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The role of iron and reactive oxygen species in cell death

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The transition metal iron is essential for life, yet potentially toxic iron-catalyzed reactive oxygen species (ROS) are unavoidable in an oxygen-rich environment. Iron and ROS are increasingly recognized as important initiators and mediators of cell death in a variety of organisms and pathological situations. Here, we review recent discoveries regarding the mechanism by which iron and ROS participate in cell death. We describe the different roles of iron in triggering cell death, targets of iron-dependent ROS that mediate cell death and a new form of iron-dependent cell death termed ferroptosis. Recent advances in understanding the role of iron and ROS in cell death offer unexpected surprises and suggest new therapeutic avenues to treat cancer, organ damage and degenerative disease.

Iron is the fourth most abundant element in the Earth's crust and is essential for most life on the planet[1]. Of relevance to this review, iron is crucially involved in the formation and destruction of ROS, a blanket term for a collection of partially reduced oxygen-containing molecules, including superoxide ($O_2^{\bullet-}$), peroxides (H_2O_2 and ROOH) and free radicals ($HO^{\bullet}$ and $RO^{\bullet}$) (**Fig. 1a,b**). Although certain amounts of iron and ROS are crucial for normal cell and organismal function[1,2], the aberrant accumulation of iron, ROS or both is linked to a staggering number of acute traumas and chronic degenerative conditions[3]. It is therefore of great interest to understand how iron and ROS contribute to cell death and whether the levels of these species can be manipulated to achieve desirable therapeutic effects.

Sources of iron-catalyzed ROS within the cell

Iron and iron derivatives (for example, heme or iron-sulfur [Fe-S] clusters) are incorporated into and are essential for the function of ROS-producing enzymes such as nicotinamide adenine dinucleotide phosphate hydride (NADPH) oxidases (NOXs), xanthine oxidase, lipoxygenases (LOXs), cytochrome P450 enzymes and subunits of the mitochondrial electron transport chain (**Fig. 1a,b**). Iron is also found at the active site of the H_2O_2-destroying enzyme catalase, found in the peroxisome (**Fig. 1b**). Small pools (<20 μM[4]) of labile ('loosely coordinated', 'redox-active') Fe^{2+} reside in the cytosol and the mitochondrial matrix (of eukaryotic cells); the lysosome also contains a pool of redox-active iron derived from extracellular sources as well as the catabolism of ferritin and iron-rich intracellular organelles such as mitochondria[5]. These redox-active iron pools are capable of directly catalyzing damaging free radical formation via Fenton chemistry[3] (**Fig. 1a,b**). For this reason, iron chelators such as deferasirox, deferiprone, deferoxamine and clioquinol have been implemented or proposed as treatments for diverse pathologies linked to iron or ROS accumulation[3] (**Fig. 1c**). Both iron-dependent ROS-producing enzymes and labile iron are thought to contribute to ROS-dependent cell damage and death. How this occurs is an emerging area of study, in large part owing to the difficulty in defining the targets and effects of ROS that are relevant to death and the effects of iron accumulation on cell function. Below we consider recent progress on these fronts.

Direct targets of ROS that impair growth and viability

Targets of superoxide. Studies of the damaging effects of superoxide and other ROS have progressed furthest in bacteria such as *Escherichia coli*. Here it is possible to grow cells under defined environmental conditions in the absence of specific antioxidant enzymes, allowing superoxide to accumulate from endogenous sources. The growth of mutants lacking superoxide dismutase (SOD) activity is inhibited owing to impairment of amino acid synthesis[6]. This growth inhibition is due to the attack of superoxide on the enzymatically essential, solvent-exposed [4Fe-4S] cluster of α,β-dihydroisovalerate dehydrogenase, leaving the remaining $[3Fe\text{-}4S]^+$ cluster in an inactive state[7] (**Fig. 2**). Superoxide attack also releases free iron, which can bind DNA and catalyze Fenton chemistry–dependent DNA damage[7] (**Fig. 2**). In eukaryotic organisms, it seems likely that mitochondrial ROS accumulation triggers cell death and mitochondrial DNA damage in a similar manner by interfering with essential metabolism (for example, inactivation of the [Fe-S]-dependent dehydratase aconitase[8] in the citric acid cycle) and the release of iron[9].

Consistent with the existence of highly conserved pathways of oxidant-induced damage, mice lacking mitochondrial superoxide dismutase 2 ($Sod2^{-/-}$) die shortly after birth with a variety of biochemical defects that can be traced to superoxide-mediated inactivation of mitochondrial enzymes, including aconitase and complex I and II of the electron transport chain[10,11]. However, the targets of endogenous superoxide (or its dismutation product H_2O_2) derived from aberrant activation of NOX enzymes during processes such as excitotoxic cell death[12] or bacteria-induced colonic epithelial cell death[13] are, for example, entirely unknown and a major open research question.

Targets of H_2O_2. Studies in bacteria have recently elucidated targets of H_2O_2, whose inactivation compromises cellular growth and viability under a variety of conditions. The development of an *E. coli* mutant strain (Hpx^-) deficient in all three main H_2O_2-degrading enzymes, the catalases KatG and KatE and the peroxide-scavenging enzyme Ahp, has made it possible to examine the effects associated with the accumulation of endogenously produced H_2O_2 (typically ~1 μM)[14]. Hpx^- cells grown in complete medium under aerobic

