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To eat or not to eat – the metabolic flavor of ferroptosis

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Ferroptosis is a newly defined iron-dependent, non-apoptotic mode of cell death with necrotic morphology. Distinctive from other death mechanisms, ferroptosis requires cellular iron and lipid peroxides, and is dictated by specific cellular metabolic processes. Importantly, ferroptosis has been implicated in a plethora of human diseases. This paper reviews the recent advances and outstanding questions of the field by focusing on the role of cellular metabolism in ferroptosis. The relevance of ferroptosis to disease and therapy is also discussed.

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Introduction

Death is the common fate of all living matters, including every cell in our bodies. Although often detrimental and a sign of deterioration, cell death can also be an integral part of life and be harnessed, or programmed, to benefit the multicellular organism, thus the concept of ‘programmed cell death’ (PCD). For example, spatially and temporally orchestrated PCD is critical in shaping the structure and function of developing tissues and organs, and precise PCD of immune cells enables proper immune response without yielding autoimmune disorders. Conversely, malfunction of PCD contributes to the development of various diseases, such as cancer, immune diseases, and neurodegeneration [1–5].

Two criteria need to be satisfied for the strict qualification of PCD: (1) the death process is genetically programmed, that is, the function of certain gene product(s) is required for the execution (NOT only inhibition) of cell death; and

(2) the death process benefits the organism under specific biological contexts. However, in this review and numerous other publications in the PCD field, the term ‘PCD’ is used in a more loose way to refer to genetically regulated cell death processes (the first criteria). A reasonable, although quite naïve, assumption is: if a delicate and highly regulated molecular pathway is required for the execution of a particular cell death process, this process most likely is relevant to, and contributes to, normal biology of the organism; and final validation of such life-benefiting functions of the death process might simply be a matter of time.

Historically, cell death had been divided into three categories based on their distinctive morphological features: type I (apoptosis), type II (autophagy), and type III (necrosis) [6]. Apoptosis and autophagy were considered to be ‘programmed’, while necrosis was believed to be passive, unregulated, and pathological [6]. In early days, programmed cell death had been synonymous with apoptosis, owing to the elegant discoveries made by Horvitz and colleagues using *Caenorhabditis elegans* model system, as well as subsequent studies in other organisms [7,8]. These studies unveiled the precise molecular program of apoptosis and its crucial function in a plethora of normal biological processes. More recently, it has been demonstrated that there are multiple forms of PCD in addition to apoptosis. Importantly, growing evidence shows that even necrosis, previously believed to be ‘non-programmed’, can also be molecularly programmed and might even play physiologically beneficial roles under specific biological conditions [9–12]. One such example is RIPK3-dependent necrosis, also known as necroptosis [13–15]. In this review, we will focus on another type of cell death known as ferroptosis, which is a blend of necrosis (morphologically) and autophagy (autophagy promotes ferroptotic death, as described later).

The emergence of the concept of ferroptosis

In 2012, the Stockwell lab reported that a small molecule, erastin, which they identified by a high throughput screening, can induce potent cell death in oncogenic KRas-expressing fibroblasts [16,17,18]. Distinctive from apoptotic cell death, erastin-induced cell death does not require caspase activation, and during cell death there is no typical apoptotic morphological features such as chromatin condensation or nuclear fragmentation. Their counter screening led to the discovery of iron chelators, among other chemical compounds, as inhibitors of such erastin-induced cell death, suggesting iron is essential for this non-apoptotic cell death. The term of ferroptosis is thus coined to describe this iron-dependent, non-

apoptotic cell death process. Further, the molecular target of erastin has been identified as glutamate-cystine antiporter system Xc⁻. System Xc⁻ is a plasma membrane-localized amino acid transporter, which mediates the import of cystine in exchange of the export of glutamate. Once inside the cell, cystine is reduced to cysteine, providing substrate for glutathione syntheses and maintaining cellular redox homeostasis. Upon erastin treatment, the system Xc⁻ is inactive and import of cystine is blocked, leading to cysteine starvation and glutathione depletion; and this is the cause of ferroptotic cell death [18^{**},19^{**}].

