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Review article

The development of the concept of ferroptosis

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ABSTRACT

The term ferroptosis was coined in 2012 to describe an iron-dependent regulated form of cell death caused by the accumulation of lipid-based reactive oxygen species; this type of cell death was found to have molecular characteristics distinct from other forms of regulated cell death. Features of ferroptosis have been observed periodically over the last several decades, but these molecular features were not recognized as evidence of a distinct form of cell death until recently. Here, we describe the history of observations consistent with the current definition of ferroptosis, as well as the advances that contributed to the emergence of the concept of ferroptosis. We also discuss recent implications and applications of manipulations of the ferroptotic death pathway.

1. Introduction

Death is a common fate of all life, from organisms to cells. The understanding that cell death can be regulated by molecular mechanisms and can yield physiological benefits and pathological consequences for multicellular organisms emerged early in the 1960s, with the concept of 'programmed cell death' [1–3]. It is now established that such programmed cell death is essential for normal development and homeostasis and, when dysregulated, contributes to a variety of pathological conditions.

Regulated cell death is defined as a death process that relies on dedicated molecular machinery, and that thus can be modulated (delayed or accelerated) by specific pharmacological and genetic interventions. Programmed cell death refers to physiological instances of regulated cell death that occur in the context of development and tissue homeostasis, in the absence of any exogenous perturbations. Programmed cell death is therefore a subset of regulated cell death. Regulated cell death is used to describe cell death that originates from perturbations of the intracellular or extracellular microenvironment, executed by molecular mechanisms when other adaptive responses are incapable of restoring cellular homeostasis [4]. Regulated cell death is thus mechanistically distinct from classic necrosis, or unregulated cell death caused by overwhelming stresses, such as dramatic heat shock, use of detergents, pore forming reagents, or highly reactive alkylating agents.

One may ask whether induced loss, through chemical or genetic perturbation for example, of an essential cellular factor represents regulated cell death or simple loss of the homeostasis needed for life. For example, deletion of the *Mdm2* gene, or inhibition of the MDM2 protein, results in cell death in many cell lines, and in embryonic lethality in mice due to the lack of this essential protein [5–9]. However, this death and embryonic lethality can be entirely reversed by the simultaneous deletion of the *TP53* tumor suppressor gene, which is the major target of MDM2 [6,8,10]. Thus, in this case, the idea that normal life requires MDM2, and that loss of MDM2 represents a sabotaging of life's homeostatic machinery is not accurate. Indeed, cells and mice can exist, albeit not as well, without MDM2, as long as they also lack the p53 protein. Similarly, one might wonder if the cell death caused by deletion of an essential metabolic gene represents the loss of an essential factor needed for life (*e.g.*, hexokinases [11]). However, in this case, again, such cells may survive if they are provided with the product of the metabolic enzyme, or an alternative means to satisfy this metabolic need. The important point is that the "normal" requirements for life depend on the network of other cellular and environmental factors present at a given point in time.

One might nonetheless suppose that although the cellular factors needed for normal homeostasis are context-dependent, the *mechanism* of death resulting from the loss of a context-dependent essential factor may not be of interest *per se*. However, if the cell death mechanism in such a situation reveals the relationship between the missing factor and cellular regulators of its essential function, then this mechanism may indeed be of interest. Moreover, it is possible in some cases to trigger regulated cell death for therapeutic benefit, such as in some cancers. Some pathological processes also act through depletion of essential

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factors and the resulting cell death process, and inhibition of such death mechanisms may be beneficial.

Finally, the surprising finding over the last two decades is that there appears to be a limited number of execution mechanisms for cell death, despite the wealth of possible triggers of cell death. Although there are thousands of essential genes in most organisms, cell death after loss of such genes occurs by unregulated necrosis, or converges on one of a handful of execution mechanisms involving activation of proteases, permeabilization of the plasma membrane, or overwhelming lipid peroxidation. In summary, the concept of regulated cell death encompasses the notions that normal homeostasis is context-dependent, that the mechanisms driving cell death are intrinsically of interest, and that such mechanisms converge on a limited number of execution mechanisms.

Historically, cell death was divided into three main categories, based on distinctive morphological features: type I cell death, referred to as apoptosis, type II cell death, referred to as cell death involving autophagy, and type III cell death, referred to as necrosis. Regulated cell death was considered synonymous with apoptosis [12], and only more recently was it demonstrated that there are multiple forms of regulated cell death, which are distinct in their molecular mechanisms and morphological characteristics.

In contrast to type I and type II cell death, which were considered to be 'programmed' and 'regulated', necrosis was categorized as an 'accidental cell death', and believed to be passive, unregulated, and commonly associated with pathological death caused by exposure to severe insults of a physical, chemical, or mechanical nature that could not be reversed by molecular perturbations [12].

The Nomenclature Committee of Cell Death (NCCD) recommended in 2012 that researchers replace the morphologic classification of cell death with a new classification based on molecular events associated with cell death [13]. This change contributed to a classification of regulated cell death that uses biochemical mechanisms. For instance, even though the notion that death with a necrotic morphology can be molecularly regulated began to emerge in the late 1980s [14], it took two decades until it was widely accepted that 'necroptosis' should be classified as a form of regulated cell death [15,16]. Contributing to this notion were the discoveries of the involvement of molecular mechanisms such as death receptors (*e.g.,* Fas, TNFR1 [17,18]) and specific kinases (*e.g.*, RIP1, RIP3 [19]) that can be genetically or chemically inhibited to delay or inhibit necroptosis [20]. Necroptosis also has normal physiological functions, such as the modulation of inflammation and the maintenance of adult T-cell homeostasis [15,21,22], and may thus be categorized as programmed cell death [23].

We focus here on the historical discoveries that led to the development of the concept of ferroptosis. Ferroptosis is defined as an iron-dependent form of regulated cell death, which occurs through the lethal accumulation of lipid-based reactive oxygen species (ROS) when glutathione (GSH)-dependent lipid peroxide repair systems are compromised [24]. Ferroptosis involves genetic, metabolic, and protein regulators, triggers, and execution mechanisms that for the most part do not overlap with other forms of regulated cell death [25]. Ferroptotic cell death can be inhibited by lipophilic antioxidants, iron chelators, inhibitors of lipid peroxidation, and depletion of polyunsaturated fatty acyl phospholipids (PUFA-PLs), which are prime substrates driving lethal lipid peroxidation [24,26].