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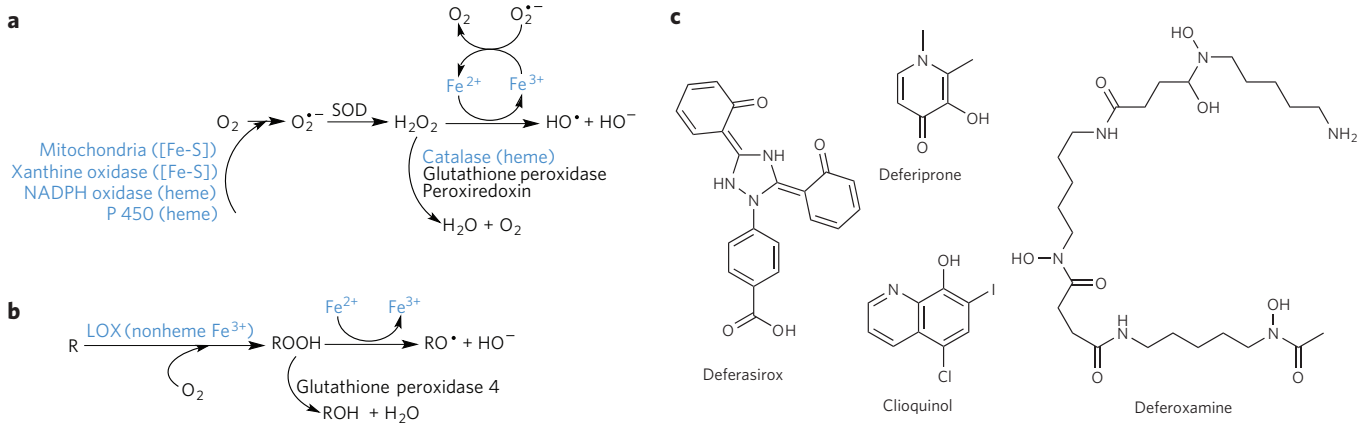

Figure 1 | The role of iron in ROS metabolism. (**a**,**b**) Pathways of formation of select soluble (**a**) and lipid (**b**) ROS. The reaction of peroxides with Fe^{2+} to yield soluble hydroxyl (HO•) or lipid alkoxy (RO•) radicals is referred to as the Fenton reaction. Key enzymes that contribute to ROS formation or detoxification are shown. Those whose function is iron dependent are colored blue, and the relevant iron species is in parentheses. (**c**) Key clinical (deferoxamine, deferiprone and deferasirox) and clinical candidate (clioquinol) iron chelators used to attenuate the toxic effects of iron overload and frequently deployed in experimental studies of the role of iron in cell death.

conditions arrest their growth owing to Fenton chemistry–dependent DNA damage; they die if either the H_2O_2 stress–responsive transcription factor OxyR or the DNA repair enzyme RecA is deleted in the Hpx⁻ background[15]. In minimal medium, Hpx⁻ cells do not proliferate owing to oxidative inactivation of isopropylmalate isomerase, a [4Fe-4S] cluster–containing dehydratase involved in leucine biosynthesis[16]. Other [Fe-S]-containing dehydratases (6-phosphogluconate dehydratase and fumarase A and B) are likewise inactivated in Hpx⁻ cells[16]. In Hpx⁻ cells, H_2O_2 also directly damages enzymes responsible for the synthesis of [Fe-S] clusters (the Isc system)[17] and inactivates a class of mononuclear iron enzymes, including ribulose-5-phosphate 3-epimerase, peptide deformylase, threonine dehydrogenase and cytosine deaminase, which are now known to use iron *in vivo*[18,19]. Given that the intracellular metalloproteome is poorly defined[18,20], it is likely that additional iron-containing enzymes susceptible to ROS-mediated inactivation remain to be discovered. The inactivation of these additional iron-dependent enzymes is likely to arrest growth or trigger death in a context-specific manner.

In eukaryotes, the direct targets of peroxide stress that lead to cell death are more difficult to discern. Exposure of cultured cancer cells to exogenous H_2O_2 or to the peroxide bridge–containing small molecule artesunate can trigger iron-dependent (i.e., can be inhibited by an iron chelator) apoptotic cell death through a process requiring lysosomal iron[21,22], but the *in vivo* relevance of this pathway remains unclear. Although there is currently no equivalent to the bacterial Hpx⁻ strain that would allow for an examination of the effects of endogenous H_2O_2 accumulation on cell viability, the effects of lipid peroxide accumulation can be studied in mice lacking the essential lipid peroxide–metabolizing enzyme glutathione peroxidase 4 (Gpx4)[23]. Results suggest that in the absence of Gpx4, unrestrained lipid peroxide accumulation triggers a nonapoptotic cell death pathway involving the iron-dependent LOX enzymes[23] (**Fig. 1b**).

ROS as a mediator of cell death

In bacteria, ROS accumulation can arrest cell growth or trigger death by directly inhibiting specific essential metabolic enzymes and by causing DNA damage. In eukaryotes, the picture is more complicated, as ROS can serve as a mediator of cell death initiated by other enzymes. Here, we review some of the different ways that ROS have been found to contribute to death in this way.

Aberrant accumulation of mitochondrial superoxide. In several eukaryotic species, mitochondria-derived ROS are directly linked to the induction of cell death. In the single-celled eukaryote *Saccharomyces cerevisiae*, a point mutation in the highly conserved AAA ATPase Cdc48/VCP ($CDC48^{S565G}$) specifically disrupts the interaction between Cdc48 and Vms1, which is needed to localize Cdc48 to damaged mitochondria[24,25]. In the absence of proper Cdc48 mitochondrial localization, damaged mitochondria produce high levels of ROS that ultimately cause cell death with many features reminiscent of apoptosis[24–26]. Clonogenic growth in the $CDC48^{S565G}$ mutant is rescued by treatment with the free radical spin traps *N-tert*-butyl-α-phenylnitrone or (2,2,6,6-tetramethylpiperidin-1-yl)oxyl (TEMPO), by inhibitors of the mitochondrial electron chain complex III, by elimination of mitochondrial DNA (mtDNA) or by incubating cells in an oxygen-depleted environment[26,27], demonstrating that even in simple eukaryotes, cell death can be triggered by ROS accumulation. How excess mitochondrial ROS production triggers death in this system is unclear, but a better understanding of this problem may be relevant to understanding human disease associated with Cdc48/VCP mutation[28].