Ferroptosis is reminiscent of a form of metabolically controlled cell death described decades earlier. In 1950s, it was reported that extracellular cysteine and cystine are essential for growth of various types of cultured cells, cystine starvation leads to glutathione depletion and triggers cell death [20]. As expected, this death can be prevented by the antioxidant atocopherol [21^{*}]. Intriguingly, iron chelators, such as deferoxamine, were found to be able to prevent cystine deprivation-induced cell death [22]. Taken together, what these early investigators had observed is probably the cell death process we now know as ferroptosis.

Another effort to define the role of metabolism in cell death and cell survival also led to the discovery of ferroptosis [23^{**},24]. The discovery started with a surprising and counter-intuitive observation: in multiple types of cultured cells, total amino acid starvation can induce rapid and potent cell death, which is non-apoptotic and non-necroptotic, but only in the presence of serum. Subsequently, it was revealed that deprivation of cystine was sufficient (as total amino acid starvation) to induce such cell death in the presence of serum, and serum iron carrier protein transferrin is essential for the killing [23^{**},24]. These findings naturally led to the idea, and subsequent experimental validation, that the death mode is ferroptosis. Importantly, another serum factor, the amino acid glutamine (which is present in regular medium but not in total amino acid-free medium) as well as its cellular metabolism (glutaminolysis), was also found to be essential for ferroptosis induced either by amino acid starvation plus serum or by erastin.

Remarkably, both transferrin and glutamine are crucial for cell viability under normal conditions, but they are nonetheless essential for the execution of ferroptotic cell death under specific conditions. Taken together, this work further demonstrated an intimate communication between ferroptosis and cellular metabolism [23^{**},24].

Below we will discuss the role of various cellular metabolic processes in ferroptosis by focusing on the metabolism of reactive oxygen species (particularly lipid peroxides), iron, and amino acids (Figure 1).

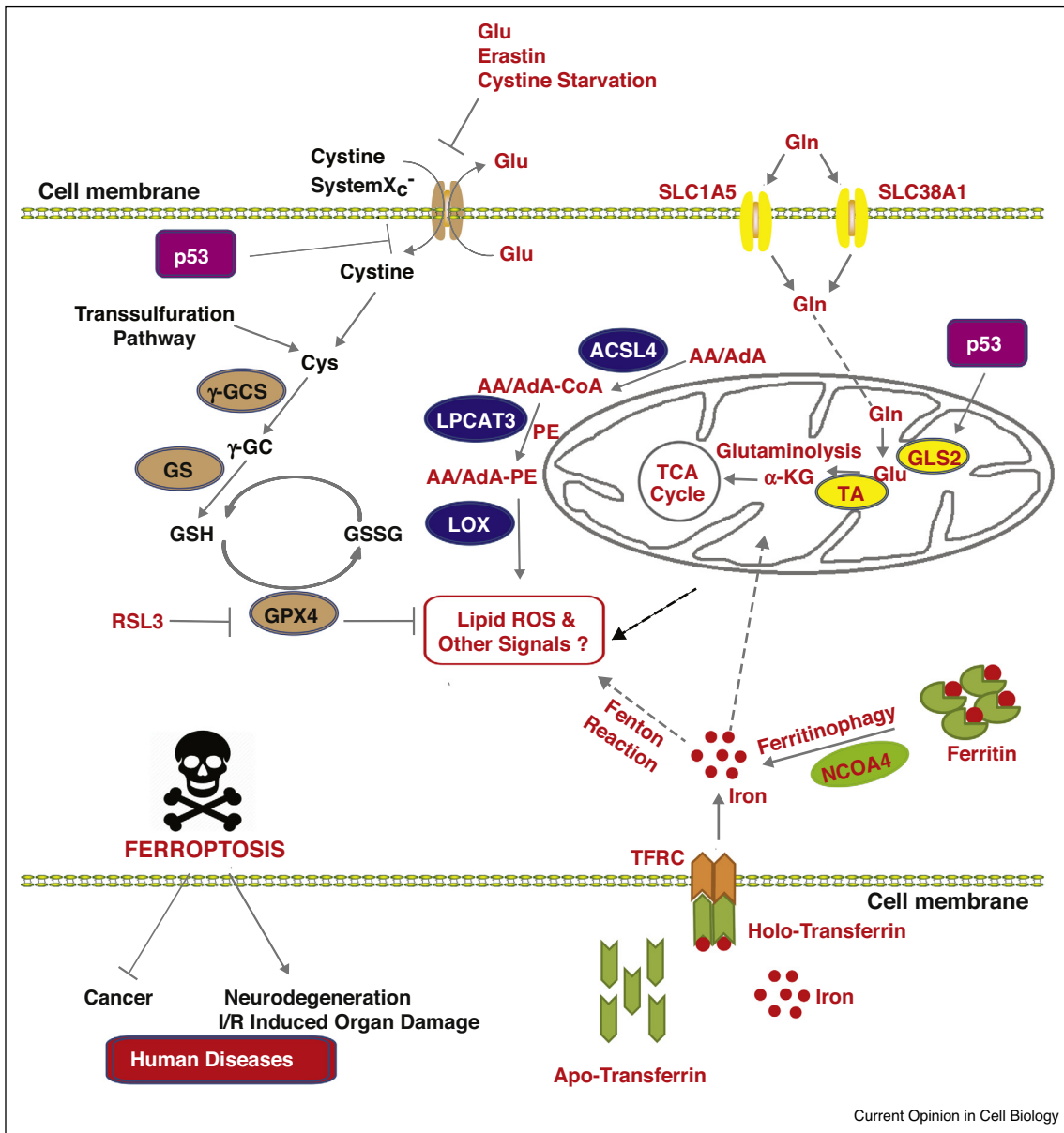
ROS and lipid peroxidation in ferroptosis

