REVIEW ARTICLE

Cellular and Molecular Biology

Ferroptosis in colorectal cancer: a future target?

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Colorectal cancer (CRC) is the third leading cause of cancer deaths worldwide and is characterised by frequently mutated genes, such as *APC*, *TP53*, *KRAS* and *BRAF*. The current treatment options of chemotherapy, radiation therapy and surgery are met with challenges such as cancer recurrence, drug resistance, and overt toxicity. CRC therapies exert their efficacy against cancer cells by activating biological pathways that contribute to various forms of regulated cell death (RCD). In 2012, ferroptosis was discovered as an iron-dependent and lipid peroxide-driven form of RCD. Recent studies suggest that therapies which target ferroptosis are promising treatment strategies for CRC. However, a greater understanding of the mechanisms of ferroptosis initiation, propagation, and resistance in CRC is needed. This review provides an overview of recent research in ferroptosis and its potential role as a therapeutic target in CRC. We also propose future research directions that could help to enhance our understanding of ferroptosis in CRC.

INTRODUCTION

Worldwide, colorectal cancer (CRC) is a leading cause of mortality and morbidity and the third most common cause of death from cancer [1]. For 2022, the estimated number of newly diagnosed CRC cases was 151,030 [2]. The aetiology of CRC is complex, however, its development is likely due to a combination of environmental influences (such as, dietary exposures, smoking and alcohol use) and the presence and interplay of pathogenic microbiota, genetic susceptibility, and immune response mechanisms [3–6]. Current treatments for CRC include surgery, radiation, and chemotherapies. Present chemotherapies used to treat CRC include (1) bevacizumab, an anti-vascular endothelial growth factor-A (anti-VEGF-A) antibody, (2) aflibercept, a VEGF-A, VEGF-B, and placental growth factor inhibitor, (3) regorafenib, a multi-kinase inhibitor and (4) cetuximab and panitumumab, anti-epidermal growth factor receptor (anti-EGFR) antibodies [7]. However, not all patients respond to these therapies. For example, patients with tumours that present on the right side of the colon have little benefit from panitumumab therapy [8], and cetuximab and panitumumab do not work as well for tumours with RAS mutations [9, 10].

Ferroptosis is a nonapoptotic and iron-dependent form of cell death that was discovered in 2012 [11]. Since its discovery, three hallmarks of ferroptosis have been established: (1) oxidation of polyunsaturated fatty acid (PUFA)-containing phospholipids, (2) iron dependency and (3) the loss of lipid peroxide repair mechanisms [12]. In addition to its potential roles in CRC, ferroptosis has been linked to the development of other cancers: breast, cervical, gastric, hepatocellular, lung, ovarian, prostate and renal carcinomas, as well as lymphoma and melanoma [13]. It has also been linked to mechanisms of lung fibrosis, stroke, traumatic brain injury, Alzheimer's disease and Huntington's disease [14]. Numerous studies have shown that modulating the ferroptotic cell death pathway can enhance the antitumor effects of drugs for CRC treatment. Similarly, in vitro and in in vivo experiments have revealed that the use of pharmacologics that trigger ferroptosis-induced cancer cell death (Erastin and Ras-selected lethal 3 (RSL3)) have promise in CRC [15, 16]. Yet there is still an urgent need to understand the mechanisms of how ferroptosis is regulated in CRC, and how these pathways and pharmacologics can be used for potential clinical translation.

This review aims to discuss the mechanisms of ferroptosis and its resistance pathways in cancer. We summarise what is currently known about ferroptosis in CRC tumorigenesis and metabolism and the use of ferroptosis inducers as treatments for CRC. We also offer concluding thoughts about the therapeutical potential of targeting ferroptotic pathways.

MECHANISMS OF FERROPTOSIS INDUCTION

Ferroptosis is distinct from other types of RCD as its mechanism is dependent on active ferrous ions and the accumulation of phospholipid hydroperoxides (PLOOHs) [17]. It is also dependent on important proteins that play various roles in normal cellular homeostasis, helping to regulate lipid recycling, iron metabolism, and to maintain redox status [18]. Table 1 illustrates the main molecular contributors to ferroptosis [19–38]. Mechanisms of ferroptosis which are tightly associated with CRC will be discussed

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Table 1. Main molecular regulators of ferroptosis.

Gene	Name	Function	Reference
Cystine uptake			
SLC7A11	Solute carrier family 7 member 11 cystine/glutamate antiporter xCT	Cystine uptake in exchange for glutamate	[19, 148]
SLC3A2	Solute carrier family 3 member 2	Maintains SLC7A11 stability	[149, 150]
ATF3	Activating transcription factor 3	Suppresses xCT by binding to the SLC7A11 promoter	[151]
OTUB1	Ovarian tumour family member	Stabilises SLC7A11 expression	[152, 153]
Redox homoeostasis regulators			
GPX4	Glutathione peroxidase 4	Prevents lipid hydroperoxide formation	[154–156]
NRF2	Nuclear factor erythroid 2-related factor 2	Regulates antioxidant response by increasing target gene transcription	[29]
Lipid metabolism			
ACSL4	Acyl-CoA synthetase long-chain family member 4	Converts AA/AdA into AA CoA/AdA CoA	[26, 31, 157]
LPCAT3	Lysophosphatidylcholine acyltransferase 3	Esterifies AA CoA/ AdA CoA into PEs	[31]
LOXs	Lipoxygenases	Generate lipid ROS by oxidising AA-PE and AdA-PE	[59, 158]
FSP1	Ferroptosis suppressor protein	Reduces CoQ10 to ubiquinol	[159, 160]
SCD1	Stearoyl-CoA desaturase	Involved in fatty acid biosynthesis	[161]
POR	Cytochrome P450 oxidoreductase	Promotes PUFA peroxidation	[162]
SQLE	Squalene epoxidase	Causes squalene accumulation	[74]
SCF2	Sterol carrier protein 2	Mediates trafficking of lipid ROS to mitochondria	[28]
GCH1	Cyclohydrolase-1	Increases antioxidant BH4 and CoQ10 abundance	[163]
Iron metabolism			
TFR1	Transferrin receptor	Imports iron into cells	[39, 115, 164]
HSPB1	Heat-shock 27-kDa protein 1	Reduces cellular iron uptake	[165]
IREB2	Iron response element binding protein 2	Limits iron uptake	[166]
NFS1	Nitrogen fixation 1	Supplies sulfur for iron–sulfur cluster synthesis	[167]
STEAP3	Six-transmembrane epithelial antigen of prostate 3	Converts Fe^{3+} to Fe^{2+}	[168]
DMT1	Divalent metal transporter 1	Mediates the release of Fe^{2+} into a labile iron pool	[169, 170]
FTH1	Ferritin heavy chain 1	Stores intracellular iron	[167, 171]
Energy metabolism			
SLC1A5	Solute carrier family 1 member 5	Controls glutamine uptake	[115]
GLS2	Mitochondrial glutaminase	Converts glutamine to glutamate	[172]
GOT1	Glutamic-oxaloacetic transaminase 1	Blocks the synthesis of a-ketoglutarate	[173]

below: iron metabolism, lipid peroxidation, redox status, lipid reprogramming and amino acid metabolism.