2. Early observations consistent with ferroptosis

Ferroptosis has been observed a number of times over the years prior to the detailed molecular understanding of this cell death process, and the concept that it exists (summarized in Fig. 1 and Table 1). Yet, until it was termed as such in 2012, reports describing what we now know as cell death with ferroptotic characteristics were attributed to other cell death mechanisms, or not recognized as being biologically significant. For example, metabolic dependencies leading to cell death were noted in the decades before ferroptosis was discovered: early in the 1950's, studies were performed by Harry Eagle and colleagues to test the requirements for specific metabolites, such as amino acids, vitamins and other nutrients, to support growth and proliferation of mammalian cells in culture [27–29]. These reports showed that starvation of just a single amino acid out of 13 different amino acids tested inhibited the growth of human and mouse cells in culture: cells deprived of cystine exhibited a unique microscopic morphology that was different from the morphologies apparent upon deprivation of other amino acids, which the authors speculated to be similar to death caused by viral infection [28].

In 1959, these investigators found that in cystine-free medium, incorporation of cysteine in *de novo* protein biosynthesis did not suffice to restore cell growth, thus concluding that cystine entails an additional metabolic function besides incorporation into protein. These studies linked cystine deprivation with the disappearance of glutathione and showed that glutathione supplementation could promote growth in cystine-free media [29,30]. Presumably, cysteine did not suffice because it is taken up by cells through different mechanisms than cystine. In the early 1970's, there were reports of hepatic necrosis (that today would be referred to as ferroptosis) in mice, which was accompanied by glutathione depletion and could be rescued by pretreatment of glutathione or cysteine [31].

In the 1970's, Shiro Bannai and colleagues showed that cystine starvation led to reduction in cellular glutathione and cell death [32]. Supporting the contribution of reactive oxygen species (ROS) accumulation in the induction of cell death was the investigators' observation that this cystine-deprivation-induced death could be rescued by the addition of the lipophilic antioxidant α-tocopherol (a component of vitamin E), without restoring glutathione levels [32]. These results implied that cysteine, derived from reduction of cystine, was needed to sustain glutathione levels and to prevent lipid-ROS-based toxicity, which could also be prevented by lipophilic antioxidants.

In the following years, several studies confirmed the crucial role of cellular cysteine deprivation and glutathione depletion in inducing cell death, and demonstrated that both iron chelators (or serum-deprivation due to lack of iron) and lipophilic antioxidants could block such death from occurring [33–41], which are now recognized as the cardinal features of ferroptosis. These common dependencies and features were demonstrated in many types of mammalian cells, including human embryonic fibroblasts [42], hybridomas and myelomas [33], cortical neurons [34–36], oligodendroglia [37], oligodendrocytes [38,39] and hair cells [43]. Remarkably, these numerous studies recognized reactive oxygen species as drivers of the death process, as well as several distinct triggers and rescuers of cell death, but were nonetheless not interpreted as evidence for a distinct cell death process that does not overlap with apoptosis or necrosis, although it was speculated upon in some cases (*e.g.,* [44]). The situation is similar to the history of protein degradation, which was assumed to be largely unregulated prior to the pioneering studies of Avram Hershko, Aaron Ciechanover and Irwin Rose [45]. Indeed, numerous pieces of the ubiquitin-dependent pathway of protein degradation were known, but not recognized as such [46–49].

At the heart of ferroptosis is a process of lethal lipid peroxidation, which is the oxidative addition of molecular oxygen (O_2) to lipids, such as polyunsaturated fatty acyl tails in phospholipids. The first descriptions of such enzymatic reactions were in 1955 by Peterson and colleagues [50] and Rothberg and colleagues [51] independently; since then, lipid peroxidation by lipoxygeneses and other mechanisms for the peroxidation of lipids have received a great deal of attention in diverse biological contexts [52–54]. The first suggestion of lipid peroxidation as a prime cause of cellular damage was in 1965, when two separate groups studying drug toxicology showed increases in lipid peroxidation in the liver of CCl_4-treated rats [55,56]. By the 1980s, it was well established that lipid peroxidation is one of the major forms of oxidative damage through destruction of unsaturated lipid moieties of cell membranes, lipoproteins and other structures [57,58], and was

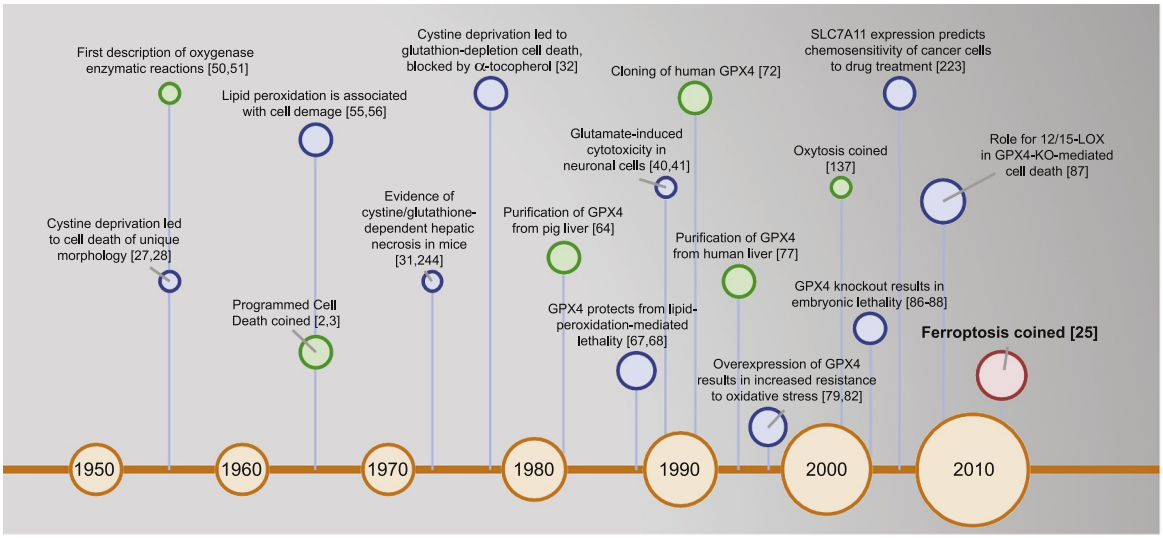

Fig. 1. Ferroptosis-related scientific discoveries before it was termed. Schematic timeline highlights key discoveries that contributed to the emergence of the concept of ferroptosis (green) and important observations that are consistent with the current definition of ferroptosis (blue), before it ferroptosis termed.

correlated with a number of pathological conditions [59]. However, these events were associated with other cellular damage mechanisms and not recognized as a cell death mechanism *per se*.

Due to the dual contribution of cellular ROS to signaling mechanisms and cell lethality, enzymatic control of redox regulation is an essential regulatory mechanism for normal cell homeostasis [60]. Redox-sensitive cysteine residues in enzymes exploit the unique ability of sulfur to cycle between oxidation states. Selenoproteins that carry a catalytically active selenocysteine can also contribute to redox control [61].