In mammals, evidence for a mitochondrial ROS-mediated, pro-death pathway was recently obtained from a study of maternally inherited deafness owing to a point mutation in the mtDNA (A1555G, in the 12S rRNA gene), which leads to the apoptotic death of inner ear neurons *in vivo*[29]. The A1555G mutation results in hypermethylation of mitochondrial ribosomes, which interferes with normal mitochondrial translation, leading directly to enhanced mitochondrial superoxide production[29]. ROS subsequently activate a retrograde, mitochondria-to-nucleus signaling pathway comprising AMP-activated protein kinase (AMPK) and the transcription factor E2F1 (ref. 29) (**Fig. 3a**). AMPK activation, E2F1 upregulation and apoptotic death in cells harboring the A1555G mutation are suppressed by overexpression of mitochondrial SOD2, demonstrating that mitochondrial superoxide is essential for triggering this lethal pathway[29]. Remarkably, in a transgenic mouse model that mimics the effects of A1555G mutation throughout the animal, activation of this lethal pathway seems to be restricted to the cells that undergo death[29]. It will be interesting to determine whether the activation of other ROS-dependent retrograde signaling pathways[30] can induce cell death in other cell types or situations *in vivo*.

Regulation of apoptosis by mitochondrial ROS. At the molecular level, apoptosis is subdivided into two major branches, the intrinsic and extrinsic pathways. In the intrinsic apoptotic pathway, the point of no return involves mitochondrial outer membrane

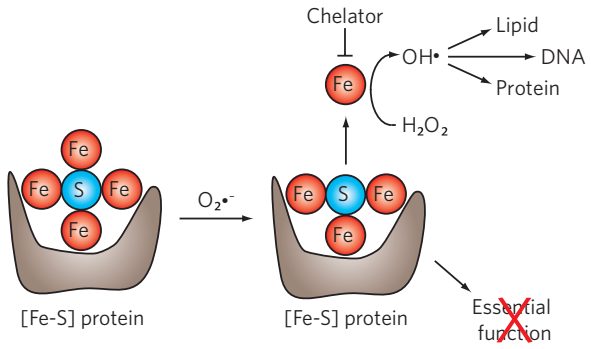

Figure 2 | Oxidative inactivation of essential enzymes and labile iron release can lead to death. Superoxide (as well as H_2O_2) can damage [4Fe-4S]-containing proteins, leading to the release of Fe^{2+}. This has two effects: First, the remaining enzyme is inactivated, and if this enzyme carries out a function essential for growth or viability, the cell will stop growing or die. Second, liberated Fe^{2+} can participate in Fenton chemistry, resulting in damage to various biomolecules (red X) that can also be toxic (for example, DNA damage leading to unrepaired DNA double strand breaks).

permeabilization (MOMP) by the pro-death proteins BAX and BAK. MOMP results in the release of mitochondrial pro-death effectors, such as the hemoprotein cytochrome *c*, which triggers formation of the apoptosome, leading to activation of caspase-3 and caspase-7. Cytochrome *c* is normally bound to cardiolipin, a lipid present on the outer surface of the mitochondrial inner membrane. Oxidation of this lipid by the peroxidase activity of the cytochrome *c* hemoprotein itself is observed following apoptotic stimuli and proposed to be required for subsequent cytochrome *c* release, caspase activation and death execution[31] (**Fig. 3b**). Indeed, cardiolipin oxidation is required for activation of caspase-3/7–dependent neuronal apoptosis during traumatic brain injury in rats[32]. A major question remains the nature of the signal that initiates cardiolipin oxidation. It has been suggested that BAX insertion into the mitochondrial outer membrane enhances the peroxidation activity of cytochrome *c*[33], but further work is required to define this mechanism.

A complicating factor when trying to understand the role of ROS in the execution of apoptosis is that they most likely have roles both upstream (described above) and downstream of cytochrome *c* release and caspase activation (**Fig. 3b**). Mitochondrial ROS production is enhanced downstream of caspase activation by caspase-mediated cleavage of the mitochondrial electron transport chain complex I subunit NDUFS1 (p75), an Fe/S-containing protein[34,35], at least *in vitro*. Cleaved NDUFS1 generates ROS, which act to amplify apoptotic signaling in the mitochondria, and expression of a noncleavable NDUFS1 protein slows, but does not prevent, the onset of apoptotic death[34]. Furthermore, it has been observed that cytochrome *c* release, once it has begun in one portion of a cell, is highly synchronous[36]. It was recently suggested that synchronous cytochrome *c* release is coordinated by waves of ROS emanating from mitochondria that sweep through apoptotic cells[36]. The sources, targets and effects of these synchronizing ROS remain to be defined, but a plausible model is that superoxide derived from NDUFS1 subunits cleaved early in the apoptotic process in one region of the cell enhance cardiolipin peroxidation and cytochrome *c* release in other regions of the cell.

Iron and ROS as executioners of cell death

Necroptosis. Necroptosis is a recently described form of nonapoptotic cell death that can be triggered in certain cells in response to tumor necrosis factor (TNF), FAS ligand and other stimuli and that appears especially relevant to the pathological cell death observed following ischemia reperfusion injury (IRI) to the brain and kidney[37,38]. In cultured mouse cells, necroptosis may involve enhanced mitochondrial ROS production as a terminal event (refs. 39,40). Two processes have tentatively been proposed to explain the enhanced production of mitochondrial ROS in these cells: (i) a receptor interacting protein kinase 1 (RIP1) phosphorylation–dependent interaction between signal transducer and activator of transcription 3 (STAT3) and the mitochondrial electron transport chain complex I subunit GRIM-19 (also known as NDUFA13), leading to enhanced mitochondrial respiration and ROS production[41], and (ii) physical interactions between RIP3 and the enzymes glutamate-ammonia ligase (GLUL), glutamate dehydrogenase 1 (GLUD1) and glycogen phosphorylase (PYGL), which possibly enhance energy metabolism and mitochondrial ROS production[39] (**Fig. 4a**). Others have suggested that cell surface NOX enzyme complexes interact with the TNF receptor and are in fact the more important source of death-inducing ROS during necroptosis, at least in TNF-stimulated cells[42,43]. Regardless of the source, how these ROS promote cell death remains to be determined. There is also some evidence that labile iron levels modulate necroptosis[44], but these results are largely restricted to one cell type (mouse L929 fibrosarcoma) studied *in vitro*. Finally, as data from human Jurkat and other cells suggest that necroptosis can occur without ROS[37], iron and ROS may have a cell