Iron metabolism

The connection between iron metabolism and ferroptosis was discovered through the observation that deferoxamine (DFO), an iron chelator, inhibits Erastin- and RSL3-induced cell death [39]. Iron levels are carefully regulated through coordinated processes such as iron storage, import, export, and recycling. Iron increases lipid peroxidation through two mechanisms. First, it can directly oxidise lipids via the Fenton reaction, in which the reaction between ferrous (Fe^{2+}) or ferric (Fe^{3+}) iron and hydrogen peroxide (H_2O_2) are involved ($Fe^{2+} + H_2O_2 = Fe^{3+} + OH^- + HO\cdot$, $Fe^{3+} + H_2O_2 = Fe^{2+} + HOO\cdot + H^+$) [11]. Iron also contributes to lipid peroxidation indirectly by serving as a cofactor for enzymes such as the lipoxygenases (LOXs) that directly oxidise lipids [40, 41]. Therefore, increased levels of labile Fe^{2+} enhance the sensitivity of cells to ferroptosis, whereas Fe^{3+} is incorporated into proteins or stored by ferritin, reducing ferroptosis sensitivity. Ferritin is composed of 24 polypeptides that are either ferritin light chain (FTL) or ferritin heavy chain (FTH1) [42]. FTH1 is the main regulator of ferritin activity and controls the oxidation of Fe^{2+} to Fe^{3+}. In RAS-mutant cells, it has been shown that decreased ferritin expression contributes to ferroptosis sensitivity by increasing iron uptake and decreasing iron storage [39]. Ferritinophagy, mediated by

nuclear receptor coactivator 4 (NCOA4), is the process by which ferritin is degraded and Fe^{2+} is released into the labile iron pool. Increased ferritinophagy has been shown to promote ferroptosis.

Transcription factors also regulate ferroptosis by controlling the expression of genes involved in iron-related metabolism. Among them is nuclear factor erythroid 2-like 2 (NFE2L2/NRF2), a key regulator of the cellular antioxidant response. NRF2 controls several aspects of antioxidant, iron and cell metabolic status, including regulation of the cystine-glutamate antiporter (xCT: SLC7A11) and glutathione peroxidase 4 (GPX4), which are two critical targets for the induction of ferroptosis.

It is also likely that iron regulates ferroptosis through its roles in hypoxia and DNA methylation. Hypoxia modulates ferroptosis via hypoxia-inducible factors (HIFs) and their interactions with NRF2 [43]. HIF prolyl hydroxylases are sensitive to iron concentrations in addition to hypoxia [44]. Increased NRF2 activity under hypoxia facilitates haem oxygenase-1 (HO-1) expression, protecting cells from ferroptosis [45, 46]. Expression of proteins such as Stearoyl-CoA desaturase 1 (SCD1) also increases under hypoxia. SCD1 has ferroxidase activity, which could potentially attenuate ferroptosis by limiting intracellular Fe^{2+}. Lastly, NCOA4 expression decreases under hypoxia, inhibiting ferritinophagy and protecting cells from ferroptosis [47, 48].
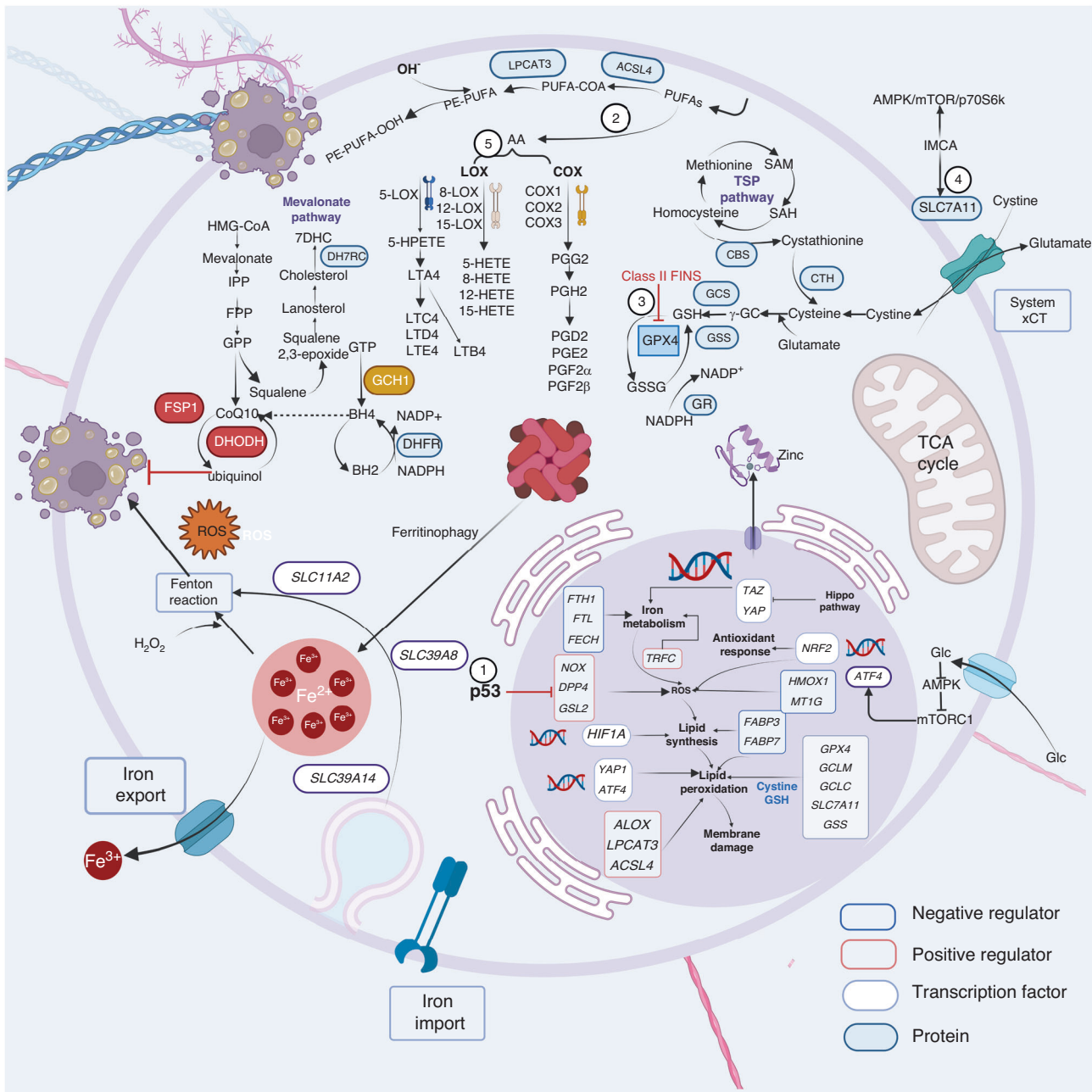
Fig. 1 The regulation and targeting of ferroptosis in CRC. The following nodes are proposed as potential therapeutic targets for CRC: ① p53: p53 negatively regulates ferroptosis by inhibiting DPP4 in CRC cells. ② ACSL4: ACSL4 plays a crucial role in the induction of ferroptosis in KRAS mutant CRC. ③ GPX4: Several molecules have been reported to induce ferroptosis by targeting GPX4 in CRC. ④ SLC7A11: As a subunit of system xCT, inhibited can induce ferroptosis. ⑤ AA: AA metabolism might be a potential target in CRC. LOXs also oxidise AA at different carbon sites and regulate cellular redox homoeostasis. Evidence showed that LOXs are important regulatory mechanisms of ferroptosis and its inhibitors such as the vitamin E family (tocopherols and tocotrienols) are effective in preventing ferroptotic death. ACSL4 acyl coenzyme A synthetase long-chain family member 4, AA arachidonic acid, BH2 dihydrobiopterin, BH4 tetrahydrobiopterin, CoA coenzyme A, DHFR dihydrofolate reductase, DHCR7 7-dehydrocholesterol reductase, Fe iron, FSP1 ferroptosis suppressor protein 1, FPP farnesyl pyrophosphate, GSH glutathione, GSSG glutathione disulfide, GCH1 GTP cyclohydrolase-1, GCS glutamylcysteine synthetase, GLS glutaminase, GPX4 glutathione peroxidase 4, GPP geranyl pyrophosphate, HMG-CoA 3-hydroxy-3-methylglutaryl CoA, HETE hydroxyeicosatetraenoic acids, HPETE hydroperoxyeicosatetraenoic acid, IPP Isopentenyl pyrophosphate, LPCAT3 lysophosphatidylcholine acyltransferase 3, LPCAT lysophosphatidylcholine acyltransferase, LTA4 leukotriene A4, LTB4 leukotriene B4, LTC4 leukotriene C4, NRF2, nuclear factor E2-related factor 2; OOH, hydroperoxide; PE-PUFA, phosphatidylethanolamine polyunsaturated fatty acid, PUFA polyunsaturated fatty acid, PGD2 prostaglandin D2, PGE2 prostaglandin E2, PGF2α prostaglandin F2α, PGF2β prostaglandin F2β, SAM S-Adenosylmethionine, SAH S-Adenosylhomocysteine, TCA cycle tricarboxylic acid cycle, TFRC transferrin receptor, TSP pathway transsulfuration pathway, γ-GC γ-glutamylcysteine.