In a seminal discovery, the membrane-associated 'phospholipid hydroperoxide glutathione peroxidase 4' (PHGPX or GPX4) was isolated as the second known selenoperoxidase, after the identification of the cytosolic glutathione peroxidase (GPX1) [62,63]. GPX4 was first isolated and purified from pig liver in 1982 by Urisini and colleagues [64] and was identified on the basis of its ability to inhibit iron-catalyzed lipid peroxidation in microsomes. The enzyme was first given the functional name 'peroxidation-inhibiting protein', that was later changed to its current name [65]. This enzyme was described as a glutathione peroxidase that protects phosphatidylcholine-containing liposomes and biological membranes from peroxidative degradation, and is now known to be the key enzymatic inhibitor of ferroptosis. Early work also showed a similar protective role for the lipophilic antioxidant α-tocopherol (vitamin E) in rat liver mitoplasts and microsomes, which was supported by previous similar observations [66]. In the late 1980s, GPX4 was shown to have lipid-peroxidation-protective effects in

Table 1
Main findings describing cell death of ferroptotic nature, before ferroptosis was coined.

	Year	Main finding	Ref
Lipid peroxidation			
	1962	Iron contributes to lipid peroxidation-associated pathology in rats	[121]
	1965	CCl_4-mediated lipid peroxidation is associated with cell death in rat liver	[55,56]
	1984	Lipid peroxidation is linked to multiple pathological conditions	[57–59]
	2005	Ceramide induces non-apoptotic ROS-dependent cell death	[104]
Cystine deprivation			
	1955	First observation that cystine deprivation lead to cell death of unique morphology	[27,28]
	1973	Cystine- and glutathione-dependent hepatic necrosis in mice	[31,245]
	1977	Cystine deprivation lead to glutathione depletion-mediated cell death in fibroblasts, which is rescued by α-tocopherol	[32]
	1979	Scavenging deleterious oxidative radicals protect from oxidative necrosis induced by glutathione depletion in fibroblasts	[42]
	1989	Glutamate-induced cytotoxicity in neuronal cells is due to inhibition of cystine uptake, resulting in lowered glutathione levels leading to oxidative stress and cell death	[40,41]
	1994	Cystine deprivation leads to death of embryonal cortical neurons due to reduced formation of glutathione	[34,35]
	1994	Serum deprivation diminished glutathione-depletion-induced death in embryonal cortical neurons	[36]
	1996	Cystine deprivation lead to reduced glutathione-mediated death of oligodendroglia, rescued by antioxidants and iron chelator	[37]
	2005	SLC7A11 expression predicts chemosensitivity cells to drug treatment in cancer cell lines	[223]
GPX4			
	1982	GPX4 purified from pig liver and characterized as protective of liposomes and biomembranes from peroxidative degradation	[64]
	1987	GPX4 protects mammalian spermatozoa from lipid peroxidation-mediated lethality	[67,68]
	1990	GPX4, but not GPX1, reduces lipid hydroperoxides in membranes	[73,74]
	1991	GPX4 protects against lipid hydroperoxides-mediated lethality in murine lymphoid leukemia cells	[246]
	1991	Cloning of GPX4 reveals its distinct nature compared to other GPXs	[72]
	1996–2004	Overexpression of GPX4 *in vitro* and *in vivo* results in increased resistance to oxidative stress-induced death	[79–82,91,97]
	2003–2008	GPX4 knockout induces embryonic (E7.5) lethality in mice	[86–88]
	2003	Heterozygous GPX4 mice are more sensitive to oxidative stress-mediated death	[88,89]
PUFAs			
	2006	Inhibition of 12/15-lipoxygenase protects from death by vascular injury in the ischemic brain	[93,94]
	2008	Critical role for 12/15-LOX in GPX4-knockout-mediated distinct form of cell death	[87]

mammalian spermatozoa, which were known to be sensitive to the deleterious effects of oxygen free radicals, due to their rich polyunsaturated fatty acid content [67,68]. This finding was in line with the notion that selenium is essential for male fertility [69,70], and was further demonstrated by the identification of GPX4 as a major structural component of the mitochondrial capsule, which embeds sperm mitochondria and is thus required for structural sperm stability [71].

The cloning of GPX4 in 1991 revealed its similarity to the classical cytosolic glutathione peroxidase (GPX1), but also its unique properties in inhibiting peroxidation of lipids due to its hydrophobic nature and monomeric form [72]. By the beginning of 1990s, there were several lines of evidence supporting the notion that GPX4 plays a unique role in protecting cells against the damaging and lethal effects of lipid peroxidation [73–76].

Shortly after the isolation of human GPX4 from human liver in 1994 [77], several groups demonstrated that overexpression of this enzyme in human cells results in resistance to lipid peroxide cytotoxicity compared to parental cells [78–82], an effect that was consistently attributed to being protective of oxidative-stress-induced apoptotic cell death [83]. Transgenic and knockout mouse models of glutathione-dependent peroxidases stressed the importance of GPX4 in protecting against cell lethality, as GPX4 knockout was the only one (out of GPX1-, GPX2-, GPX3- [84,85] and GPX4-knockout mouse models) to induce embryonic lethality [86–88]. Heterozygous *GPX4* mouse models (*GPX4*$^{+/-}$) have contributed to the understanding of its protective role against a unique form of cell death, as these mice were shown to be more sensitive to death induced by γ-irradiation and *tert*-butyl hydroperoxide [88,89]. It was surprising that these heterozygous *GPX4* knockout mice live longer than wild-type mice due to delayed pathologies such as fatal lymphoma [90], that can now be explained as increased sensitivity of such nascent cancer cells to ferroptotic death (see below), implying that GPX4 may be oncogenic. In line with GPX4 overexpression in cell culture, overexpression of GPX4 in mouse models was also shown to be protective from oxidative-stress-induced death [91].

A breakthrough in the understanding of the unique properties of GPX4-downregulation-induced cell death was accomplished when Seiler et al. in 2008 demonstrated the role of 12/15-lipoxygenase (12/15-LOX), a polyunsaturated fatty acid metabolizing enzyme [92], in the execution of GPX4-knockout-mediated cell death [87]. This finding was supported by previous evidence of LOX involvement in deleterious pathologic situations [93–96], and provided a mechanistic explanation for several observations of what was then considered to be 'oxidative-stress-induced apoptosis', which was not dependent on activation of the Bcl-2 family proteins [97–99]. This led to the notion of a unique cell death mechanism linking membrane lipid peroxidation, arachidonic acid metabolism, glutathione peroxidase activity (or lipophilic antioxidants; vitamin E) and oxidative stress.

3. The emergence of the concept of ferroptosis

Throughout the years, there have been several puzzling reports of non-apoptotic caspase-independent cell death with necrotic-like morphology, that seemed to be an active form of cell death that could be regulated [100–103]. One example is ceramide-induced cell death that was shown to involve the accumulation of ROS and did not exhibit apoptotic characteristics, but could be rescued by radical scavengers in human glioma cells [104]. This observation was supported by earlier descriptions of ceramide-induced cell death [105,106]; here too, investigators recognized the distinct nature of this cell death, but tried unsuccessfully to fit it into the existing framework of cell death modes. Ceramide may be involved in the induction of ferroptosis, although this remains unclear [107]. The main discoveries towards characterization of ferroptotic cell death are summarized in Table 2.