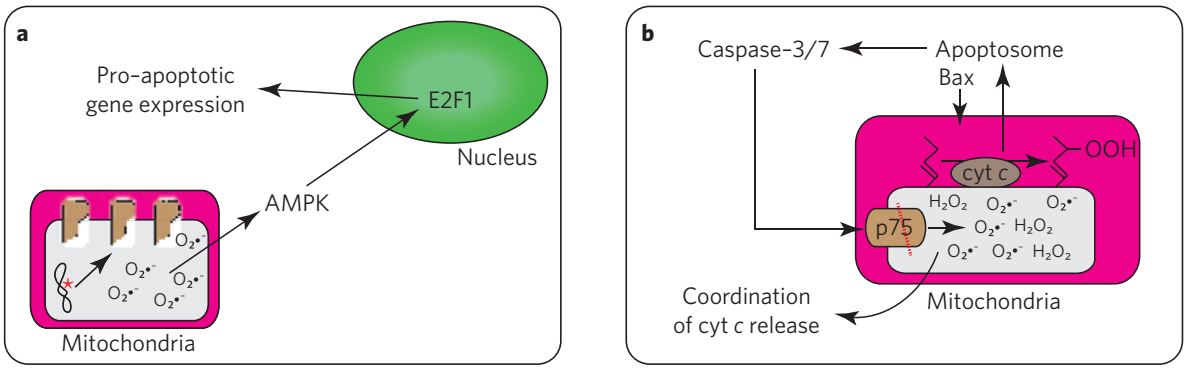

Figure 3 | Involvement of ROS in apoptotic cell death. (**a**) In mammalian cells, mutation of mitochondria DNA (red star) results in defective mitochondrial translation and enhanced superoxide production, presumably due to defective stoichiometry or assembly of mitochondrial electron transport chain complexes (brown). Superoxide accumulation results in activation of AMP-activated protein kinase (AMPK), which can upregulate the transcription factor E2F1, leading in turn to transcriptional upregulation of pro-apoptotic genes. (**b**) The hemoprotein cytochrome *c* (cyt *c*) can catalyze peroxidation of cardiolipins, possibly in response to Bax insertion into the mitochondrial outer membrane, leading to cyt *c* release and, ultimately, the activation of caspase-3 and caspase-7. These caspases can, in turn, cleave the p75 subunit of complex I of the mitochondrial electron transport chain (red dashed line), resulting in the generation of ROS that amplify apoptotic signaling, possibly through the coordination of cyt *c* release between mitochondria.