Epigenetic regulators such as DNA methylation, histone acetylation and miRNA actively regulate cellular iron homoeostasis. Several studies have reported significant DNA methylation alterations upon increases in intracellular reactive oxygen species (ROS) and iron [49–51]. Remarkably, methylcytosine dioxygenase Tet1 was first identified as an iron-dependent regulator of multiple downstream target genes through its ability to oxidise and demethylate 5-methylcytosines in DNA, and ubiquitinate

ferroportin [52]. In addition, DNA methylation changes in ferroptotic myeloma cells also demonstrated enrichment of CpG probes located in genes associated with cell cycle progression and senescence via increased iron levels, such as nuclear receptor subfamily 4 group A member 2 (NR4A2) [53]. More recently, Fe^{2+} stress was found to induce DNA methylation and genomic instability [54]. Future studies that continue to explore the relationship between DNA methylation, hypoxia and iron metabolism might provide important insight into ferroptosis biology. In terms of treatments that could target iron metabolism, DFO and deferiprone are iron chelators that are undergoing trials for treating conditions associated with increased inflammation, including Parkinson's disease. Whether these iron chelators play a direct role in ferroptosis and how they could be implemented in CRC warrants investigation [55].

Lipid peroxidation

The role of lipid peroxidation in ferroptosis has been reviewed thoroughly elsewhere [56–58]. However, we emphasise here that lipid peroxidation is an important hallmark of ferroptosis, and regulation of lipid peroxidation by LOXs and Acyl-CoA Synthetase Long-Chain Family Member 4 (ACSL4) present as potential therapeutic targets, especially in CRC (Fig. 1).

The mechanism of how the oxidation of polyunsaturated fatty acids (PUFAs) to PUFA hydroperoxides (PUFA-OOHs) triggers ferroptosis is under debate. LOX, particularly 12/15-LOX (i.e., ALOX15), has been repeatedly suggested to play a central role in lipid peroxidation and ferroptosis [28]. Yang et al. observed that PUFA oxidation by LOX via a Phosphorylase Kinase Catalytic Subunit Gamma 2 (PHKG2)-dependent iron pool is necessary for ferroptosis, and the covalent inhibition of the catalytic selenocysteine in GPX4 prevents elimination of PLOOHs [28]. Furthermore, it has been indicated that the initial accumulation of PLOOH regardless of the contribution of LOX is the actual driver of ferroptosis [59]. Recent evidence has revealed that ACSL4 contributes to ferroptosis by upregulating the production of PUFAs and specifically aiding the incorporation of PUFAs into the cell membrane. In 2017, ACSL4 was reported to drive ferroptosis via the accumulation of oxidised cellular membrane phospholipids [26]. These oxidised cellular membrane phospholipids were subsequently identified as oxidised phosphatidylethanolamines (PEs), which are produced via ACSL4 activity [60]. ACSL4 catalyzes the ligation of an arachidonoyl or adrenoyl (AdA) moiety to produce arachidonoyl and AdA acyl-CoA derivatives, respectively. These are then esterified into arachidonoyl-PEs and AdA-PEs by lysophosphatidylcholine acyltransferase 3 (LPCAT3). Subsequently, arachidonoyl-PE and AdA-PE are oxidised by 15-LOX to generate lipid hydroperoxides, the proximate executors of ferroptosis. Supporting this, it has been suggested that lipid peroxidation at the cell membrane is a common requirement for both Erastin and RSL3-induced ferroptosis. ACSL4, however, is only required for RSL3-induced ferroptosis [61]. Why ACSL4 is dispensable for Erastin-induced ferroptosis and whether it is necessary for other modes of ferroptosis induction are important questions and areas for future research.

Another key question in ferroptosis biology is where is the precise location of lethal lipid peroxidation in the cell? While the endoplasmic reticulum, lysosome, and mitochondrial membrane are plausible suggestions, current evidence suggests that the cell membrane is the most probable. It is plausible that extensive lipid peroxidation, resulting from the accumulation of free PUFAs, might alter the chemical make-up of the lipid bilayer. This can compromise membrane integrity and affect barrier function. Attacks on PUFA-containing phospholipids (PUFA-PLs) at their bis-allylic sites by LOXs, radicals and oxygen, drive cell death. This later phenomenon is partly reconcilable with the prior hypothesis because it suggests that free PUFAs must be incorporated into

phospholipids in a reaction catalysed by ACSL4, a protein involved in lipid biosynthesis [14, 62].