In 2001–2003, the Stockwell Lab performed a screen for small molecules that could selectively kill human BJ fibroblasts that had been engineered to be tumorigenic, but not their otherwise isogenic parental precursors. This screen was designed to identify lethal compounds with selectivity for cells expressing oncogenic mutant HRAS, as well as the large and small T oncoproteins [108–110]. The most selectively lethal compound to emerge from the screen was a novel compound from a newly generated small molecule combinatorial library with no known activity. Stockwell and colleagues named this compound "erastin" because of its apparent ability to Eradicate RAS-and Small T transformed cells. The lab members became interested in determining the mechanism of action for this new compound erastin, as it might illuminate a way of selectively killing RAS-transformed cancer cells. The other compounds that emerged from this screen induced apoptosis; the default assumption was that erastin would as well. However, when Dolma and Stockwell performed typical apoptosis assays with erastin in these engineered tumor cells, they consistently found no evidence of caspase activation, no cleavage of caspase substrates, no Annexin V staining, no nuclear morphological changes, or any other hallmarks of apoptosis. Nonetheless, erastin's lethality could be potently suppressed by iron chelators and lipophilic antioxidants. Thus, the lab members began to consider the possibility that erastin induced a regulated, but non-apoptotic, form of cell death [108,111], which was a somewhat heretical notion at the time.

Yang and Stockwell screened additional small molecule libraries in the same assay and identified another compound, which was named RAS synthetic lethal 3 (RSL3), that induced a similar form of non-apoptotic, iron-dependent cell death [109]. This confirmed that erastin was not unique in its ability to activate this type of cell death, and that perhaps this form of cell death was a more generally important phenomenon. A series of experiments by Dixon and Stockwell led to the idea that erastin acted by inhibiting the cystine/glutamate antiporter, system X_c^-, which reduces cysteine-dependent synthesis of reduced glutathione (GSH) [25,112]. Reinforcing that conclusion was the finding that the unique cell death pathway induced by erastin was similar to cell death induced by sulfasalazine (SAS) [25], a known system X_c^- inhibitor [113]. This was the first mechanistic insight into a trigger for what became known as ferroptosis, showing that erastin inhibits cystine import, leading to deregulated cellular redox homeostasis. Distinct from apoptotic cell death, this cell death mechanism did not require caspase activation or the involvement of other apoptotic effectors, such as BAX or BAK, and was not accompanied by apoptotic morphological features or biochemical processes. Moreover, there was no inhibitory effect on erastin- and RSL3-induced cell death by either small molecule inhibitors of necroptosis (necrostatin-1) or autophagy (chloroquine or 3-methyladenine) [25,114,115].

Following these discoveries, the term ferroptosis was coined in 2012 [25], to describe this iron-dependent, non-apoptotic form cell death induced by erastin and RSL3. This discovery was accompanied by the development of the first small molecule ferroptosis inhibitor, termed ferrostatin-1, and the demonstration of glutamate-induced ferroptosis in organotypic rat brain slices, suggesting the potential function of ferroptosis in neurodegeneration.

Elucidation of the RSL3 mechanism of action provided the next major insight in the regulation of the emerging mechanism of ferroptosis. Chemoproteomic studies using (1 S,3 R)-RSL3 (the active diastereomer out of the four possible RSL3 stereoisomers) as an affinity reagent identified the crucial role for the glutathione-dependent selenoprotein glutathione peroxidase 4 (GPX4) in the regulation of ferroptosis [114].

Subsequently, numerous other studies began identifying a similar ferroptotic process underlying diverse phenomena. In multiple cell types, amino acid starvation was reported to induce cell death that is non-apoptotic and non-necroptotic, but only in the presence of serum [116,117]. It was shown that deprivation of cystine was sufficient to induce the same serum-dependent cell death pathway, and that the serum component crucial for cell killing was transferrin, an iron carrier. Deprivation of cystine was thus equivalent to system X_c^- inhibition. These findings led to the confirmation that the cell death mechanism

Table 2
Main discoveries towards characterization of ferroptotic cell death.

Year	Main finding	Ref
2003	Erastin was discovered through a synthetic lethal high-throughput screen to selectively kill engineered tumorigenic cells in a non-apoptotic manner	[108]
2007	Erastin treatment causes the appearance of oxidative species	[111]
	Erastin-induced cell death is inhibited by lipophilic antioxidants	[111]
2008	RSL3 was discovered through a synthetic lethal screening of small molecules to have increased lethality in the presence of oncogenic RAS, through a death mechanism similar to erastin	[109]
	Iron chelation and lipophilic antioxidants inhibit RSL-3 induced cell death	[109]
2011	Modulatory profiling identifies common death induction mechanisms for erastin and RSL3, which are distinct from death mechanisms of other death inducers	[115,247]
2012	Erastin was found to inhibit system x_c^-	[25,112]
	Ferrostatin-1 was identified as inhibitor of ferroptosis	[25]
	Iron accelerates erastin-induced ferroptosis	[25]
	Erastin-treated cells show smaller mitochondria with increased membrane density	[25]
	Mitochondrial DNA is not required for ferroptosis	[25]
2013	Sorafenib induces ferroptotic cell death	[159,160]
2014	GPX4 was identified as the target for RSL3	[114]
	GPX4 knockout triggers ferroptosis-induced acute renal failure in mice	[144]
	Arachidonic acid (AA) is the most frequently depleted PUFA in cells undergoing ferroptosis	[144,145]
	Liproxstatin-1 was identified as inhibitor of ferroptosis	[144]
2015	The iron carrier transferrin and L-glutamine are the two serum components required for cystine-depletion-mediated ferroptosis	[117]
	ACSL4 and LPCAT3, involved in the insertion of PUFAs into membrane phospholipids, contribute to ferroptosis induction	[146,147]
	imidazole ketone erastin (IKE), a potent inhibitor of system X_c^-, was designed	[164]
2016	Peroxidation of polyunsaturated fatty acids by lipoxygenases drives ferroptosis	[124]
	RSL3 inhibit GPX4 by covalently targeting the active site selenocysteine, leading to accumulation of PUFA hydroperoxides	[124]
	'Ferritinophagy' contributes to ferroptotic death trough increase in LIP	[128–130]
	Upregulation of the transsulfuration pathway (biosynthesize cysteine from methionine) can inhibit ferroptosis when induced by system X_c^- inactivation	[151]
	Nrf2 protects against ferroptosis in HCC	[155]
	FIN56 was identified as a ferroptosis inducer through depletion of GPX4 and CoQ_{10}	[172]
	Vitamin E protects from ferroptosis in GPX4 null mice	[170]
2017	Phosphatidylethanolamines (PEs) are the fatty acids oxidized in the ER through ferroptosis	[26]
2018	Mitochondria are not required for ferroptosis	[135]

induced by amino acid starvation in the presence of an exogenous iron source is ferroptosis.

