents can induce ferroptosis in some contexts: buthionine sulfoximine (BSO) depletes glutathione, and in some cases is capable of inducing ferroptosis (Yang et al., 2014); CCl_4 may induce ferroptosis in the liver (Guo et al., 2017), artesunate can induce ferroptosis in pancreatic cancer cells (Eling et al., 2015), cisplatin induces both ferroptosis and apoptosis in several tissues (Jennis et al., 2016), and a derivative of artemisinin (Greenshields et al., 2017) induce both ferroptosis and apoptosis.

A variety of pharmacological and genetic inhibitors of ferroptosis have also been reported (Angeli et al., 2017; Conrad et al., 2016; Doll et al., 2017). Inhibitors of lipid metabolism that suppress PUFA incorporation into phospholipid membranes have been reported, such as knockdown or knockout of *ACSL4* or *LPCAT3,* and thiazolidinediones, which inhibit ACSL4 (Dixon et al., 2015; Doll et al., 2017). Inhibitors of lipid peroxidation, such as LOX inhibitors, also suppress ferroptosis (Yang et al., 2016). Inhibitors of iron metabolism and iron chelators (*e.g.,* deferoxamine (DFO) and ciclopirox (CPX)) suppress ferroptosis by reducing availability of iron (Yang and Stockwell, 2008). Inhibition of glutaminolysis, as noted above, suppresses ferroptosis, although the mechanism of action is not known (Gao et al., 2015). The nitroxide XJB-5-131 is a potent inhibitor of ferroptosis, probably by blocking lipid peroxidation in relevant membranes (Krainz et al., 2016). Similarly, ferrostatins and liproxstatins inhibit lipid peroxidation, possibly by acting as radical-trapping antioxidants (Zilka et al., 2017), similarly to the lipophilic antioxidants BHT, BHA and vitamin E. In the latter case, tocotrienols are more effective than tocopherols (Kagan et al., 2017). Selectively bis-allylic deuterated PUFAs suppress propagation of lipid peroxidation and have been found to suppress ferroptosis induced by RSL3 and erastin, although they are more effective against the former than the latter (Yang et al., 2016). Finally, the protein synthesis inhibitor cycloheximide suppresses ferroptosis induced by system x_c^- inhibition (Yagoda et al., 2007).

Given different mechanisms of ferroptotic inhibition, the characterization of new inhibitors should be accompanied by an evaluation of antioxidant or iron-chelating

activity; otherwise, the mechanism of action of inhibitors that appear to act through distinct mechanisms may be misinterpreted. For example, many LOX inhibitors and the MEK inhibitor U0126 exhibit antioxidant activity, which is probably the basis of their ability to suppress ferroptosis. In addition, the necroptosis inhibitor necrostatin-1 has off-target activity, which can suppress ferroptosis at high concentrations (Friedmann Angeli et al., 2014), but this is unrelated to necroptosis.

Measuring lipid peroxidation is essential for evaluating whether ferroptosis occurs in specific contexts. C11-BODIPY and Liperfluo are lipophilic ROS sensors that provide a rapid, indirect means to detect lipid ROS (Dixon et al., 2012). Liquid chromatography (LC) / tandem mass spectrometry (MS) analysis has lower throughput but can be used to detect specific oxidized lipids directly (Friedmann Angeli et al., 2014; Kagan et al., 2017). In addition, isoprostanes have been used to measure lipid peroxidation (Milne et al., 2007), although not yet in the context of ferroptosis.

Other useful assays for studying ferroptosis include measuring iron abundance and GPX4 activity. The former can be detected using inductively coupled plasma-MS or calcein AM quenching, as well as other specific iron probes (Hirayama and Nagasawa, 2017; Spangler et al., 2016), while the latter can be detected using phosphatidylcholine hydroperoxide reduction in cell lysates using LC-MS (Yang et al., 2014).

Since ferroptosis susceptibility is so closely tied to the metabolic state of the cell, it is important in in vivo and in vitro studies to control the composition of chow and serum

composition, respectively, which can vary between lots, laboratories, and experiments, by altering the levels of key ferroptotic regulators, such as selenium, iron, vitamin E, cysteine, glutathione and PUFAs in experimental samples. In addition, redox conditions vary between cell-based/organoid cultures and in vivo conditions, which is illustrated by the frequent requirement for system x_c^- in cell culture, whereas system x_c^- knockout mice are viable (McCullagh and Featherstone, 2014). This can be addressed to some extent by measuring the impact of system x_c^- both in vivo and in culture, and by modulating oxygen concentrations in culture (Dixon et al., 2014).

Given the sensitivity of ferroptosis to small variations in the levels of so many different molecules and metabolites (iron, PUFAs, cysteine) it is possible that dozens or hundreds of genes and proteins impact ferroptosis sensitivity by having small effects on the levels of one or more metabolites. Such connections do not imply a direct role in the execution of ferroptosis, and while important to document, should be distinguished from core regulators of iron-dependent lipid peroxidation, such as ACSL4, system x_c^-, and GPX4.