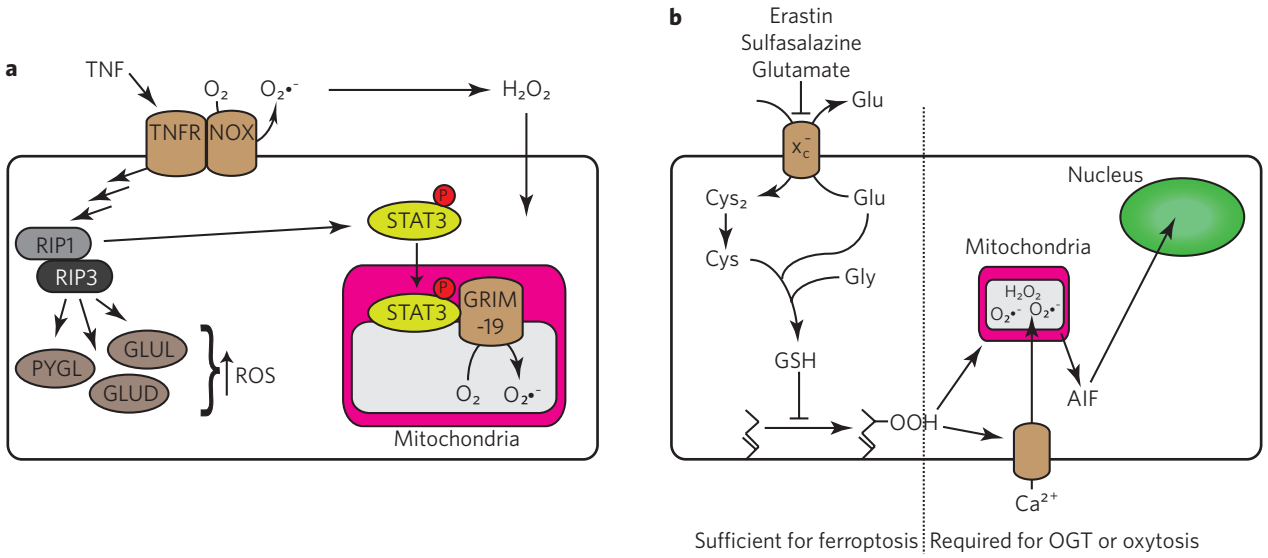

Figure 4 | The role of iron and ROS in necroptosis and ferroptosis. (**a**) In response to TNF and other ligands, certain cells undergo necroptotic death. TNF receptor (TNFR) activation triggers a cascade of events (not depicted) that results in activation of the protein kinases RIP1 and RIP3. Enhanced ROS production may involve RIP1-mediated phosphorylation of STAT3 and STAT3 interaction with the mitochondrial electron transport chain complex I subunit GRIM-19 or activation of the metabolic enzymes (GLUL, GLUD and PYGL) downstream of RIP3, leading to enhanced intracellular metabolism and ROS production. Cell surface NOX enzyme complexes can interact with the TNFR and may also provide a source of death-inducing ROS. (**b**) Treatment of certain cancer cells with erastin or sulfasalazine triggers ferroptotic cell death by inhibiting the function of the cell surface cystine (Cys_2)/glutamate antiporter system x_c^-. Depletion of intracellular cysteine results in lower reduced glutathione (GSH) levels, leading to enhanced formation of lipid ROS species, possibly through activation of iron-dependent LOX or other enzymes (not shown). A related pathway is also triggered in certain neurons, where it is referred to as oxidative glutamate toxicity (OGT) or oxytosis. This pathway is similar to ferroptosis, but death execution also requires Ca^{2+} influx, damage to mitochondria and the release of AIF downstream of lipid ROS formation.

type–specific role in death execution during necroptosis. Further elucidation of the necroptotic pathway promises to be one of the most active areas of cell death research in coming years.

Ferroptosis. A form of iron-dependent, oxidative cell death termed ferroptosis was recently described in mammalian cancer cells and fibroblasts[45]. Ferroptosis, which can be triggered by structurally diverse small molecules (for example, erastin, sulfasalazine and RSL3), is morphologically, biochemically and genetically distinct from apoptosis, autophagy and reported forms of necrosis[45–47]. Ferroptosis is prevented by lipophilic antioxidants, such as trolox and vitamin E, and by iron chelators such as deferoxamine but not by well-known small-molecule inhibitors of apoptosis, necrosis or autophagy; this pattern of sensitivity defines this type of death in all contexts examined so far[45–47]. Interestingly, iron chelation via deferoxamine also prevents H_2O_2 and artesunate-induced, lysosomal iron–mediated death (described above), yet these two lethal triggers do not cause ferroptosis[45]. This highlights an important point: that not all iron chelator–sensitive cell death in mammalian cells involves identical lethal processes. A likely explanation is that different iron species (for example, labile versus enzyme bound [Fe-S] and heme) or iron pools (for example, lysosomal versus mitochondrial versus cytosolic) are involved in mediating unique cell death phenotypes in response to different lethal stimuli.

Mechanistically, erastin and sulfasalazine trigger ferroptosis by inhibiting cystine uptake by system x_c^-, a cell surface cystine and glutamate antiporter[45,48] (**Fig. 4b**). Inhibition of cystine uptake results in the depletion of the endogenous antioxidant tripeptide glutathione (γ-glutamyl-cysteinyl glycine), whose synthesis is dependent upon cysteine, the reduced form of cystine[49]. It is hypothesized that glutathione depletion leads to the iron-dependent accumulation of ROS, especially lipid ROS[45], that themselves are sufficient to kill the cell. The precise role of iron in ferroptosis is unclear. Inhibition of heme-dependent NADPH oxidase enzymes is able to prevent ferroptosis in some cell types[45], accounting for one role for iron in this process. Intriguingly, cobalt chloride inhibits ferroptosis[50] as well as other reported forms of small molecule–induced, iron-dependent death[51]. Cobalt can displace iron from the active site of iron-charged mononuclear enzymes[18], and we speculate that one or more Fe-containing enzymes essential for ferroptosis are inactivated by either iron chelation or displacement of active site iron by the less reactive cobalt atom. One interesting candidate in this connection is PHD1 (also known as EGLN2), an O_2-, 2-oxoglutarate– and iron-dependent enzyme. In a neuronal cell death model that mimics many features of ferroptosis (described below), PHD1 was shown to be essential for cell death and a candidate target to account for iron chelation–mediated inhibition of death[52]. Whether and how the iron-dependent hydroxylase activity of PHD1 contributes to ferroptosis requires further analysis, but in the study described above it was shown to be independent of effects upon the canonical target of this enzyme, HIF-1 (ref. 52). Another candidate is the LOX family of iron-dependent, lipid-modifying enzymes (**Fig. 1b**). These enzymes are activated by glutathione depletion[53] and inhibited by iron chelation[54]. LOX enzyme activation is also implicated[53] in the related cell death pathway described next.

Oxidative glutamate toxicity. Ferroptosis seems closely related to another death phenotype termed oxidative glutamate toxicity (or oxytosis)[55]. Oxidative glutamate toxicity is observed when certain brain cells are cultured in the absence of cystine or treated with high concentrations of glutamate (or glutamate analogs), thereby inhibiting system x_c^- function[49,55]. Similarly to what occurs with erastin and sulfasalazine treatment of tumor cells, high levels of glutamate result in glutathione depletion and oxidative cell death that can be suppressed by iron chelation[55,56]. However, oxidative glutamate toxicity, as observed in neuronal cells, requires additional events downstream of membrane lipid damage for cell death to occur, including LOX activation, Ca^{2+} influx into the cell, enhanced mitochondrial ROS production and fragmentation

and the release of mitochondrial apoptosis-inducing factor (AIF)[57–59] (**Fig. 4b**). These latter events are not known to be necessary in cancer cells and fibroblasts undergoing ferroptosis[45,46]. One possibility is that differences in the availability of oxidizable membrane lipids or the activity of membrane repair enzymes between cell types or both explain the requirement for these additional downstream events in oxidative glutamate toxicity compared to ferroptosis.