Reactive oxygen species (ROS) are partially reduced oxygen-containing molecules, including superoxide (O₂^{+·}), peroxides (H₂O₂, ROOH) and free radicals (HO₂[·] and RO₂[·]) [25]. Although a low and controlled level of ROS is crucial for normal cellular and organismal function, the aberrant accumulation of ROS is linked to a number of acute organ injuries and chronic degenerative conditions. Mitochondria are one of the most important organelles where significant amounts of ROS are generated from normal metabolism and energy production through the electron transport chain (ETC).

The observation that cystine deprivation (or equivalently, system Xc⁻ inhibition) can trigger ferroptosis immediately suggested a role of (ROS) in ferroptosis [18^{**},23^{**}]. Indeed, ferroptosis can be inhibited by various antioxidants and ROS scavengers. But what is the exact source of lethal ROS production upon ferroptosis induction? This is still a highly debated question. For example, the role of mitochondria in ferroptosis-associated ROS generation is controversial. On one hand, oligomycin, a mitochondrial oxidative phosphorylation inhibitor was reported to partially inhibit ferroptosis, suggesting ETC is one of ROS sources [23^{**}]. On the other hand, cells with mitochondrial DNA depleted can still undergo potent ferroptosis, suggesting that mitochondrion is dispensable for ferroptosis [18^{**}].