Connections between ferroptosis and other cell death pathways

Both ferroptosis and necroptosis contribute to ischemic injury, such that prevention of ferroptosis and necroptosis at the same time provides maximum benefit (Linkermann et al., 2014). Moreover, some modulators affect both apoptosis and ferroptosis, such as p53: whereas the role of p53 in apoptosis has been investigated for more than two decades, its role in ferroptosis has only been reported recently (Jiang et al., 2015). For

the most part, however, ferroptosis appears to be independent of other known cell death pathways. Ferroptosis proceeds even in the absence of key effectors of apoptosis, such as BAX, BAK and caspases. Likewise, ferroptosis proceeds in the absence of the key components of necroptosis, such as MLKL, RIPK1 and RIPK3.

Assessing ferroptosis in diverse biological contexts

Ferroptosis is a process that has likely been discovered and re-discovered a number of times over the years before a specific molecular understanding of this phenotype was solidified (Dixon and Stockwell, 2013). Since 2012, rapid progress has been made in defining the molecular regulation of ferroptosis. We anticipate that additional regulators and biological contexts will emerge for ferroptosis in the future. We suggest here basic criteria for concluding that ferroptosis is occurring in a particular experimental system, while acknowledging that these criteria may vary depending on the mechanism by which ferroptosis is triggered. In general, ferroptosis should be suppressed by both an iron chelator (e.g., DFO or CPX) and a lipophilic antioxidant (e.g., ferrostatin, liproxstatin, vitamin E, BHT), and should involve accumulation of lipid hydroperoxides. Ferroptosis induced by system x_c^- inhibition should be suppressed by β-mercaptoethanol, which reduces extracellular cystine to cysteine, bypassing the requirement for system x_c^- for import, as cysteine is imported through other mechanisms (Dixon, 2012). System x_c^- inhibition should result in glutathione depletion. Gene expression markers associated with cells undergoing ferroptosis include increases in *CHAC1* and *PTGS2* mRNA and ACSL4 protein (Table 2), but these changes may not be observed in all experiments. In addition, inhibitors of apoptosis (*e.g.,* caspase

inhibitors or deletion of BAX/BAK) or necroptosis (*e.g.,* necrostatins or necrosulfonamide) should be examined to rule out these mechanisms as being required for cell death. Cells dying by ferroptosis primarily exhibit shrunken and damaged mitochondria by electron microscopy, with few other morphological changes evident prior to the point of cell death (Yagoda et al., 2007). In contrast, apoptosis typically involved fragmentation and margination of chromatin, as well as generation of apoptotic bodies and plasma membrane blebbing. Using the criteria and reagents described herein will allow the scientific community to determine the role of ferroptosis in a wide variety of systems, diseases and biological processes in the years to come.

Acknowledgements

We thank The Banbury Center at Cold Spring Harbor Labs for hosting a meeting of the authors that served as the genesis of this manuscript. We thank the following funding organizations: NIH (R35CA209896 to BRS, R01CA171101 to FT, R01CA188025 to ST, CA102184 to MEM, HL114453, U19AI068021, NS076511, NS061817 to VEK and HB), JSPS KAKENHI (JP16K15257 and JP17H04064 to ST). CSR is an employee of and has a profit interest in Collaborative Medicinal Development, LLC. KN is a paid consultant of and has a profit interest in Collaborative Medicinal Development, LLC. AIB is a shareholder in Prana Biotechnology Ltd, Mesoblast Ltd, Grunbiotics Pty Ltd, Cogstate Ltd, and is a paid consultant for and has a profit interest in Collaborative Medicinal Development, LLC.

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Figure Legends

Figure 1. GPX4 uses glutathione to eliminate lipid peroxides formed in phospholipids containing polyunsaturated fatty acids. System x_c^- imports cystine, which is reduced to cysteine and used to synthesize glutathione, a necessary cofactor of GPX4 for eliminating lipid peroxides.

Figure 2. Pathways controlling ferroptosis. The indicated pathways regulate ferroptosis sensitivity.

Table 1. Reagents that modulate ferroptosis. Numerous reagents have been found to modulate sensitivity to ferroptosis.

Table 2. Genes involved in ferroptosis. Numerous genes have been found to regulate ferroptosis or to serve as markers of ferroptosis.

Table 3. Features of dementia consistent with ferroptosis. The listed features of dementia are consistent with a role for ferroptosis in the disease pathogenesis.

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Stockwell, BR; Angeli, JPF; Bayir, H; Bush, AI; Conrad, M; Dixon, SJ; Fulda, S; Gascon, S; Hatzios, SK; Kagan, VE; Noel, K; Jiang, X; Linkermann, A; Murphy, ME; Overholtzer, M; Oyagi, A; Pagnussat, GC; Park, J; Ran, Q; Rosenfeld, CS; Salnikow, K; Tang, D; Torti, FM; Torti, SV; Toyokuni, S; Woerpel, KA; Zhang, DD

Title:
Ferroptosis: A Regulated Cell Death Nexus Linking Metabolism, Redox Biology, and Disease

Date:
2017-10-05

Citation:
Stockwell, B. R., Angeli, J. P. F., Bayir, H., Bush, A. I., Conrad, M., Dixon, S. J., Fulda, S., Gascon, S., Hatzios, S. K., Kagan, V. E., Noel, K., Jiang, X., Linkermann, A., Murphy, M. E., Overholtzer, M., Oyagi, A., Pagnussat, G. C., Park, J., Ran, Q. ,... Zhang, D. D. (2017). Ferroptosis: A Regulated Cell Death Nexus Linking Metabolism, Redox Biology, and Disease. CELL, 171 (2), pp.273-285. https://doi.org/10.1016/j.cell.2017.09.021.