Redox status, lipid metabolism and amino acid metabolism

Redox status, lipid metabolism, and amino acid metabolism are also important in the regulation of ferroptosis, and we briefly introduce them here. The thioredoxin system is essential for redox homoeostasis, which can protect DNA from lipid peroxidation and oxidative stress-related damage [63]. One recent study suggested that Auranofin/buthionine sulfoxime and Erastin/buthionine sulfoxime cotreatment alters redox homoeostasis by increasing levels of NRF2 and HO-1, and by decreasing GPX4 levels to induce ferroptosis [64].

As aforementioned, lipid peroxidation occurs in PUFAs on specific phospholipids, and various lipid metabolic pathways are involved in lipid peroxidation and ferroptosis. One of the core questions remaining is how the cells acquire and maintain fatty acid levels in cells? While monounsaturated fatty acids and saturated fatty acids can be generated from acetyl-CoA in cells, long-chain PUFAs such as arachidonic acid (AA) and AdA can be synthesised from dietary essential fatty acids such as linoleic acid in cells. In addition, long-chain PUFAs can be obtained extracellularly. Further, fatty acid uptake and release are tightly regulated by cellular states [65]. Together, these factors determine the levels of fatty acid in cells, which ultimately affect ferroptosis sensitivity [65].

Evidence has demonstrated that amino acid metabolism is critical for ferroptosis; for example, the metabolism of cyst(e)ine, glutamine, branched-chain amino acids (including leucine, valine and isoleucine), tryptophan, and other amino acids (such as arginine, serine, glycine, lysine, etc.). As basic nutrients and energy sources, amino acids contribute to homoeostasis and tumour proliferation significantly as intermediates in glucose, lipid, and nucleotide metabolic pathways. Although amino acid metabolism has been a recent target for newer cancer treatment strategies, a full understanding of amino acid metabolism and ferroptosis in cancer is needed which will help guide drug development and cancer therapy.

MECHANISMS OF FERROPTOSIS SUPPRESSION

Since the initial identification of GPX4 as the covalent target for RSL3, the study of suppression systems in ferroptosis has continued to expand. As this point in time, the four key suppression systems that neutralise lipid peroxides include (1) the GPX4-glutathione (GSH) system, (2) the ferroptosis suppressor protein 1 (FSP1)-ubiquinol (CoQH2) system, (3) the dihydroorotate dehydrogenase (DHODH)-CoQH2 system and (4) the GTP cyclohydrolase I (GCH1)-tetrahydrobiopterin (BH4) system. These four systems have been reviewed in detail elsewhere [66]. Here, we discuss the potential ferroptosis-suppressing functions of two new emerging systems, the transsulfuration pathway (TSP) and mevalonate pathway.

Transsulfuration pathway

Cellular cysteine is a critical metabolite for ferroptosis. It is primarily acquired through the xCT, which consists of subunits SLC7A11 and SLC3A2 and imports cystine in exchange for glutamate. Intracellularly, cystine is reduced to cysteine, which is used for the synthesis of GSH and detoxification of ROS [67]. In addition to extracellular uptake of cystine, another source of cysteine is the TSP, in which cysteine is synthesised and regulated by cystathionine-B-synthase (CBS) and cystathionine-γ-lyase (CGL) [68]; methionine is used as a sulfur donor and converted into cysteine through homocysteine and cystathionine intermediates (Fig. 1). The TSP thus provides a compensatory source of cysteine when extracellular uptake through xCT system is inhibited [69]. Ferroptosis inducers, that block

cystine uptake during glutamate exchange, such as erastin, might be lethal to cancer cells with a defective TSP [70].

Mevalonate pathway

The mevalonate pathway, which regulates the generation of isopentenyl pyrophosphate (IPP), CoQ_{10}, squalene, and cholesterol, impacts ferroptosis in several ways (Fig. 1). IPP modulates selenocysteine (Sec) transfer RNA during the process of GPX maturation [71, 72]. Blocking the rate-limiting enzyme of the mevalonate pathway 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase with statins compromises the efficient translation of GPX4 and consequently sensitises cells to ferroptosis [73]. Squalene has been implicated to act as an anti-ferroptotic agent in a subset of cancer cells. Cells devoid of squalene monooxygenase activity show squalene accumulation and resistance to ferroptotic inducers, which disappears when squalene synthase (SQS) is disrupted [74].

Both 7-dehydrocholesterol (7-DHC) and cholesterol are potential modulators of lipid peroxidation and ferroptosis [75]. RSL3-triggered ferroptosis was shown to accumulate exogenous PUFAs in a cholesterol-independent manner [28]. Further studies are needed to explore the relationship between the mevalonate pathway, cholesterol metabolism and ferroptosis induction.

FERROPTOSIS IN CRC TUMORIGENESIS

CRCs harbour a high frequency of mutations, and tumours between patients are highly heterogenous in immune responses and metabolism [76]. The most common CRC activation pathway is triggered by the inactivation of the tumour suppressor gene adenomatous polyposis coli (APC), which promotes adenoma formation. Subsequent mutations to Kirsten rat sarcoma virus (KRAS) and TP53 promote the formation of carcinomas [77]. Here, we discuss how each of these events might impact ferroptosis sensitivity in CRC.

Mutations in APC, a member of the destruction complex consisting of GSK-3β and Axin, occur in about 80% of all sporadic CRCs [78]. APC is a negative regulator of Wnt/β-catenin signalling, and biallelic loss of APC causes an initiated epithelium that is "over-charged" and progresses to the adenoma stage. Elevated β-catenin levels enhance transcriptional activity of proto-oncogenes such as MYC and cell cycle markers, including cyclin D1 [79, 80]. There is currently a dearth of information on the functional significance of APC loss in ferroptotic cell death. However, pre-treatment of HeLa cells with a GSK-3β inhibitor prevented erastin-induced ferroptosis cell death [81], and it is possible that loss of APC elicits a similar effect. It is also likely that the effect of APC mutations on ferroptosis sensitivity will depend on the overall metabolic changes that accompany such mutations. These are intriguing questions that warrant further laboratory experimentation.

Subsequent activation of oncogenes such as KRAS occurs in about 30–40% of colon adenomas and further enables progression to carcinoma [82]. The KRAS oncogene is one of the most frequent mutations found in CRCs, and tumours harbouring these mutations are also the most difficult to treat. Emerging evidence suggests that mutant KRAS protects cancer cells from ferroptosis through several mechanisms. In CRC, KRAS mutations are associated with increased expression of FSP1 and relative ferroptosis resistance [83]. Notably, FSP1 aids in cellular transformation and tumour initiation, and inhibiting FSP1 sensitised KRAS-mutant cells to ferroptosis. In lung cancer, mutant KRAS was also found to regulate the synthesis of ferroptosis-suppressing monounsaturated fatty acids, establishing a targetable vulnerability in those cells [84]. It has also been suggested that KRAS could regulate glutaminolysis, mitochondrial ROS production, and glucose uptake in CRC [85], and all three of these processes have indirect effects on ferroptosis.