Regardless of the particular mechanistic relationship, a better understanding of the ferroptotic and oxidative glutamate toxicity mechanisms are likely to have important medical implications. An iron-dependent, oxidative cell death phenotype is observed in premature oligodendrocytes exposed to high glutamate or deprived of cystine[60]. Within the nervous system, the loss of these premyelinating cells gives rise to periventricular leukomalacia (PVL), a disease that is characterized by white matter lesions within the brain and is a precursor to the development of cerebral palsy[60]. Within the brain, system x_c^- expression is highest in periventricular regions adjacent to where white matter lesions are typically observed in PVL[60,61], perhaps to support enhanced defense against oxidative stress imposed by the highly oxygenated cerebrospinal fluid. Inhibition of system x_c^-–mediated cystine import and glutathione synthesis in these regions by excess glutamate may lead to ferroptotic death in premature infants; specifically targeting this process therefore represents a possible avenue for the treatment of PVL.

Iron as a trigger or mediator of cell death signaling

Although the role of specific ROS-producing enzymes in cell death is becoming increasingly clear, the role of iron itself is not. Below we highlight examples of where iron seems to have a direct role as an initiator or mediator of cell death.

Chronic intracellular iron accumulation in hereditary hemochromatosis. In humans, recessive mutations in *HFE* (hemochromatosis), *HFE2* (hemochromatosis type 2 (juvenile)), *TFR2* (transferrin receptor 2) and *HAMP* (hepcidin antimicrobial peptide) result in excessive dietary iron absorption and tissue iron loading, leading to a range of adverse consequences including cirrhosis of the liver, heart failure and cancer[62]. This spectrum of pathologies, most notably the cardiomyopathy, overlaps to a degree with those observed in mice lacking mitochondrial SOD (*sod2* (ref. 11)) as well as in animals lacking the mitochondrial iron chaperone frataxin[63], leading to the plausible suggestion that iron-dependent, ROS-mediated damage to mitochondrial enzymes and mitochondrial function could lie at the root of iron overload–induced pathology[64]. This model is consistent with recent reports demonstrating that liver toxicity associated with dietary iron overload can be ameliorated with a mitochondrially targeted antioxidant[65]. Importantly, however, high levels of iron are not universally harmful to cell proliferation and viability and are in fact associated with a number of cancers, including hepatocellular cancers observed in some patients suffering from hereditary hemochromatosis itself[66]. It will be important to understand and differentiate between the effects of intracellular iron overload that promote cell death and those that enhance cell proliferation or survival in certain tissues and cell types, whether through cell-nonautonomous effects on the tissue microenvironment or cell-autonomous effects on mutation status, iron-dependent enzyme function or cell signaling[66].

Chronic intracellular iron accumulation in neurodegeneration. A large and growing number of neurodegenerative diseases are associated with the accumulation of aberrant levels of iron within neurons[67]. The role of iron in Parkinson's disease is perhaps best understood. In the case of Parkinson's disease, iron accumulation has been linked to depletion of the Tau protein, which is required for iron export via the ferroportin–amyloid precursor protein (APP) complex[68,69]. The levels of Tau protein, both in humans with Parkinson's disease and in mice treated with a neurotoxin (1-methyl-4-phenyl-1,2,3,6-tetrapyridine (MPTP)) that promotes Parkinson's-like symptoms, are inversely correlated with the levels of intracellular iron. Iron accumulation is causally linked to disease progression, as iron chelator treatment or overexpression of ferritin can attenuate cell loss and ameliorate disease symptoms in Tau knockout or MPTP-treated animal models[68,70]. As with hereditary hemochromatosis, a major outstanding question is how the accumulation of iron actually promotes cell death, whether via enhanced Fenton chemistry–mediated damage, the activation of an iron-dependent enzyme or an alternative means.

A related question concerns the exquisite cell type–specific cell death observed in Parkinson's disease and other neurodegenerative conditions. It was recently observed that the dopaminergic neuronal populations susceptible to degeneration in Parkinson's disease generate especially high levels of mitochondrial ROS[71]; we speculate that these mitochondria-derived ROS synergize with increased iron levels to push these cells selectively toward death. Interestingly, the iron chelators deferiprone and deferoxamine promote mitochondrial destruction via autophagy (mitophagy)[72]. Iron chelator–triggered mitophagy could conceivably prevent neuronal degeneration in Parkinson's and other diseases by enhancing the removal of mitochondria that are responsible for producing damaging levels of ROS. This represents an intriguing 'indirect' mechanism that could account for the ability of iron chelators to attenuate oxidative stress in certain circumstances.

Acute extracellular iron entry in excitotoxicity. An important role for iron as a mediator of death signaling emerged from the analysis of the excitotoxic death of mouse primary cortical neuronal populations in response to *N*-methyl-D-aspartate (NMDA). This process was shown to require iron import from outside the cell via the iron transporter divalent metal transporter 1 (DMT1)[73,74] both *in vitro* and most likely *in vivo*[73,74]. NMDA treatment results in enhanced ROS production and cell death, both of which are attenuated by iron chelation[74]. It will be interesting to reconcile and integrate these results with data showing that NDMA-induced death also requires NOX-derived superoxide production[12]. An interesting possibility is that iron import and NOX-derived ROS synergize to enhance toxic ROS production, leading to neuronal death.

Acute lysosomal iron release as a mediator of necrosis. Another important role for iron as a mediator of death signaling is emerging from the analysis of cyclophilin D–dependent necrotic death. Cyclophilin D (encoded by the gene *PPIF*) is a peptidyl-prolyl *cis-trans* isomerase that is localized to the mitochondrial matrix and required for the execution of nonapoptotic, necrotic cell death in a variety of contexts *in vitro* and *in vivo*[38,75]. Cyclophilin D controls death by modulating the opening of the mitochondrial permeability transition pore complex (mPTPC), and a role of lysosomal iron in mPTPC opening has recently been posited. In different death scenarios, such as drug-induced hepatotoxicity and IRI, iron released from lysosomes is taken up by mitochondria and serves as a stimulus for cyclophilin D–dependent permeability transition pore opening and, ultimately, death[76–77]. Such a model accounts for the ability of iron chelators to protect from IRI and related injuries, but innumerable questions remain unanswered. For example, the nature of the signal that triggers lysosomal iron release during IRI, how iron enters the mitochondria, and how excess mitochondrial iron actually enhances permeability transition pore opening all remain to be resolved.