It was later found that not all ROS function equally in ferroptosis, and that lipid peroxidation is the main driver for ferroptotic death [118], supported by the previous identification of lipophilic antioxidants as suppressors of ferroptotic death induced by erastin and other compounds [111].

4. Identification of positive regulators of ferroptosis

Iron has an essential role in life on Earth dating back billions of years. However, emergence of iron-dependent oxygen utilization and polyunsaturated fatty acid metabolism to drive processes in living cells created a deadly paradox: while these processes of energy production, lipid metabolism, and signaling are valuable for the existence of complex life forms, they are also associated with generation of harmful and ultimately lethal species. The oxidation of organic substrates by iron (II) with hydrogen peroxide (H_2O_2) is referred to as Fenton chemistry (or the Fenton reaction) and was first described by Fenton in 1894 [119] (reviewed in [120]). This reaction partially explains the dependency of ferroptosis on iron, as redox-active iron pools are able to directly catalyze propagation of lipid peroxidation to form damaging species that lead to death.

Early in the 1960s, iron was shown to contribute to lipid peroxidation-associated pathological changes in rats, that could be prevented by vitamin E [121]. As implied by the name 'ferroptosis', the existence of high levels of intracellular iron is a requirement for the execution of this type of cell death. An indication of this necessity is that ferroptotic death, whether induced by system X_c^- inhibition, direct GPX4 inhibition, cystine deprivation, or extracellular glutamate, can be suppressed by iron chelators, knockdown of the iron transporter transferrin or its receptor, or the lack of iron in serum [25,109,114,122,123], as well as inhibition of iron availability to lipoxygenases [124], which drive ferroptosis through peroxidation of PUFA-PLs (see below). Moreover, addition of iron to the growth medium [25], as well as of iron-bound transferrin [117], were shown to accelerate erastin-induced ferroptosis, and administration of a bioavailable iron form enhances ferroptotic death in mouse models defective in system X_c^- [125].

Mechanistically, intracellular redox-active iron promotes ferroptosis by catalyzing the formation of soluble lipid radicals that can initiate or propagate oxidative PUFA fragmentation, enzymatically and non-enzymatically [126]. Intracellular iron homeostasis is strictly regulated by the iron-binding and mRNA-regulatory proteins named iron-regulatory proteins 1 and 2 (IRP1 and IRP2), that can sense the cellular concentration of free iron and respond by altering the expression of proteins governing iron export, import, storage and release [127]. Ferroptotic death is often linked to the disruption of delicate iron homeostasis, which causes an undesired increase of free cellular iron concentration (Fe^{2+}; also known as the 'labile iron portion' or 'LIP'). This fine-tuning of iron levels is mostly attributed to an impaired activity of IRP2, coupled with increased expression of the iron carrier transferrin, and transferrin receptor [117].

One of the main mechanisms enabling recycling of intracellular redox-active iron is lysosomal degradation of ferritin in the process of autophagy, often referred to as 'ferritinophagy' [128]. Autophagy was suggested to contribute to ferroptotic cell death by promoting the

degradation of iron storage proteins such as ferritin, which results in release of free iron [129,130]. Reinforcing this hypothesis, recent evidence indicates that ferroptotic induction requires the presence of active lysosomes [123]. The importance of autophagy (specifically 'ferritinophagy') for inducing ferroptosis is demonstrated by the complete blockage of ferroptotic death by inhibition of NCOA4, a specific autophagy cargo receptor that mediated the delivery of ferritin to lysosomes [129]. Confirming the central role of lysosomal iron in ferroptosis, is the ability of the membrane impermeable iron chelator deferoxamine (DFO) to inhibit erastin-induced and RSL3-induced death [25,109]. DFO is taken up by the cell through endocytosis and accumulates in lysosomes [131], suggesting that it prevents ferroptosis by chelation of the 'redox active' lysosomal iron pool or by inhibiting specific iron-dependent lipid-ROS promoting enzyme.

One of the central organelles where significant amounts of ROS can be generated is mitochondria, where ROS are formed as a result of normal metabolism and energy production through the electron transport chain. There is some evidence of possible mitochondrial involvement in processes supporting ferroptotic death, starting from the first description of a distinctive morphological feature of erastin-treated cells that showed smaller mitochondria with increased membrane density [25]. Moreover, several mitochondrial genes were found to be associated with ferroptotic cell death, and there is a suggestion of peroxidation of cardiolipin, a mitochondria-specific phospholipid, linking mitochondrial lipid peroxidation to ferroptosis [132–134]. Nevertheless, there are other cellular sources for ROS, and cells with mitochondrial DNA depleted can still undergo potent ferroptosis [25]. Recently, it was found that removing mitochondria from cells does not prevent ferroptosis, suggesting that mitochondria are not needed for ferroptosis [135].

Both glutamate and glutamine play important roles in ferroptosis induction. High concentrations of extracellular glutamate can block cystine uptake through system X_c^- and induce oxidative-stress-mediated cell death which can be rescued by α-tocopherol supplementation [40,136]. This cell death mechanism was described in cells of the central nervous system and shown to be distinct from apoptosis, thus it was termed 'oxidative glutamate toxicity' or 'oxytosis' [137]. Glutamate-induced cell death shares several characteristics with ferroptosis, mainly in the mechanism of initiation by cystine deprivation and glutathione depletion, leading to accumulation of lipid-based ROS [25]. However, the terminal phases of death by glutamate toxicity may involve apoptotic, and not ferroptotic features, in some neuronal cells, and are dependent on calcium rather than iron. This death mechanism may overlap with ferroptosis in other cells [138]. A more general analysis of the degree of overlap between these two death mechanisms requires further investigation.

The role of glutamine in ferroptosis is complex. Although glutamine can be converted to glutamate by glutaminases (GLS1 and GLS2), high concentrations of extracellular glutamine alone cannot induce ferroptosis. Instead, glutamine was shown to drive ferroptosis through glutaminolysis, in combination with cystine deprivation in MEFs [116,117]. Moreover, inhibition of glutamine uptake and of glutaminolysis was recently suggested to contribute to ferroptosis resistance in cancer cells [139]. 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 necessity for glutaminolysis in ferroptosis is further reinforced 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 was shown to be required for ferroptosis [117]. These glutaminases differ in their intracellular localization: GLS1 is a cytosolic protein, whereas GLS2 localizes in the mitochondria [140], suggesting that, at least in this cellular context, the induction of ferroptosis seems to be dependent on mitochondrial GSL2. Bearing in mind that mitochondria were shown to not be essential for ferroptosis induction in other cellular models [25,135], determination of the centrality of mitochondria-mediated ROS production in ferroptosis requires further study. Of note, glutaminolysis is considered to be a mitochondrial process that promotes (mainly cancer) cell survival by maintaining ATP production and inhibiting ROS production [141]. Thus, data suggesting an involvement of glutaminolysis as a driver of ferroptosis through the activity of mitochondrial GLS2 [117], was surprising and counterintuitive. However, glutaminolysis was also shown to stimulate autophagy, which can drive ferroptotic death as discussed above.