Given an increased role for ferroptosis in anticancer immune responses, there is also a possible connection between pro-inflammatory metabolites, ferroptosis and immune modulation caused by KRAS that leads to immune escape in CRC. KRAS can induce chemokines, cytokines and signalling pathways that promote tumorigenesis in CRC [86]. Knockdown of mutant KRAS in a poorly immunogenic CRC mouse cell line led to an improved immune response and tumour regression [87]. KRAS might also sustain high iron levels for CRC growth by regulation of HIF-2α and Janus kinase (JAK)-signal transducer and activator of transcription (STAT) pathway signalling, which promotes tumour growth through HIF-2α-mediated iron import via divalent metal transporter 1 (DMT1) [88, 89]. Analysis of data from The Cancer Genome Atlas (TCGA) revealed that KRAS potentiates the expression of iron importers in CRC [88].

As described previously, RSL3 is a potent small-molecule compound that induce ferroptosis. RSL3 has been shown to exert cytotoxicity on RAS-mutant cancer cell lines (such as KRAS-mutant HCT116 cell lines) and relies on the cellular iron pool for this process [90]. These findings explain the rationale for a recent study that explored the therapeutic efficacy of cetuximab in combination with RSL3 in cancer cell lines. Yang and colleagues showed that cetuximab administration to KRAS-mutant cell lines is insufficient to increase lipid peroxides or trigger ferroptosis [91]. However, when cetuximab was used in combination with RSL3, it enhanced the cytotoxic effect of RSL3 by upregulating ROS production and malondialdehyde (MDA), a lipid peroxide adduct [91]. Similarly, cetuximab increased tumour MDA levels in a KRAS-mutant CRC xenograft mouse model, suggesting that this phenomenon is reproducible in vivo [91]. These findings further strengthen the clinical potential of ferroptosis inducers as part of combination therapies to target tumour antioxidant status and treat CRC. Given that metastatic RAS-mutant CRC is typically unresponsive to cetuximab and particularly difficult to treat, novel combination strategies are urgently needed.

Following APC and KRAS mutations, the last step in CRC malignant transformation is often the loss of the tumour suppressor protein function of p53 [92]. To date, p53 has been implicated as both a positive and negative modulator of ferroptosis [93–98]. It was shown that p53 promotes ferroptosis by repressing the expression of SLC7A11 and inhibiting cystine uptake [99]. Strikingly, a mutant form of p53 that retains the ability to repress SLC7A11 expression but is unable to halt cell cycle progression and induce apoptosis, prevents tumour growth comparably to wild-type p53. On the contrary, over-expression of SLC7A11 in cancer cells eliminated p53-mediated tumour suppression. It was further demonstrated that ALOX12 is critical for p53-mediated ferroptosis and inhibition of cancer growth in a CRC cell line (HCT116) and other human cancer cell lines [93]. With this in mind, ALOX12 expression may be regarded as a prognostic signature of ferroptosis; a recent analysis of gene expression data from two publicly available databases (TCGA and NCBI GEO) identified ALOX12 as a prognosis-related gene that correlated ferroptosis with survival in colon cancer patients [100]. Conversely, p53 has also been shown to have opposite effects on ferroptosis. In human CRC cells, p53 constrained erastin-stimulated ferroptosis through the inhibition of dipeptidyl-peptidase-4 (DPP4). Silencing of p53 facilitated plasma membrane-associated DPP4-dependent lipid peroxidation and thus prevented nuclear accumulation of DPP4 (Fig. 1) [94]. Given the context-dependence of ferroptosis regulation, it remains unclear whether p53 always plays a ferroptosis-suppressing role in CRC or if its overall function is dependent on other cellular metabolic and signalling pathways.
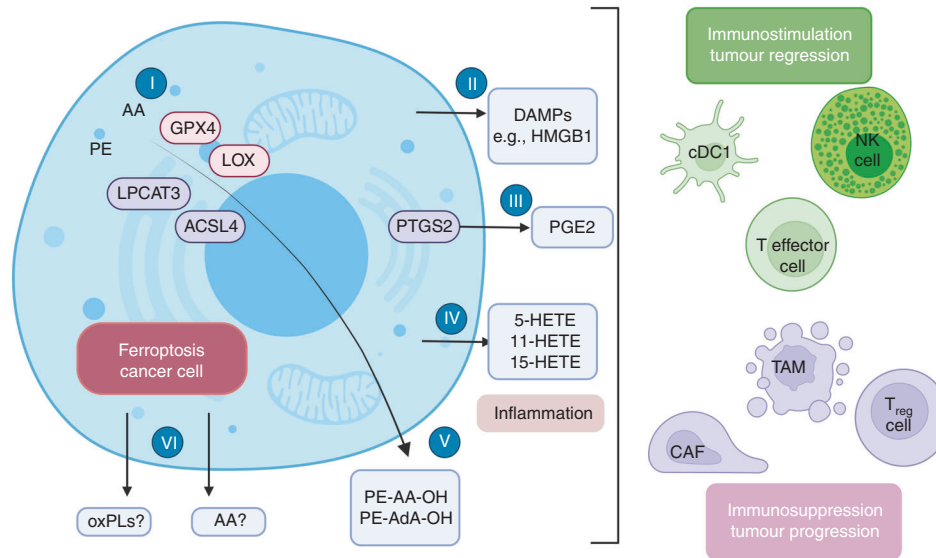
Fig. 2 Modulation of ferroptosis cancer cell immunity. GPX4, ACSL4, LPCAT3 and LOXs (labelled (I)) mediate sensitivity to ferroptosis. ACSL4 is a key regulator of ferroptosis. It catalyzes the esterification of AA or AdA into PE, whereas LOXs can oxidise PE-AA and PE-AdA to PE-AA-OH and PE-AdA-OH (labelled (V)), further promoting ferroptosis. Ferroptosis can induce the attraction and activation of innate immune cells such as neutrophils, which are effectively engulfed by phagocytic cells. HMGB1 (labelled (II)), a damage-related molecular patterns (DAMP), has been shown to be released in response to ferroptosis [146]. It has been shown that in addition to PGE2 (labelled (III)), siderophiles also release 20-alkane compounds, such as 5-HETE, 12-HETE and 15-HETE (labelled (IV)), in response to induction of GPX4 depletion [147]. ACSL4 acyl coenzyme A synthetase long-chain family member 4, AA arachidonic acid, AdA adrenic acid, CAF cancer-associated fibroblasts, CDC1 conventional type 1 dendritic cells, GPX4 glutathione peroxidase 4, HMGB1 high-mobility group box 1, LPCAT3 lysophosphatidylcholine acyltransferase 3, LOX Lipoxygenase, NK natural killer, oxPLs oxidised phospholipids, PE phosphatidylethanolamine, 5-HETE 5-hydroxy-eicosatrienoic acid, 12-HETE 12-hydroxy-eicosatrienoic acid, 15-HETE 15-hydroxy-eicosatrienoic acid, PTGS2 prostaglandin-endoperoxidase synthase 2, PGE2 prostaglandin E2, TAM tumour-associated macrophages, Treg cells regulatory T cell.