Iron-dependent death without ROS

In the examples considered so far, iron is known or assumed to trigger cell death via ROS accumulation. Studies in yeast suggest

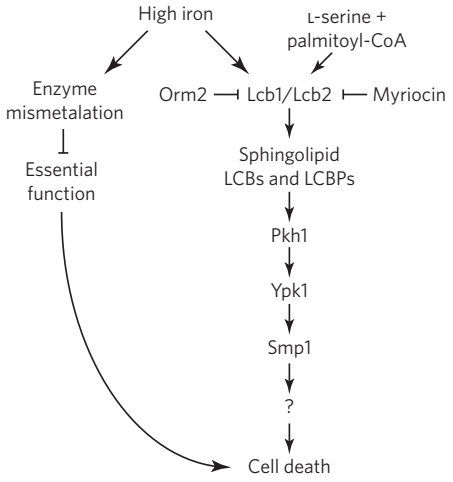

Figure 5 | Iron overload death in in absence of ROS in *S. cerevisiae*. In the yeast *S. cerevisiae,* high levels of iron may cause death by favoring the mismetallation of an essential enzyme with iron, leading to enzyme inactivation and cell death. High levels of iron also stimulate the activity of the myriocin- and Orm2-sensitive serine palmitoyl transferase (SPT) complex containing Lcb1 and Lcb2. Sphingolipid long-chain bases and long-chain base phosphates activate a kinase signaling cascade that leads to death. How iron enhances the activity of the SPT complex and which downstream targets of the kinase pathway mediate death are unknown. Activation of this nonoxidative pathway may explain the ability of high iron to trigger cell death under anaerobic conditions.

that this need not always be the case. Wild-type *S. cerevisiae* cells can grow normally under aerobic conditions in the presence of up to 10 mM iron in the growth medium[78–80], indicating that exposure to such a high concentration is not necessarily toxic to eukaryotic cells. Within cells, high concentrations of iron activate the transcription factor Yap5, leading to expression of the vacuolar iron transporter Ccc1, which promotes the sequestration of iron within vacuoles, structures that are functionally equivalent to metazoan lysosomes[81]. Yeast lacking Ccc1 (*ccc1Δ* mutants) accumulate high levels of cytosolic iron and cannot grow in the presence of high concentrations of exogenous iron[78,79]. Intriguingly, the viability of *ccc1Δ* mutants cannot be restored by overexpression of antioxidant enzymes, such as Sod1, Sod2 or catalase, or growth in anaerobic conditions, suggesting that iron-catalyzed ROS production may not account for the lethal effects of iron overload in this organism[79].

Two potential explanations for these observations have been proposed. First, high levels of cytosolic iron could favor the mismetallation and inactivation of essential enzymes (for example, insertion of iron in place of similar metals such as manganese)[79]. Second, iron overload–induced death may activate a specific lethal pathway (**Fig. 5**). Recently, it was found that the lethality of 7 mM $FeCl_3$ was suppressed by treatment with the natural product myriocin or overexpression of the endoplasmic reticulum–resident proteins Orm2 and Orm1 (ref. 82). Myriocin is a natural product inhibitor of the serine palmitoyltransferase complex, the first enzyme in the sphingolipid production pathway. Orm2 and Orm1 proteins are highly conserved negative regulators of serine palmitoyltransferase function[83]. High iron levels promote the accumulation of sphingolipid long-chain bases and long-chain base phosphate, and these increases are counteracted by Orm2 overexpression or myriocin treatment[82]. High levels of sphingolipid long-chain bases stimulate a Phk1-Ypk1-Smp1 protein kinase pathway that is essential for lethality, independent of established yeast death effectors such as Mca1 and Mma111 (ref. 82). It remains to be determined how high iron activates sphingolipid production, how sphingolipids enhance kinase pathway activity and how activation of this kinase pathway triggers death. Given that the function of Orm2 and Orm1 in sphingolipid biosynthesis is conserved in human cells[83], it will be interesting to determine whether a similar pathway could contribute in mammalian cells to the toxicity associated with pathological iron overload.

New therapeutic opportunities

An improved understanding of the role of iron and ROS in cell death is creating new opportunities for therapeutic intervention. Below we highlight two areas of recent progress.