Not all ROS molecules function equally in ferroptosis. It is generally accepted that eventual executioners of ferroptosis are lipid peroxides, and it takes excess lipid peroxide generation to cause plasma membrane damage and ultimately, ferroptotic cell death [26]. Several commonly used ferroptosis inhibitors, including the original ferrostatin-1, are believed to function by trapping lipid peroxides. Consistently, inhibition of phospholipid glutathione peroxidase 4 (GPX4, an antioxidant enzyme that uses glutathione as a cofactor to catalyze the reduction of lipid peroxides) genetically or pharmacologically can lead to ferroptosis even when cells have ample supply of cystine/cysteine [19^{**},27^{**}]. Inhibition of GPX4 results in uncontrolled polyunsaturated fatty acid (PUFA) oxidation and fatty acid radical generation, thereby leading to ferroptotic cell death [28^{*}] (Figure 1). More recently, a study using a genome-wide CRISPR-based genetic screen and microarray analysis of ferroptosis-resistant cell lines unveiled that acyl-CoA synthetase long-chain family member 4 (ACSL4) drives ferroptosis via accumulating oxidized cellular membrane phospholipids [29^{**},30^{**}]. Further, a series of experiments suggest the following specific lipid metabolic pathway plays a central role in ferroptosis: ACSL4 promotes ferroptosis by producing oxidized phosphatidylethanolamines (PE) in endoplasmic reticulum-associated oxygenation center; ACSL4 catalyzes the ligation of an arachidonoyl (AA) or adrenoyl (AdA) to produce AA or AdA acyl-Co-A derivatives; these

Figure 1



Regulation of ferroptosis by cellular metabolism. Ferroptosis is characterized by iron-dependent lipid peroxidation. Under normal conditions, glutathione synthesis axis, consisted of system Xc^- - γ -GCS-GS/GPX4, counteracts lipid peroxidation and thus prevents ferroptosis. Ferroptosis inducers inhibit the system Xc^- - γ -GCS-GS/GPX4 axis, leading to accumulation of lipid ROS (which is generated by the ACSL4/LPCTA3/LOX lipid peroxidation pathway). Transferrin receptor-mediated iron transportation and NCOA4-mediated ferritinophagy promote ferroptosis by sustaining cellular iron availability. Glutamine metabolic pathway, glutaminolysis, plays crucial roles in the death process. Glutaminolysis may contribute to lipid peroxidation by providing precursors towards fatty acid or lipid synthesis. *Abbreviations:* AA/AdA, arachidonic acid or adrenic acid; AA/AdA-CoAA, arachidonic acid or adrenic acid Coenzyme A; AA/AdA-PE, arachidonic acid or adrenic acid-phosphatidyl-ethanolamine; ACSL4, acyl-CoA synthetase long-chain family member 4; Cys, L-cysteine; γ -GC, gamma-glutamylcysteine; γ -GCS, γ -glutamylcysteine synthetase; GPX4, glutathione peroxidase 4; Gln, L-glutamine; GLS2, glutaminase 2; GS, glutathione synthetase; GSH, reduced glutathione; GSSG, di-glutathione; Glu, L-glutamate; I/R induced organ damage, ischemia/reperfusion induced organ damage; α -KG, α -ketoglutarate; LPCAT3, lysophosphatidylcholine acyltransferase 3; LOX, lipoxygenase; NCOA4, nuclear receptor coactivator 4; PE, phosphatidyl-ethanolamine; lipid ROS, lipid reactive oxygen species; TA, transaminases; TCA, tricarboxylic acid cycle; TFRC, transferrin receptor.

derivatives are then esterified into phosphatidylethanolamines (AA-PE and AdA-PE) by lysophosphatidylcholine acyltransferase 3 (LPCAT3), and AA-PE and AdA-PE are subsequently oxidized by 15-lipoxygenase (15-LOX) to generate lipid hydroperoxides, which execute ferroptosis [29^{••},30^{••}] (Figure 1).

Although the essential and necessary role of lipid peroxides in ferroptosis has been established beyond doubt, there is no definitive evidence proving that this class of ROS is the most downstream molecules that drive ferroptosis. Therefore, the jury is still out on what the ultimate molecular executioner of ferroptotic cell death is (as caspases for apoptosis).

It should be noted that recent evidence indicates a role of the antioxidant defensive system, coordinated by the transcriptional factor NRF2, in the regulation of ferroptosis [31,32[•]]. This is not unexpected, considering the central role of ROS in ferroptosis.

Iron metabolism and ferroptosis

As the name ‘ferroptosis’ infers, the requirement of intracellular iron is a fundamental property of ferroptosis. Be the death triggered from the most upstream of the pathway (such as by erastin or direct cystine deprivation) or at a downstream step (such as by inhibiting GPX4), ferroptosis and associated lipid peroxide accumulation can always be completely abrogated by iron chelators [22].