THE POTENTIAL CONNECTION BETWEEN FERROPTOSIS AND METABOLISM IN CRC
Arachidonic acid metabolism
As mentioned previously, oxidation of PUFAs to PLOOHs can drive ferroptosis. AA is a key PUFA which can be metabolised enzymatically by COXs and LOXs to form eicosanoids (e.g., prostaglandins, thromboxanes, lipoxins and leukotrienes) and non-enzymatically through autooxidation to generate lipid peroxides which activate ferroptosis as described above [57, 101]. This is important to note in CRC, as COX-2 mediated AA metabolism is well-established as a having roles in CRC progression [102]. A recent report showed that the elongation of very long-chain fatty acids-like 5 (ELOVL5), a rate-limiting enzyme involved in de novo PUFA synthesis, drives sensitivity towards ferroptosis-induced cancer cell death [103]. ELOVL5 is upstream of AA and adrenic acid metabolism. Lipid profiling revealed that mesenchymal gastric cancer cells expressing high ELOVL5 levels are able to synthesise PUFAs, indicating a possible explanation for their ferroptosis sensitivity [103]. Additional reports have shown that AA can also directly induce ferroptosis in certain tumour cells [104]. Most notably, new evidence revealed that T cell-derived interferon-γ in combination with AA induces immunogenic tumour ferroptosis, serving as a mode of action for CD8 + T cell (CTL)-mediated tumour killing [105]. This, combined with previous studies that identified roles for ferroptosis in the mechanism of action of both immunotherapy and radiotherapy, suggests that ferroptosis resistance could represent a key mechanism by which CRCs proliferate despite treatment[34, 106]. Despite these findings, it is not completely understood how normal cells and cancer cells differentially regulate the intracellular pools of AA. It is possible that cancer cells might simply take up or synthesise more PUFAs at baseline (likely related to their growth needs and overall changes in lipid metabolism). Alternatively, if there are no differences in the amounts of

PUFAs between normal cells and cancer cells, then there might be changes in where the PUFAs are located: e.g., cytosol, lipid droplet storage, membrane conjugated to PLs, etc. It will also be important to further characterise what other signalling molecules work in combination with AA to induce ferroptosis, as AA is insufficient to induce ferroptosis on its own at physiologic concentrations.

Under normal conditions, AA is released from intracellular phospholipids by cytosolic phospholipases (cPLA) such as cPLA2α [107]. Two studies have identified the calcium-independent phospholipase A2 (iPLA2β) as a major ferroptosis suppressor [108, 109]. Although suppressing ferroptosis by increasing AA production might appear paradoxical, iPLA2β likely accomplishes this by reducing the quantity of AA available at critical cellular membranes. Future studies, however, are required to confirm this and determine whether other phospholipases also modify ferroptosis.

A two-fold increased abundance of AA has been shown in patients with ulcerative colitis (UC) compared to healthy mucosa, suggesting a role for AA in CRC development [110]. While this finding might also appear counterintuitive, elevated AA can also promote the synthesis of eicosanoids, such as the pro-inflammatory hydroxyeicosatetraenoic acid (HETE) oxylipids 5-HETE, 11-HETE and 15-HETE, which drive cancer progression and immune evasion (Fig. 2) [102]. It is possible that during tumorigenesis, the synthesis of these mediators counteracts any ferroptosis-promoting actions of AA, whereas once tumorigenesis occurs the metabolic adaptations associated with it heighten cellular vulnerability to ferroptosis. Under certain conditions, AA metabolites might also cooperate with endogenous antioxidants to prevent ferroptosis. The hydrophilic antioxidant N-acetylcysteine (NAC), for instance, synergises with PGE2, a COX-derived prostaglandin, to confer a ferroptosis-resistant state in neuronal cells in a haemorrhagic stroke model [111]. The enhancing effects of exogenous antioxidants on ferroptosis

Table 2. Inducers of ferroptosis in CRC.

Compound	Model	Target	Mechanism	Ref.
Talaroconvolutin A	HCT116, SW480, and SW620; xenografts mice	SLC7A11	Talaroconvolutin A induces ferroptosis by targeting ROS accumulation and SLC7A11 in CRC	[174]
Erastin	HT-29	SLC7A11	Targeting SLC7A11 specifically suppresses the progression of CRC stem cells via inducing ferroptosis	[175]
IMCA	DLD-1, HCT116; xenografts mice	SLC7A11	IMCA induces ferroptosis mediates by SLC7A11 through the AMPK/mTOR pathway in CRC	[176]
Andrographis	HCT116, SW480; xenografts mice	β-catenin/Wnt-signalling pathways	Activation of ferroptosis and suppression of β-catenin/Wnt-signalling pathways were the key mediators for the anticancer and chemosensitizing effects of andrographis	[177]
ACADSB	SW620, LoVo	GPX4	ACADSB induces ferroptosis of CRC cells by negatively regulating GPX4	[128]
Apatinib	HCT116	ELOVL6/ACSL4	Apatinib promotes ferroptosis in CRC cells by targeting ELOVL6/ACSL4	[130]
Bromelain	Kras wild-type (Caco2; NCI-H508) or mutant (HCT116, G13D; DLD-1, G12D); KRASG12D mutant heterozygous mice	ACSL4	Bromelain effectively exerts cytotoxic effects in KRAS-mutant CRC cells by targeting ACSL4	[135]
Iron	HCT116, HT-29; xenografts mice	Warburg effect, NRF2 pathway	Iron promotes Warburg Effect, induce ROS production and activate the NRF2 pathway in CRC	[178]
Resibufogenin	HT-29, SW480; xenografts mice	GPX4	Resibufogenin inhibits CRC cell growth through triggering ferroptosis mediated by GPX4 inactivation	[179]
RSL3	HCT116, LoVo, and HT-29	GPX4	RSL3-induced cell death in CRC cells	[126]
Vitamin C	DiFi cell; CRC organoids	ROS	Vitamin C regulates iron metabolism, increased ROS production and induce ferroptosis	[180]
β-elemene, cetuximab	HCT116, LoVo; xenografts mice	Epithelial–mesenchymal transformation	KRAS-mutant CRC cells are sensitive to the combination treatment of β-elemene and cetuximab by inhibiting EMT and inducing ferroptosis.	
Cisplatin	HCT116	GPX4	Depletion of reduced glutathione caused by cisplatin and the inactivation of GPX4 plays the vital role in CRC cells to induce ferroptosis	[181]

IMCA benzopyran derivative 2-imino-6-methoxy-2H-chromene-3-carbothioamide, ACADSB acyl-CoA dehydrogenase, short/branched-chain, EMT epithelial–mesenchymal transformation.

resistance may be due to the increased activity of GPX4 [112]. This would be consistent with previous studies that showed *GPX4* activity is required to suppress AA metabolism [113].