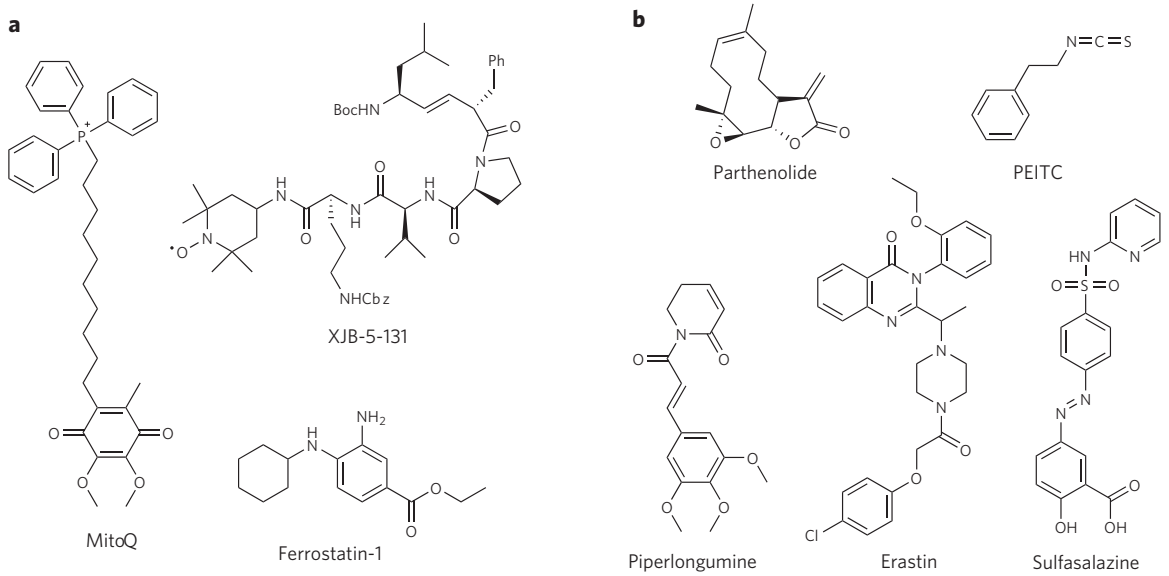

Figure 6 | Small-molecule modulators of ROS- and iron-dependent death. (**a**) Structures of targeted antioxidants used to prevent the ROS-dependent cell death. MitoQ and XJB-5-131 accumulate in the mitochondrial matrix, whereas Fer-1 seems to specifically target ROS in nonmitochondrial membranes. In XJB-5-131, Boc and Cbz refer to the standard *tert*-butyloxycarbonyl and carboxybenzyl protecting groups, respectively, which are maintained in the final compound. (**b**) Structures of small molecules that promote ROS accumulation by antagonizing glutathione-mediated antioxidant pathways.

Targeted antioxidants. An emerging strategy to combat the toxic effects of ROS accumulation is to design or identify small molecules that preferentially scavenge ROS at specific intracellular sites (**Fig. 6a**). In one approach, the mitochondrial inner membrane potential is harnessed to drive antioxidants into the mitochondrial matrix to scavenge mitochondrial ROS. The compound MitoQ is a fusion of ubiquinone, the endogenous electron carrier of the mitochondrial electron chain, and a positively charged triphenylphosphonium ion that accumulates in the mitochondrial matrix[84]. MitoQ treatment can improve memory function and delay apoptotic neurodegeneration in a mouse model of Alzheimer's disease[85] and improve organ function in a rat model of sepsis-induced organ failure[86]. Another agent, whose accumulation is independent of the inner membrane potential, is XJB-5-131, a conjugate of the small-molecule radical spin trap TEMPO and a peptide mitochondria-targeting sequence (Leu–D–Phe–Pro–Val–Orn) derived from the antibiotic gramicidin S[87]. XJB-5-131 seems to ameliorate mitochondrial function and prevent cell death by preventing the oxidation of mtDNA and mitochondrial cardiolipins[32,88], the latter event previously linked to the induction of apoptosis (described above). XJB-5-131 has shown promise in attenuating traumatic neuronal brain injuries[32] and motor neuron damage in a mouse model of Huntington's disease[88].

In addition to the mitochondria, other sites of ROS production and accumulation are likely to be important for cell death in different pathological scenarios, and targeting agents to these sites may prove beneficial. The synthetic small molecule ferrostatin-1 (Fer-1) was discovered in a phenotypic screen for inhibitors of ferroptosis and subsequently shown to prevent glutamate toxicity in rat hippocampal slice cultures[45]. Structure activity relationship analysis suggested that the N-cyclohexyl moiety serves as a lipophilic membrane anchor, whereas the two amines most likely act to donate reducing equivalents[45]. Fer-1 seems to be tuned in some way to the ferroptotic lethal mechanism as it does not inhibit other forms of cell death, including staurosporine-induced apoptosis and iron-dependent, H_2O_2-induced death[45]. We speculate that Fer-1 is targeted to a specific non-mitochondrial membrane whose oxidation is required for ferroptosis.

Anticancer agents that perturb ROS homeostasis. Another potential application of iron- and ROS-dependent death is in the treatment of cancer. Recent studies in a variety of genetically engineered mouse models of cancer suggest that alterations such as KRAS and p53 mutation can, in certain cancers, promote high NADPH production and low ROS levels and that this is essential for tumor cell proliferation and survival[89–91]. NADPH is required to maintain glutathione homeostasis, and therefore one interpretation of these and many previous results is that tumor cells require a highly reduced intracellular environment to survive. This provides a rationale for testing whether decreasing the levels of glutathione and/or increasing the levels of oxidative stress could specifically push cancer cells toward senescence or death[92]. One promising approach is to enhance lethal ROS accumulation by incapacitating the antioxidant defenses of the cell. Promising preclinical results have been obtained with small molecules targeting nodes in the cellular glutathione antioxidant network, including the catalytic subunit of glutamate cysteine ligase, glutathione peroxidase and glutathione itself (parthenolide[93] and phenyl ethylisothiocyanate[94]), glutathione S-transferase pi 1 and carbonyl reductase (piperlongumine[95]) and system x_c^- (sulfasalazine and erastin[45,96]) (**Fig. 6b**). These compounds may be especially valuable as agents to target certain cancer stem cell populations that have been shown to display an intrinsic resistance to radiotherapy-induced, ROS-mediated cell death owing to high expression of enzymes in the glutathione synthesis pathway[97].

Future directions

In many cases, we lack answers to important questions, such as how iron promotes death, either via enhanced ROS production or alternative means, and the sources, types and targets of ROS that are relevant to cell death. In many organisms, from bacteria to mammals, the role of iron and ROS in cell death continues to generate controversy[98–100]. A more detailed understanding of these questions will undoubtedly benefit from the application of new probes, such as the mitochondrially targeted, ratiometric MS probe MitoBoronic acid[101] and the genetically encoded sensor Orp1-roGFP2 (ref. 102), which will enable ROS levels to be correlated with cell death *in vivo*. Finally, the development of new probes that can report on free iron levels with the cell (for example, iron probe 1 (ref. 103)) will facilitate future studies of the role of iron in cell death. Further progress along these lines will ensure that the role of iron and ROS in the life and death of cells remains an important area of investigation into the future.

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Competing financial interests

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