Intracellular iron metabolism and homeostasis is under delicate regulation. A sophisticated network, involving iron-binding and mRNA-regulatory proteins IRP1 and IRP2 (iron-regulatory protein 1 and 2), can directly sense the concentration of free iron (Fe^{2+}) in cells, and respond by altering the synthesis of a series of proteins governing iron export, import, storage and release [33]. During ferroptosis, this fine-tuned iron homeostasis is probably disrupted, and an undesired increase of free cellular iron contents (also known as ‘labile iron portion’ or ‘LIP’) occurs. Such abnormal increase of LIP requires transferrin and transferrin receptor (to import iron from extracellular environment), as well as autophagic-lysosomal degradation of ferritin (to release the stored intracellular iron) [34^{••},35[•]] (Figure 1).

The finding that autophagy promotes ferroptosis is also important for the field of autophagy. It has been a long-standing and highly debated question in the autophagy field whether autophagy, commonly a stress-responsive mechanism for cell survival, can also be pro-death as the term ‘autophagic cell death’ implies. The finding that autophagic degradation of ferritin (also known as ferritinophagy, mediated by the specific autophagy cargo receptor NCOA4 [36,37]) mediates ferroptosis provides a defined mechanism that indeed autophagy can promote

cell death under specific biological conditions [34^{••},35[•]] (Figure 1).

Although intracellular iron is essential for ferroptosis, the key question — what is the exact function of iron in ferroptosis — remains to be answered. One proposed model is that iron is involved in the generation of lipid ROS, either through Fenton chemistry, or via the action of iron-dependent oxidases. Since iron is a cofactor of various metabolic enzymes (e.g., multiple enzymes in the TCA cycle), the requirement of iron for ferroptosis may simply reflect the role of these enzymes in ROS generation. It should be noted though, that a redox-independent role of iron has not been completely ruled out, and that iron might have multiple functions in ferroptosis.

The essential role of iron in life can be dated back to billions years ago when oxygen just became available to drive central metabolic process in living cells. The oxygen/redox-centered metabolism, almost always with the involvement of iron (due to the unique physical chemical property of this transition metal), created a paradox: while iron-dependent biochemical reactions are essential for life, these highly efficient processes are associated with generation of ROS, which can be harmful and even lethal. Conversely and speculatively, organisms may take advantage of such ROS/iron-driven cell death and ‘program’ it for their own benefit, that is, can ferroptosis be the most ancient form of PCD? And if so, under what contexts life can be benefitted from ferroptotic cell death?

Amino acid metabolism and ferroptosis

Amino acids play a central role in ferroptosis. Ferroptosis is induced by cystine/cysteine depletion when cells are treated with erastin or other system Xc^- inhibitors [18^{••},23^{••}]. Some cells can use the transsulfuration pathway to biosynthesize cysteine from methionine when the system Xc^- is inactivated; as a result, these cells are resistance to ferroptosis induced by system Xc^- inhibitors [38]. Knockdown of cysteinyl-tRNA synthetase (*CARS*) results in upregulation of the transsulfuration pathway and resistance to erastin-induced ferroptosis. As expected, *CARS* knockdown cells are still sensitive to ferroptosis induced by GPX4 inhibitors [38] (Figure 1).

Both glutamate and glutamine play important roles in ferroptosis. High concentration of extracellular glutamate can block cystine uptake by inhibiting the activity of system Xc^- , thus inducing ferroptosis [18^{••},39]. The role of glutamine in ferroptosis is more complexed [23^{••}]. Although glutamine can be converted to glutamate by glutaminases (GLS1 and GLS2) in cells, high dose of extracellular glutamine alone cannot induce ferroptosis. Instead, glutamine drives ferroptosis in combination of cystine deprivation. Glutamine fuels ferroptosis through its specific metabolism, glutaminolysis [23^{••}] (Figure 1). In the absence of glutamine, or when glutaminolysis is