Glutaminolysis, glutamine metabolism and ferroptosis

As the most abundant amino acid in the body, glutamine supplies nitrogen for the biosynthesis of other amino acids, lipids, and TCA intermediates; glutaminases (GLS) are rate-limiting for the latter [114]. Under situations of amino acid starvation, supplementation with glutamine induces ferroptosis [115]. The induction of ferroptosis through glutamine relies on glutaminolysis, in which GLS converts glutamine to glutamate. In 2015, Gao et al. showed that GLS2, and not GLS1, is indispensable for ferroptosis even though these two enzymes both hydrolyse glutamine [115]. This discrepancy is explained by the role of increased mitochondrial ROS in ferroptosis [116]. GLS1 is a cytosolic protein, whereas GLS2 localises in the mitochondria, where it contributes to mitochondrial ROS. This is explained by later work from the same group demonstrating the crucial role of mitochondria in cysteine deprivation- and erastin-induced ferroptosis but not RSL3-induced ferroptosis [115]. Cysteine deprivation leads to mitochondrial membrane potential hyperpolarization and lipid peroxide accumulation [117]. The therapeutic importance of this was demonstrated in an ex vivo cardiac model of ischaemia/reperfusion injury-induced ferroptosis, whereby inhibition of GLS improved left ventricular developed pressure (LVDP) and reduced the number of myocardial infarcts compared to hearts treated with vehicle [115].

As discussed above, the role of the mitochondria in ferroptosis may be broadly due to its supply of TCA intermediates from glutamine but also by the tumour suppressor fumarate hydratase/fumarase (FH) [114]; renal cancer cells lacking FH were less sensitive to cysteine-deprived induced ferroptosis. Furthermore, CRISPR-Cas9-mediated knockout of GPX4 promoted ferroptotic cell death in human fibrosarcoma HT1080 cells regardless of mitochondrial depletion, indicating that the role of the mitochondria in ferroptosis is context-dependent [117]. Nonetheless, it is possible that the upstream supply of small molecules by the substrate glutamine is needed for oxidative phosphorylation and ROS production, which may influence ferroptosis.

Hypoxia-inducible factor 1-alpha (HIF-1α)

Hypoxia commonly occurs in solid tumours and regulates metabolism, redox homoeostasis and cell proliferation via the HIF-1α and HIF-2α signalling pathway [118]. Cancer cell resistance to chemotherapy is tightly linked to hypoxia, thus implicating HIF-1/2 as potential targets for therapy. Singhal et al. showed that Cre-Lox mediated activation of HIF-2α contributed to doxorubicin resistance in a manner consistent with the role of HIF signalling [119]. In contrast, chemical induction of HIF-2α sensitises intestinal cancer cells to Erastin-induced ferroptotic death [120]. In addition, the mitochondrial metabolite dimethyl fumarate (DMF) caused cell death in HIF-2α-expressing tumour enteroids and increased the abundance of mitochondrial metabolites after DMF-treatment of colon cancer cell lines. In this context, DMF serves as an analogue of fumarate, a mitochondrial TCA intermediate, thus indicating the association between mitochondrial metabolism and HIF-induced ferroptosis. As reviewed by Li et al., HIF may transcriptionally regulate a subset of genes to limit mitochondrial metabolism and oxygen consumption suggesting that HIF is an important factor for mitochondrial-related ferroptosis [118].

As previously discussed, hypoxia signalling also intersects with iron metabolism, and the specific cellular and contextual contributions of hypoxia-related signalling molecules to ferroptosis regulation require further disentangling.

FERROPTOSIS IN CRC THERAPY

Regulators that induce ferroptosis can directly or indirectly affect GPX4 activity by modulating various metabolic pathways, resulting in a decrease of antioxidant capacity and accumulation of lipid ROS in cells, ultimately leading to ferroptotic cell death. Moreover, cancer cells have an increased demand of metabolic intermediates which are required for energy generation to maintain their rapid proliferation, which results in ROS production. In line with this, cancer cells respond to high ROS levels by boosting their antioxidant defence mechanism to resist cell death [121]; the successful use of chemotherapeutic agents (including oxaliplatin and 5-fluorouracil) is partly linked to their ROS-generating capacities and depletion of intracellular GSH [122–124]. It has also been shown that iron exposure activates the production of ROS and *NRF2*, leading to an upregulation of *SLC7A1*1 and *GPX4*. This counteracts iron-induced lipid peroxidation and rescues CRC cells from ferroptosis [125]. Thus, targeting NRF2 may be a viable strategy for ferroptosis induction in CRC.

In CRC research, studies have identified that targeting ferroptosis could be a promising therapeutic opportunity. This has been shown in vitro with the use of RSL3 which inhibits GPX4 and increases ROS production in CRC cells [126]. To date, *GPX4* and *SLC7A11* are still the main targets for induction of ferroptosis in CRC (Fig. 1). Several ferroptosis inducers and inhibitors have been tested in various CRC studies, including RSL3, resibufogenin, bromelain, apatinib, Acyl-CoA Dehydrogenase Short/Branched Chain (ACADSB), 2-imino-6-methoxy-2H-chromene-3-carbothioa-mide (IMCA), and others. Table 2 summarises the results of these studies. RSL3 suppresses CRC by inhibiting *GPX4* and generating ROS, thereby reducing cell growth [127]. IMCA decreases the viability of CRC cells in vitro and inhibits tumour growth in vivo. This is accomplished by decreasing expression of *SLC7A11* and depleting cysteine and GSH. ACADSB negatively regulated *GPX4* expression, and overexpression of ACADSB enhanced Fe^{2+}, superoxide dismutase, and lipid peroxidation in CRC cells, which induces ferroptosis [128]. Apatinib decreased the expression of GPX4 in gastric cancer [129] and promoted ferroptosis in HCT116 cells by upregulating *ACSL4* and *ECOVL6* expression; this was accompanied by the downregulation of *GPX4* and *FTH1* expression [130]. Notably, Sorafenib, which was initially considered a ferroptosis inducer, was shown to not induce ferroptosis in a series of tumour cell lines, unlike the xCT inhibitors sulfasalazine and Erastin [131].