A common feature of ferroptosis is the iron-dependent accumulation of lipid-ROS and the subsequent depletion of polyunsaturated fatty acid phospholipids (PUFA-PLs) [118]. The PUFA chains of membrane lipids are more susceptible to both enzymatic and non-enzymatic oxidation, which results in PUFA fragmentation into a variety of products [126]. Indeed, several cell types that contain relatively high levels of PUFAs, such as cells of the retina and spermatozoa, were known for a few decades to be more sensitive to lethal oxidative stress that can be reduced by vitamin E or GPX4 [68,142]. This is in line with earlier observations that inhibition of arachidonate 12-lipoxygenase (Alox12), an iron-containing lipid dioxygenase, inhibited oxidative glutamate toxicity and cell death in neurons, while treatment of cells with arachidonic acid (AA), a substrate of Alox12, further potentiated such death [143]. Arachidonic acid (AA) is now known to be the most frequently depleted PUFA in cells undergoing ferroptosis [144,145]. One study showed that deletion of crucial enzymes involved in the insertion of AA into membrane phospholipids can prevent ferroptosis induction [146]. Acyl-CoA synthetase long-chain family member 4 (ACSL4), thus drives ferroptosis by contributing to the accumulation of oxidizable cellular membrane phospholipids [26,147]. The currently suggested mechanism for the involvement of lipid metabolic pathways in ferroptosis induction is the following: ACSL4, which prefers AA as its main substrate, promotes ferroptosis by producing oxidized phosphatidylethanolamines (PE) in endoplasmic-reticulum-associated oxygenation centers. ACSL4 catalyzes the ligation of an arachidonyl (AA) or adrenoyl (AdA) to produce AA or AdA acyl Co-A derivatives. These derivatives are then esterified into PE to form AA-PE and AdA-PE by lysophosphatidylcholine acyltransferase 3 (LPCAT3), and subsequently oxidized by 15-lipoxygenase (15-LOX) to generate lipid hydroperoxides, which execute ferroptosis [26,124,147]. Although 15-LOX is thought to play a central role in catalyzing lipid peroxidation that leads to ferroptotic death, this role may be attributed to multiple LOXs, since deletion in 15-LOX fails to rescue the renal phenotype of GPX4 null mice [144]. The idea that lipid metabolism and membrane lipid composition affect susceptibility of cells to ferroptosis is further supported by molecular dynamics modeling of membrane lipid peroxidation [148].

Although the essential role for lipid peroxides in induction of ferroptotic death has been established, there is still no definitive evidence that this class of ROS is the most downstream factors that execute ferroptosis. Therefore, the question of what the ultimate molecular executioner of ferroptotic cell death is (*e.g.,* as caspases for apoptosis), remains to be resolved.

5. Identification of negative regulators of ferroptosis

The first description of ferroptosis induction, by Dixon et al. in 2012, was in the context of cystine deprivation by inhibiting cystine (Cys_2) import via the system X_c^- antiporter with the small molecule erastin [25]. System X_c^- is a plasma membrane cystine/glutamate antiporter composed of a twelve-pass transmembrane transporter protein, SLC7A11 (xCT), linked to the transmembrane regulatory protein, SLC3A2, by a disulfide bridge [149]. The lethal effect of erastin (and also of SAS, see above) can be reversed by β-mercaptoethanol (β-ME) [25,112], which bypasses the need for system X_c^- by forming mixed disulfides with Cys_2 that can be imported into the cell by a different transporter [150]. Additionally, some cells can use the transsulfuration

pathway to biosynthesize cysteine from methionine when system X_c^- is inactivated. This pathway was recently shown to also be upregulated upon knockdown of cystenyl-tRNA synthetase (*CARS*). Cells that use the transsulfuration biosynthetic pathway were thus found to be resistant to ferroptosis induced by system X_c^- inhibitors, but could still undergo ferroptosis through GPX4 inhibition [151]. Consistent with the need of cellular cystine to protect from ferroptotic death, according to a recent series of experiments, the redox-sensitive transcription factor Nrf2 [152,153] can protect cells from ferroptotic death by upregulating system X_c^- [132,154], and was found to be commonly overactivated in various cancers [155,156]. In contrast, induction of ferroptosis through inhibition of system X_c^- was suggested to be a mechanism of tumor suppression by p53 [157].

The precise mechanism by which erastin inhibits SLC7A11-mediated cystine import it still unknown. The initially proposed mechanism, by which erastin binds a related transport protein, SLC7A5, and inhibits SLC7A11 *in trans* [25], was revised soon after, and it was suggested that erastin inhibits SLC7A11 perhaps directly [112]. An additional inhibitor of system X_c^-, the FDA-approved multi-kinase inhibitor sorafenib (Nexavar), was shown to induce a GSH-depletion-mediated ferroptosis in cancer cell lines [112,158–160] and enhance ROS accumulation in cancer patients [161]. Although the clinical benefit of sorafenib was in part caspase-independent [160,161], this drug was shown to trigger classic apoptosis in some cells [162]. The specific contribution of ferroptotic cell death to the therapeutic effects of sorafenib in cancer patients is unknown [163]. A more potent inhibitor for system X_c^-, imidazole ketone erastin (IKE), was designed by introducing a stable ketone to erastin [164]. In addition to the improved potency of IKE compared to erastin, IKE was shown to have improved metabolic stability placing this compound as a good candidate for *in vivo* studies of ferroptosis induction.

Downstream of system X_c^- is a central cellular guard against lipid ROS-induced ferroptotic death, GPX4. GPX4 acts as a guardian of cell membranes and converts potentially toxic lipid hydroperoxides (L-OOH) to non-toxic lipid alcohols (L-OH) [64]. Small molecule inhibition of GPX4 was first observed with the small molecule (1 S, 3 R)-RSL3, and resulted in uncontrolled polyunsaturated fatty acid phospholipid (PUFA-PL) oxidation and fatty acid radical generation, leading to ferroptotic cell death [114,124]. Genetic or pharmacological inhibition of GPX4 leads to ferroptotic death, and hence is independent of cystine supply [114,144]; deletion of *GPX4* in mice is embryonically lethal [87]. Concomitantly, overexpression of GPX4 blocks RSL3-induced ferroptosis [114], and the lethality of *GPX4*-depleted mice could be rescued with ferroptosis inhibitors (*e.g.,* ferrostatin-1) [87,144]; further confirming that GPX4 activity is essential to prevent ferroptosis. The fact that elimination of this important cellular guard against lipid ROS is sufficient to induce ferroptotic death suggests that cells are continually exposed to the threat of radical-mediated lipid destruction. In addition to RSL3, other less potent small molecule inhibitors of GPX4 were identified (*e.g.,* altretamine [165]), yet the mechanism of action of these inhibitors is not clear [138].