inhibited, cystine starvation and blockage of cystine import cannot induce ferroptosis or the associated rapid accumulation of ROS and lipid peroxidation. The role of glutaminolysis in ferroptosis can be explained by the observation that α -ketoglutarate (aKG), a product of glutaminolysis, can replace the requirement of glutamine for ferroptosis. Interestingly, although both GLS1 and GLS2 can convert glutamine to glutamate, only GLS2 is required for ferroptosis [23**]. Since GLS1 is a cytosolic protein whereas GLS2 localizes in the mitochondria [40], it is likely that mitochondria plays a role in ferroptosis-associated ROS accumulation.

Ferroptosis and human diseases

Although a life-beneficial or physiological function of ferroptosis has yet to be uncovered (genetic deletion of GPX4 in mouse model only gives rise to developmental defect and lethality [27**,41–44]), the role of ferroptosis in various human diseases have been established.

Studies using ferroptosis inhibitors such as ferrostatin-1 and its improved analogs, as well as glutaminolysis inhibitors, have demonstrated the important role of ferroptosis in ischemia/reperfusion (I/R)-induced damage in organs such as kidney, liver, brain, and heart [18**,21*,23**,27**]. These studies suggest that ferroptosis is the major mode of cell death associated with I/R-induced organ damage, and thus ferroptosis is a promising therapeutic target for the treatment of these diseases.

Ferroptosis is also implicated in neuron degenerative diseases, as ferrostatins and iron chelators have been shown to be effective in models of Huntington's disease and Parkinson's disease [45]. Additionally, ferroptosis has been suggested to contribute to neuronal cell death triggered by high dose of glutamate, namely glutamate excitotoxicity, because cystine import can be inhibited by high dose of glutamate [18**].

Can ferroptosis pathway be explored for cancer treatment? Indeed, the original study that led to the identification of erastin aimed for selective killing of cancer cells [16*,17*,18**]. Since then, multiple lines of research suggest the important role of ferroptosis in cancer development and treatment. For example, it has been reported that a p53 mutant which loss the ability to induce apoptosis and senescence can still inhibit tumor growth by suppressing the expression of SLC7A11, a component of system x_c^- , thus activating ferroptosis [46**]. Additionally, since multiple cancer types are addicted to glutaminolysis, can these cancer cells be more sensitive to ferroptosis induction as a potential treatment? In glutaminolysis pathway, GLS2 but not GLS1 mediates ferroptosis [23**]. Consistently, GLS1 is a putative oncoprotein whereas GLS2 has been suggested to be a tumor suppressor [43,47,48]. Further, the GLS2 but not GLS1 gene is a transcriptional target of p53, and upregulation of

GLS2 contributes to p53-dependent ferroptosis [48,49]. Most recently, it was reported that a therapy-resistant, high-mesenchymal cell state depends on a druggable GPX4/lipid peroxidase pathway to evade ferroptosis, further supporting the notion that induction of ferroptosis represents a novel strategy for anti-tumor drug discovery [50*].

Conclusions and perspectives

Distinctive from other forms of cell death, ferroptosis features lipid ROS and iron dependency. Recent advance has provided insights into the precise molecular mechanisms of ferroptosis, particularly its relationship with cellular metabolism. The relevance of ferroptosis to various human diseases has also been well established. However, many critical questions remain. For example, what is the molecular executioner of ferroptosis (is it lipid peroxides, if so, any specific subclasses?)? What happens after lipid oxidation during ferroptosis, and what is the point of no-return of ferroptosis? What is the exact function of iron in ferroptosis? Further, how do the central metabolic processes in ferroptosis, namely, that of iron, redox, amino acids, and lipids, communicate with each other to dictate the output of survival versus ferroptotic cell death? Last but not the least, the normal physiological function of ferroptosis, if any, is still elusive. Answers to these questions will draw a clear picture about the ferroptotic pathway and should be instrumental in translating our knowledge of this basic cell biology to clinical settings.

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