However, a number of studies have used a combination of natural products or FDA-approved drugs to target ferroptosis in CRC. For example, treatment-triggered tumoricidal immunity was explored using a hypoxia-responsive nanoelicitor for promoting lipid peroxidation, which facilitated ferroptosis by relieving a GPX4-mediated brake [62]. Activated tumoricidal immunity was co-stimulated by chlorogenic acid (a polyphenol found in fruits, vegetables, coffee and tea) and mitoxantrone (a chemotherapeutic used to treat prostate cancer and acute nonlymphocytic leukaemia), which simultaneously inhibited the xCT system and GPX4 pathway [62]. This resulted in the accumulation of lipid peroxides, which promoted iron-initiated tumour cell damage [62]. Honokiol (HNK) was recently investigated and shown to induce ferroptosis by regulating GPX4 in a number of colon cancer cell lines [132].

Compounds that target mutant KRAS proteins have not reached the clinic yet for CRC, however, in 2021 the FDA approved a mutant KRAS (G12C) inhibitor for lung cancer [133, 134]. Studies using various drugs to target mutant KRAS in CRC have shown that they also modulate ferroptosis. For example, bromelain, a mixture of proteolytic enzymes derived from pineapple stem, suppressed mutant KRAS by stimulating ferroptosis [135]. Compared to KRAS wild-type CRC cell lines, KRAS-mutant cell lines also exhibited a strong upregulation of *ACSL4* when treated with bromelain [135]. Likewise, cetuximab, a monoclonal antibody

for epidermal growth factor receptor (EGFR), enhanced the induction of ferroptosis by RSL3 on KRAS-mutant CRC cells by inhibiting the NRF2/HO-1 axis through the activation of p38 MAPK [136]. Further, combination of cetuximab and β-elemene, a bioactive compound isolated from Chinese herb *Curcumae Rhizoma*, sensitised KRAS-mutant CRC cells by inducing ferroptosis, and was accompanied by ROS accumulation, GSH depletion, lipid peroxidation, and upregulation of HO-1 and transferrin [137]. Curcumin, a yellow pigment found primarily in turmeric, can trigger ferroptosis and suppress CRC proliferation by inhibiting PI3K/AKT/mTOR signalling pathway [138]. Collectively, these studies show that targeting EGFR, PI3K and ACSL4 could induce ferroptosis in KRAS-mutant CRCs. Conversely, Yi et al. revealed that oncogenic activation of the PI3K/AKT/mTOR pathway can confer resistance to ferroptosis [139]. In a panel of human cancer cells, it was shown that the cells which harboured *PIK3CA*-activating mutations had diminished sensitivity to RSL3-induced ferroptosis, moreover, pharmacological blockade of PI3K and AKT dramatically sensitised cells to ferroptosis and lipid peroxidation [139].

To date, most experiments have been performed in cell lines and xenograft models where the immune system cannot be studied in most cases. Therefore, future studies will indicate whether metabolites associated with ferroptosis (i.e., PE-AA-OH and PE-AdA-OH (labelled (V), Fig. 2) affect anti-tumour immunity. It is conceivable that potential immunomodulatory signals are AA oxidation products and oxPLs (labelled (VI), Fig. 2) that are released from ferroptotic cancer cells, although further studies are needed to confirm this. Recently, a nanovaccine was designed to inhibit tumour growth by inducing specific cytotoxic T lymphocyte (CTL) immune responses against tumour cells and fibroblast activation protein-α (FAP) $^+$ cancer-associated fibroblasts (CAFs) to reshape the tumour microenvironment (TME) in a colon cancer model. This nanovaccine could promote tumour ferroptosis by releasing interferon-gamma from CTLs and depleting FAP$^+$CAFs [140]. In addition, expression of SLC7A11, SLC3A2 and GPX4 were decreased compared to controls, suggesting that the nanovaccine may stimulate the antitumor immunity and initiate ferroptosis in tumours [140].

While ferroptosis-targeting therapies are intended to target cancer cells, beneficial cell populations in the TME, such as immune cells, are also vulnerable to ferroptosis. For example, a recent study showed that large amounts of lipid peroxides were detectable in CD8$^+$ T cells derived from tumours but not from lymph nodes, and identified ferroptosis as a metabolic vulnerability of tumour-specific CD8$^+$ T cells. The killing rates and numbers of CD8$^+$ T cells were reduced by GPX4 inhibitors that had no impact on the cancer cell survival, and it is likely that CD36-mediated PUFA uptake drives this sensitivity [141, 142]. In addition to CD8 + T cells, ferroptosis can impact macrophages, dendritic cells, and neutrophils. Inducible nitric oxide synthase (iNOS)/NO•-enrichment of activated M1 (but not alternatively activated M2) macrophages/microglia modulates susceptibility to ferroptosis [143]. Furthermore, ferroptosis of cancer cells impairs dendritic cell maturation and antigen presentation, limiting adaptive antitumor immune responses [144]. Ferroptosis of tumour infiltrating neutrophils has a similar effect, where release of oxygenated lipids impairs the tumoricidal activity of CD8 + T cells [145]. Overall, it is likely that the effects of ferroptosis inducers in the TME will depend on the overall cellular architecture of specific cancer types.

CONCLUDING REMARKS AND FUTURE PERSPECTIVES
Accumulating evidence has demonstrated that ferroptosis could play a role in CRC therapy. Moreover, several molecular targets had been identified in models of CRC that could have potential therapeutic application such as *GPX4, SLC7A11, ACSL4* and *TP53* (Fig. 1). However, less is known about the role of oncogenes that

commonly occur in CRC, and what their differential effects might be on ferroptosis via various signalling mechanisms. In addition, studies in other cancer types have shown that ferroptosis can modulate inflammation and immune responses, which has not yet been approached in CRC.

Our review has raised several key questions for future investigation: (1) What is the effect of genetic mutations (including KRAS) on ferroptosis sensitivity? (2) What influence does the TME have on hypoxia, nutrient availability, and ferroptosis sensitivity? (3) What is the role of AA metabolism in ferroptosis in CRC? (4) Given the context-dependency of ferroptosis regulation, are there ferroptosis induction or suppression mechanisms that are unique to CRC? (5) What is the exact cellular location of lipid peroxidation that contributes to ferroptosis? Is it on the mitochondrial membrane, endoplasmic reticulum, lysosomes or other subcellular locations?, and (6) What regulates the coupling phase of free PUFAs to phospholipids for lethal lipid peroxide formation?

With evidence mounting in the connection between ferroptosis, inflammation and immunity, PGE2, HETEs and GPX4 have received considerable attention. Undeniably, more work is warranted to understand the immunomodulatory role of ferroptotic CRC cells in antitumor immunity. Although substantial progress has been made to elucidate how oncogenic signalling drives CRC proliferation, less is known about how this drives or alters susceptibility to ferroptosis. In addition, the combined usage of novel ferroptosis drugs with other small molecules that regulate cellular and molecular pathways, including lipid peroxidation, mitochondrial respiration and redox status hold promise for future clinical testing of these drugs. Furthermore, continued research is needed to understand how KRAS mutations directly shape the metabolism of CRC and ferroptotic networks. Exploiting these networks, especially in KRAS-mutant context, could be a promising therapeutic strategy for improving treatment regimens.

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ADDITIONAL INFORMATION
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