Although both cys-mediated GSH synthesis and GPX4 activity were shown to be essential for protecting cells against ferroptotic death, alternative antioxidant pathways maintain cell survival in the absence of GSH-GPX4. Indeed, the majority of commonly used ferroptosis inhibitors, including the original ferrostatin-1 and the more recently discovered liproxstatin-1 [144], are believed to function by trapping lipid radicals [122,166]. Radical-trapping antioxidants are molecules that react with chain-carrying radicals and break the oxidation chain reaction [167]. Vitamin E, a lipophilic antioxidant, was discovered almost a century ago [168] and its beneficial antioxidant effect in human health was recognized since. The most biologically active form of vitamin E, α-tocopherol [169], was shown to inhibit ferroptotic death both *in vitro* and *in vivo* [170–173], and vitamin E deficiency was linked to ferroptosis-mediated premature onset of neurodegeneration [174,175]. In some cells, both *in vitro* and *in vivo*, high levels of SLC7A11-mediated cystine import, in conjugation with the GSH-independent thioredoxin (Txn) system, maintains endogenous α-tocopherol (vitamin E) in a reduced state and prevent lethal lipid-ROS accumulation [176–179]. Additionally, it was recently suggested that in addition to the direct antioxidant activity of vitamin E in limiting lipid-ROS, it can also act as inhibitor of lipogeneses by competing for their substrate binding site, further inhibiting ferroptosis [26].

The recent identification of new mechanisms for triggering ferroptosis, by compounds termed FIN56 and $FINO_2$, provided new insights into regulation of ferroptosis. $FINO_2$ acts through a distinct mechanism: it promotes lipid peroxidation by oxidizing iron and indirectly inactivating GPX4 [180]. FIN56 was found to trigger ferroptosis by inducing a combined effect of mediating the depletion of both GPX4 protein and the mevalonate-derived antioxidant coenzyme Q_{10} (CoQ_{10}) [172]. CoQ_{10} is an endogenously-produced lipid-soluble antioxidant, which was shown to prevent the harmful oxidation of proteins, lipids and DNA [181,182]. Importantly, in addition to its direct antioxidant activity, CoQ_{10} also contributes to regeneration of other antioxidants such as ascorbate and α-tocopherol [183]. The role for cellular CoQ_{10} pool in regulating ferroptotic death remains to be explored.

6. Possible biological functions of ferroptosis

Although a low and controlled level of lipid ROS is perhaps acceptable for normal cellular and organismal function, the aberrant accumulation of lipid ROS is associated with a number of chronic degenerative conditions and acute organ injuries. Such conditions are caused by the imbalance between radical-generating and radical-scavenging cellular systems and pathways described above, leading to oxidative stress that results in cell death. Thus, mechanisms of controlling ferroptotic cell death are being investigated in recent years as therapeutic means for multiple pathologies.

6.1. Ferroptosis in neurodegeneration

Although there has been evidence for the involvement of lipid peroxidation and oxidative stress in numerous neurological conditions for a few decades before ferroptosis was described [184–186], brain cell death in neurological and neuropsychiatric conditions was attributed to apoptosis, and acute central nervous system (CNS) cell death events such as traumatic brain injury and infection, were considered to be due to necrosis [187]. Following the discovery of ferroptosis, this paradigm is under challenge, with ferroptosis now being suggested as the main driver of neurological cell death in diseases such as Parkinson's disease (PD) and Alzheimer disease (AD) [188–191]. It is now being appreciated that dysregulation in iron homeostasis might be a central driver for such neurodegenerative diseases [192,193]. Even before the discovery of ferroptosis, there was already evidence of the central role of oxidative stress in neuropsychiatric conditions such as bipolar disorder [194], schizophrenia [195] and depression [196], disorders for which ferroptosis is now considered to be a possible driver [197]. Ferroptosis was also suggested to contribute to the toxic effect of mutant *Huntingtin* (HTT) that cause Huntington's disease [198], which was shown to correlate with dysregulation of iron, glutamate and glutathione [199,200]. Further supporting the notion that ferroptosis plays a detrimental role for cell death in Huntington's disease is the ability of the ferroptosis inhibitor ferrostatin-1 to abrogate cell death induced by mutant HTT [145].

Accumulating evidence of the correlation between elevated iron levels in the brains of Alzheimer's disease patients and cognitive decline [201–204], along with evidence of increased lipid peroxidation [205–207], have raised the hypothesis that ferroptosis is the main form of cell death in this pathology (reviewed in [191]). The development of novel ferroptosis inhibitors, with improved ADME properties (relatively to the original ferrostatin-1) [208], are currently considered promising treatments for neurodegenerative and neuropsychiatric diseases.

6.2. Ferroptosis in cancer

Cancers often exhibit high proliferation and imbalanced redox consumption and signaling, since various oncogenic pathways (such as proliferation and evading cell death) converge on redox-dependent signaling [209]. Additionally, recent epidemiological and animal studies have shown that the iron-rich microenvironment that often characterizes malignancies supports rapid proliferation and contributes to carcinogenesis [210,211]. This creates an 'addiction' of cancer cells to high iron levels and places tumors under persistent oxidative stress [212], which often drives addiction to genes and pathways that protect from ferroptotic death providing growth advantage and contributing to cancer chemoresistance. For example, various cancer types harbor somatic mutations in *Nrf2* or *Keap1* which lead to enhanced transcription of antioxidant enzymes [213], and glutathione and thioredoxin antioxidant systems are commonly activated in cancers [214,215]. Thus, a fine control of body iron levels was recently suggested as a strategy for reduction of cancer-risk in healthy populations [210,211].

A link between ferroptosis sensitivity and overexpressed oncogenic HRAS activity served as the basis for the original discovery of ferroptosis. As stated above, ferroptosis was discovered through the study of erastin and RSL3, which were found in a small molecule screen to be selectively more lethal to cancer cells harboring oncogenic RAS mutants. In addition, silencing of oncogenic mutant KRAS or BRAF was found to reduce sensitivity to erastin-induced death in two different cancer cell models [111], suggesting that the RAS-RAF-MEK pathway plays a role in determining ferroptosis sensitivity, at least in some cancer cell lines. A suggested mechanistic explanation for this relationship is that constitutive activity of the RAS signaling pathway mediates an increase in cellular iron content through elevated expression of transferrin receptor and down regulation of the iron storage protein ferritin [109]. Of note, this was only demonstrated in a model of engineered tumor cell line, while following studies showed no correlation between ferroptosis sensitivity and oncogenic RAS genes across many cancer types [114], and even increased resistance to erastin and RSL3 in some contexts [216].

Recent discoveries of ferroptosis-inducing agents and further identification of regulation mechanisms and genes involved in ferroptosis induction [217] serve as a foundation for developing strategies for cancer therapy through induction of ferroptosis [218,219]. The iron-enriched (ferroptosis-promoting) tumor environment [220], along with accumulating evidence of overexpression of ferroptosis-inhibiting mechanisms in cancer cells, is suggestive for increased sensitivity of cancer cells to ferroptosis induction [114], which is counteracted by cancer cell addiction to tumor suppressor pathways. For example, the pronounced addiction of triple-negative breast carcinoma to glutamine relates (at least in part) to its ability to drive cystine uptake via system x_c^-, implying that system x_c^- may constitute a therapeutic target in this setting [221,222]. In addition, B cell-derived lymphomas and a subset of triple-negative breast cancer cell lines were shown to acquire a strong dependency on GPX4 and system X_c^- [223,224] (reviewed in [217]).

An additional example for the role of ferroptosis in tumorigenesis is the ability of the tumor suppressor p53 to induce ferroptosis. The p53 protein is a crucial tumor suppressor that mediates cell cycle arrest, cell death and/or senescence in response to various stress conditions [225]. Recent studies link ferroptotic cell death to a non-conventional p53-mediated activity of tumor suppression [226,227]. Years before the acknowledgment that cancer cells may be more prone to ROS-induced death, and that tumor suppressor mechanisms may involve evading ferroptosis, it was suggested that p53 can regulate ROS accumulation [228,229]. The mechanism for that regulation was recently suggested by Jiang at al., which showed that p53 represses the expression of the system X_c^- component, SLC7A11, causing a reduction in cystine uptake, which results in increased sensitivity to ferroptosis [230]. The importance of this tumor suppressive role of p53 is further reinforced by the ability of SLC7A11 overexpression, observed in several forms of human cancer [223,224,231], to protect from ROS-induced death. However, p53 was also shown to prevent ROS accumulation by upregulating antioxidant genes, a function that was attributed to prevention of ROS-mediated DNA damage and tumor suppression, but also contributes to ferroptosis evasion in malignancies.

6.3. Ferroptosis in development

The occurrence of several types of morphogenetic death processes during development was demonstrated decades ago [232]. However, apoptotic death is still considered to be the main form of cell death that contributes to proper tissue and organ formation. The notion that ROS act as major intracellular signal mediators during development emerged in the 1990s [233,234]. Since then, the important functions of ROS in embryonic development, as well as the crucial role for ROS scavengers (specifically lipid ROS) has been extensively studied [235]. Observations that embryonic tissues undergoing cell death show increased levels of ROS, and that such death can be controlled by GPX4 expression and inhibited by lipophilic antioxidants (i.e., in the developing limb [236,237]), supported the involvement of lipid ROS-mediated cell death in development. To date, the role of ferroptotic death in embryonic development is considered to be more relevant for maintaining tissue integrity and homeostasis, rather than organ formation *per se*.

Recent studies also point to homeostatic and ROS-induced cell death in plants, that encompass ferroptotic characteristics [238,239]. This further demonstrates the relevance of ferroptosis to maintain organismal homeostasis and supports the evolutionary conservation of this cell death mechanism. Although it is becoming clear that ferroptotic death is an essential process for the multicellular organism, the specific roles for ferroptosis in development are currently not understood.

In summary, the concept of ferroptosis encapsulates the idea that iron-dependent lethal lipid peroxidation is a distinct form of cell death, with unique triggers, regulators, and effectors. This concept provides a framework for elucidating additional molecular controls of this process, its involvement in disease, therapeutic strategies, and its physiological and evolutionary functions.

7. Open questions

7.1. What is the exact function of iron in ferroptosis?

Iron is believed to have multiple functions in ferroptosis, and a redox-independent role for iron has not been completely ruled out. The most common 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 [122]. Nevertheless, the requirement for iron for ferroptosis may reflect the role of various metabolic enzymes in ROS generation, for which iron functions as a cofactor (e.g., the LOX family of enzymes [240] or prolyl 4-hydroxylase isoform 1, PHD1 [241]).

7.2. What is the molecular executor of ferroptosis?

The molecular events that occur downstream of lipid oxidation and the specific 'point of no return' in ferroptotic cell death are unclear. It may be that oxidative fragmentation of PUFAs and membrane lipid damage are sufficient death inducers, through permeabilization of the plasma membrane and damage to intracellular organelle membranes. Alternatively (or cooperatively), the fragmented products of oxidized PUFAs may promote death by reacting and inactivating essential cellular proteins. For example, the toxic reactive lipid intermediate 4-hydroxynonenal (4-HNE), a byproduct of oxidative PUFA fragmentation, can be detoxified by three aldo-keto reductase family 1, member C (AKR1C) [242]. The expression of AKR1C is controlled by the antioxidant master regulatory transcription factor NRF2 [243] and is

overexpressed in cell lines selected for resistance to ferroptosis [112], suggesting that the accumulation of 4-HNE may be a key ferroptotic driver. Nevertheless, we cannot exclude the possibility of a death-inducing protein or protein complex activated downstream of lipid-ROS accumulation, thus further study is required to determine the terminal executor(s) for ferroptotic cell death.

7.3. Are there molecular markers to identify cells undergoing ferroptosis?

In the study of ferroptosis, there is a great need to identify molecular markers that would classify cells undergoing this process prior to death, such as caspase-activation for apoptosis. To date, the experimental confirmation of ferroptotic process relies mainly on observation of increased cellular ROS and the ability of ferroptosis inhibitors (e.g., ferrostatin-1) and iron chelators (e.g., DFO) to block cell death. The mRNA expression of two genes, prostaglandin E synthase 2 (*PTGS2*) and ChaC glutathione-specific gamma-glutamylcyclotransferase 1 (*CHAC1*), were found to be significantly elevated in cells undergoing ferroptosis [112,114]. Yet, these markers are not suitable for use in live cells or intact tissues. Additionally, an increase in the expression levels of heme oxygenase-1 (HO-1) was observed upon erastin-mediated ferroptosis induction [244], but the generality of this marker for the occurrence of ferroptotic death in various cells and initiation pathways requires further validation. Therefore, researchers are constantly in search for additional ferroptosis markers that could be used for future in vivo studies.

7.4. Under what contexts life benefit from ferroptotic cell death?

Since iron-dependent oxidative metabolism became an essential part of life billions of years ago, one can wonder, is ferroptosis the most ancient form of programmed cell death? If so, did any evolutionary force drive organisms to take advantage such ROS/iron-driven cell death and 'program' it for their own benefit?

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